ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Biotechnology

ISSN 1682-296X
DOI: 10.3923/biotech.2016.17.25

Research Article
Phylogenetic Relationship of Cuscuses (Marsupialia: Phalangeridae) from Papua and Maluku Based on Mitochondrial Sequences of NADH Dehydrogenase Sub-unit 1 Gene

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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. This study, the phylogenetic relationship of cuscuses from Papua and Maluku based on mitochondrial sequences of NADH dehydrogenase subunit 1 gene was investigated. Whole genome DNA was extracted from 22 tissue biopsy samples from Maluku (13 individuals) and Papua (9 individuals) according to the protocol of Qiamp DNA Blood Mini Kit (Qiagen) and then it was used as template for amplification of ND1 gene by using PCR method. The PCR product gives result nucleotides of 1152 bp and sequencing product gives result nucleotides of 956 bp of the ND1 gene for phylogenetic analysis. The genetic distance between Phalanger and Spilocuscus, 14.2% was found higher than the genetic distance between cuscus from Papua and Maluku 7.8%. The genetic distance within Phalanger was 3.7% and genetic distance within Spilocuscus was 1.3%. The genetic distance within Phalanger from Papua and Maluku was 1.3% and the average genetic distance within Spilocuscus from Papua and Maluku was 1.3%. The phylogram tree using Neighbor Joining classified cuscus from Papua and Maluku in clade A (Phalanger) and clade B (Spilocuscus), respectively. Clade A and B were further subdivided into clade A1 (Phalanger from Papua), A2 (Phalanger from Maluku) and clade B1 (Spilocuscus from Papua), B2 (Spilocuscus from Maluku). Spilocuscus genera members from Ternate and Sentani islands was found five nucleotides distinguishing compared to Spilocuscus from Papua and Maluku. It was concluded that identification of Spilocuscus and Phalanger members from Maluku, Papua, Ternate and Sentani could be distinguished by mitochondrial DNA sequencing of the ND1 gene.

Key words: NADH dehydrogenase sub-unit 1, genetic distance, cuscus, DNA sequencing

Received: December 11, 2015 **Accepted:** February 01, 2016 **Published:** February 15, 2016

Citation: Rini Widayanti, Agustianti, Tito Suprayogi, Rony Marsyal Kunda and Suhendra Pakpahan, 2016. Phylogenetic relationship of cuscuses (Marsupialia: Phalangeridae) from Papua and Maluku based on mitochondrial sequences of NADH dehydrogenase sub-unit 1 gene. Biotechnology, 15: 17-25.

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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

Spilocuscus is one of marsupial genera, classified into the Phalangeridae family, spread exclusively in the Eastern part of Indonesia (Sulawesi, Maluku, Papua and Timor islands), Australia and Papua New Guinea[1-3]. In Papua, there are two genera of cuscus exist, the Phalanger (unspotted cuscus) and the Spilocuscus (spotted cuscus), whereas in Maluku island, the Phalanger and Spilocuscus genuses found in the Northern part of Maluku islands, the island of Halmahera, Bacan and Morotai[4]. Petocz[2] stated that according to the morphology, there were five species of cuscuses from Papua and Maluku islands found, the *P. gymnotis* (ground cuscus), *S. maculatus* (spotted cuscus), *P. orientalis* (Northern common cuscus), *S. rufoniger* (black-spotted cuscus) and *P. vestitus* (stein cuscus). According to Menzies[1] there is also *Spilocuscus papuensis* (Waigeo cuscus) endemic cuscus from Waigeo island of Papua and *Spilocuscus wilsoni* (spotted cuscus) the endemic cuscus of the Biak island of Papua[5].

Identification performed in this study was based on the morphological characteristics, as in the color and spots of the skin[2] even though the Spilocuscus from Papua and Maluku have the same color, the pattern could not be distinguished. Thus, the identification of the cuscus could only be specifically determined using genetic marker sequencing.

Mitochondrial DNA (mtDNA) analysis has been used in the phylogenetic relationships study[6] and evolutionary study of the animal species for more than 30 years[7]. The mitochondrial DNA (mtDNA) is a double-stranded circular DNA, composed of 37 genes and coding for mitochondrial proteins that would yield energy-rich ATP molecules. Their mutation rate is five to ten times faster than the nuclear genome. These features serve mtDNA as a specific key to predict the phylogenetic relationship of closely related species more accurately[8].

Sequencing of mtDNA genes could be utilized to assist in phylogenetic analysis, among others, COI[6], 12S rRNA for Ailurops and Strigocuscus members from Sulawesi[9] and 16S rRNA[8], Cyt b gene for Phalanger and Spilocuscus genera members from Maluku and Papua[11]. The 12S rRNA and Cyt b genes for phylogentic analysis of dasyurine marsupials[12] and the D-loop region[13].

Previous molecular sequence analysis, limited that included Ailurops and Strigocuscus[9] and Ailurops, Spilocuscus and Phalanger members from Sulawesi, Maluku and Papua[10] 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 anothers genes trees[14-16]. The phylogenetic relationship of the species were examine cuscuses from Maluku and Papua that based on morphological characters refers to as *Spilocuscus maculatus* (spotted/white cuscus), *Spilocuscus rufoninger* (black spotted cuscus), *Phalanger orientalis* (Northern common cuscus/brown-linear-black) and the *Phalanger vestitus* (stein cuscus) using sequences from NADH dehydrogenase sub-unit 1 gene (ND1).

The objective of this study is to determine the nucleotide sequences and genetic markers in ND1 gene using DNA sequencing methods to identify and distinguish the cuscus from Papua and Maluku. 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 02 March, 2015 to ending 02 June, 2015. Twenty-two tissue biopsy samples were collected from two cuscuses of native habitat in Maluku (13 samples) and Papua (9 samples). Tissue samples stored in RNA latter (Qiagen). In order to optimize the DNA preservation, the tissue samples were re-suspended in phosphate buffer saline after few days of storage. Total DNA isolation was carried out using the DNA isolation kit provided by Qiagen.

PCR amplification: Approximately 1152 bp was amplified from the 5 region of ND1 gene from mitochondrial DNA using the following primers. The primers were designed using primer 3 online programs with the 4.0 version (http://www-genome.wi.mit.edu/cgi.bin/primr3.cgi/results_from-primer3) based on data from mitochondrial genome sequences of *Phalanger vestitus* (AB241057.1) and *Trichosurus vulpecula* (AF357238).

Forward: ND1F-5′ AGC AGG CAA TTG CAT AAA AC 3′ and Reverse: ND1R-5′ AAT GTG GTG TAA TGG AAG CA 3′. About 50 µL PCR reaction mixture include 25 µL kappa ready mix (1st Base), 1 µL each primer (10 pmol), 3 µL DNA template and 20 µL nuclease free water (1st Base). Amplification was performed using an infinigen thermal cycler. The PCR program consisted of an initial denaturation at 94°C for 5 min, followed

by 35 cycles of denaturation (0.5 min at 94°C), annealing (0.5 min at 52°C) and extension (1 min at 72°C), followed final extension (72°C for 5 min) and then held at 4°C. The PCR products were visualized on 0.8% agarose gel with DNA stain (1st Base).

Sequencing and sequencing analysis: The purified PCR products were sequenced directly by 1st Base Sequencing INT (Malaysia). Comparative sequences taken from National Centre for Biotechnological Information (NCBI) for intergeneric, interspecific and intraspecific analyses. Sequences were aligned using clustal W[17] in MEGA version 6.0 software[18]. To remove any ambiguity in the sequenced bases, sequences were crossed checked with the electropherogram of the complementary strand. Sequence divergences were calculated using the kimura two parameter (K2P) distance model[19]. Neighbor Joining (NJ) trees of K2P distances were created to provide representation of the patterning of divergence between genera and species of cuscuses from Papua and Maluku[20]. Phylogram was constructed with bootstrapping of 1000 replication through MEGA version 6.0[18].

RESULTS

All twenty-two cuscus samples from Papua and Maluku resulted in excellent amplifications, with product approximately 1152 bp (Fig. 1).

A total of 22 sequences (13 sequences from Maluku, 9 sequences from Papua) were obtained. In this study 5 species from GenBank used as a comparison, to determine the genus and species of cuscus from Papua and Maluku island with access number *Phalanger vestitus* (AB241057.1), *Phalanger gymnotis* (KJ868142.1), *Spilocuscus maculatus* (KJ868160.1), *Strigocuscus celebensis* (KJ868161.1) and *Airulops ursinus* (KJ868096.1). Results of DNA sequencing based on the 1152 nucleotides sequence of the mitochondrial genome *Phalanger gymnotis* (KJ868142) at position 2625-3776, namely the partial $tRNA^{Leu}$ gene (2625...2685), gene ND1 (2687...3642), gene $tRNA^{Ile}$ (3643...3711) and $tRNA^{Gln}$ partial (3709...3776). In this study only ND1 gene sequences (956 nucleotides) were utilized for further analysis.

Genetic distance: Nine hundred and fifty-six nucleotides of the 22 individual cuscuses did not show any deletion nor insertion. By using the K2P distance, cuscus from Papua and Maluku islands were classified into two genuses, the Phalanger and Spilocuscus. The average genetic distance (K2P distance) within Phalanger was 3.7% and average genetic distance within Spilocuscus was 1.3%. The average genetic distance (K2P distance) within Phalanger from Papua and Maluku was 1.3% and the average genetic distance (K2P distance) within Spilocuscus from Papua and Maluku was 1.3%. The average of K2P distance between Phalanger and Spilocuscus was 14.2% and the average of K2P distance between cuscus from Papua and Maluku was 7.8% (Table 1).

The 22 sample of cuscuses of multiple alignment resulted in the 22 sites of nucleotides and 5 sites of amino acids that can be used as genetic markers between Phalanger from Papua and Maluku (Table 2 and 3) and there were 20 sites of nucleotides and two sites of amino acids that can be used as a marker of genetic among Spilocuscus of Papua and Maluku (Table 4 and 5). Spilocuscus from Sentani island and Tobelo, Ternate island had 5 sites of nucleotides that could be utilized to differentiate the origin of Spilocuscus Papua (Table 4).

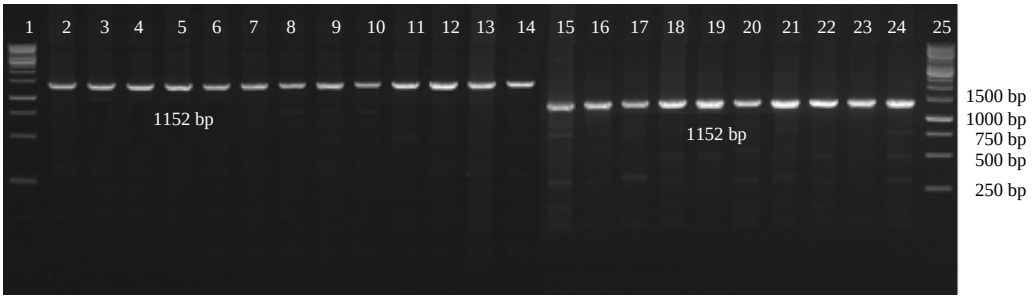

Fig. 1: PCR product ND1 gene of cuscuses on a 0.8% agarose gel, Lane 1-25: DNA ladder 1 Kb, Lane 2-24: PCR product of cuscuses samples (1152 bp) and PCR: Polymerase chain reaction

Table 1: Distribution of K2P distance (percent) for ND1 gene within the group cuscuses sequenced

Group name	A1	A2	B1	B2	*Spilocuscus maculatus*	*Phalanger vestitus*	*Phalanger gymnotis*
A1							
A2	2.7						
B1	13.9	14.3					
B2	13.3	13.7	2.1				
Spilocuscus maculatus KJ868160.1	14.2	14.2	1.0	1.7			
Phalanger vestitus AB241057.1	7.7	7.7	14.3	13.7	14.2		
Phalanger gymnotis KJ868142.1	13	13.4	13.6	13.7	13.8	13.2	

A1: Group of Phalanger from Papua, A2: Group of Phalanger from Maluku island, B1: Group of Spilocuscus from Papua and Tobelo, Ternate island, B2: Group of Spilocuscus from Maluku island

Table 2: Group diversity of nucleotide sites of *Phalanger* sp., originated from Papua and Maluku that can be used as a marker

Origin				2	2	2	2	2	2	3	3	4	4	4	5	5	6	6	7	7	9	9	9
		1	3	0	3	6	6	6	8	0	4	0	3	4	2	9	6	7	3	4	2	2	4
		8	6	1	4	0	1	5	0	3	8	5	2	4	0	7	0	2	2	0	1	8	0
Papua	MNBLB	A	A	T	A	T	C	C	C	G	C	A	A	T	C	C	C	C	G	C	T	G	G
	WNDG	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	WNBLB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	YNBLB	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Maluku	GeSiBLB	G	C	A	C	C	T	T	T	A	T	C	T	C	T	T	T	T	A	T	C	T	A
	GoSiWW	G	C	A	C	C	T	T	T	A	T	C	T	C	T	T	T	T	A	T	C	T	A
	KHiBLB	G	C	A	C	C	T	T	T	A	T	C	T	C	T	T	T	T	A	T	C	T	A
	KHiWW	G	C	A	C	C	T	T	T	A	T	C	T	C	T	T	T	T	A	T	C	T	A
	AAiWW	G	C	A	C	C	T	T	T	A	T	C	T	C	T	T	T	T	A	T	C	T	A
	LAiBLB	G	C	A	C	C	T	T	T	A	T	C	T	C	T	T	T	T	A	T	C	T	A
	LiBLB	G	C	A	C	C	T	T	T	A	T	C	T	C	T	T	T	T	A	T	C	T	A
	NMiWW	G	C	A	C	C	T	T	T	A	T	C	T	C	T	T	T	T	A	T	C	T	A
	GeSiWW	G	C	A	C	C	T	T	T	A	T	C	T	C	T	T	T	T	A	T	C	T	A

-: Homologous with MNBLB sequences

Table 3: Amino acid diversity of Phalanger groups from Papua and Maluku that can be used as a marker

Origin		87	94	247	310	314
Papua	MNBLB	I	P	T	A	V
	WNDG	-	-	-	-	-
	WNBLB	-	-	-	-	-
	YNBLB	-	-	-	-	-
Maluku	GeSiBLB	T	S	I	S	I
	GoSiWW	T	S	I	S	I
	KHiBLB	T	S	I	S	I
	KHiWW	T	S	I	S	I
	AAiWW	T	S	I	S	I
	LAiBLB	T	S	I	S	I
	LiBLB	T	S	I	S	I
	NMiWW	T	S	I	S	I
	GeSiWW	T	S	I	S	I

-: Homologous with MNBLB sequences

DISCUSSION

The phylogram (Fig. 2) generated through NJ method using K2P distance was highly reliable as the out group used (*Strigocuscus celebensis* and *Ailurops ursinus*) were segregated in separate clade as expected. The ability of the ND1 gene in determining two different genuses was proven through the phylogram as two different clades (Clades A and B). Clade A as the Phalanger and clade B as the Spilocuscus. These results are in accordance with the finding of Fatem and Sawen[21] and Latinis[4] that genus cuscus in Papua and Maluku was Phalanger and Spilocuscus. Phylogenetic signals were evident in the clade A the Phalanger of Papua was segregated from the Phalanger of Maluku with few other related spesies (strong evidence by bootstrap value 100%). Similarly for phylogenetic signals were evident in the clade B the Spilocuscus of Papua was segregated from the Spilocuscus of Maluku (strong evidence by bootstrap value 100%). Bit of phylogenetic signals were evident in the clade B spotted cuscus of Sentani island, spotted cuscus of Tobelo, Ternate island and *S. maculatus* (GenBank) was segregated from the Spilocuscus of Papua (supported by bootstrap value 90%).

In this study, 22 samples of cuscus was also used by Kunda *et al.*[11] (acceptance latter for article No. 76171-PJBS-ANSI), added one sample cuscus from Tobelo (Ternate island) (Table 6). The usage of K2P genetic distance to analyze cuscuses from Maluku and Papua revealed that between the genus sequences, the variation was high (14.2%) compared to the sequence variation within the genus (1.3%). The existence of genetic variation that is unique (Nucleotide:

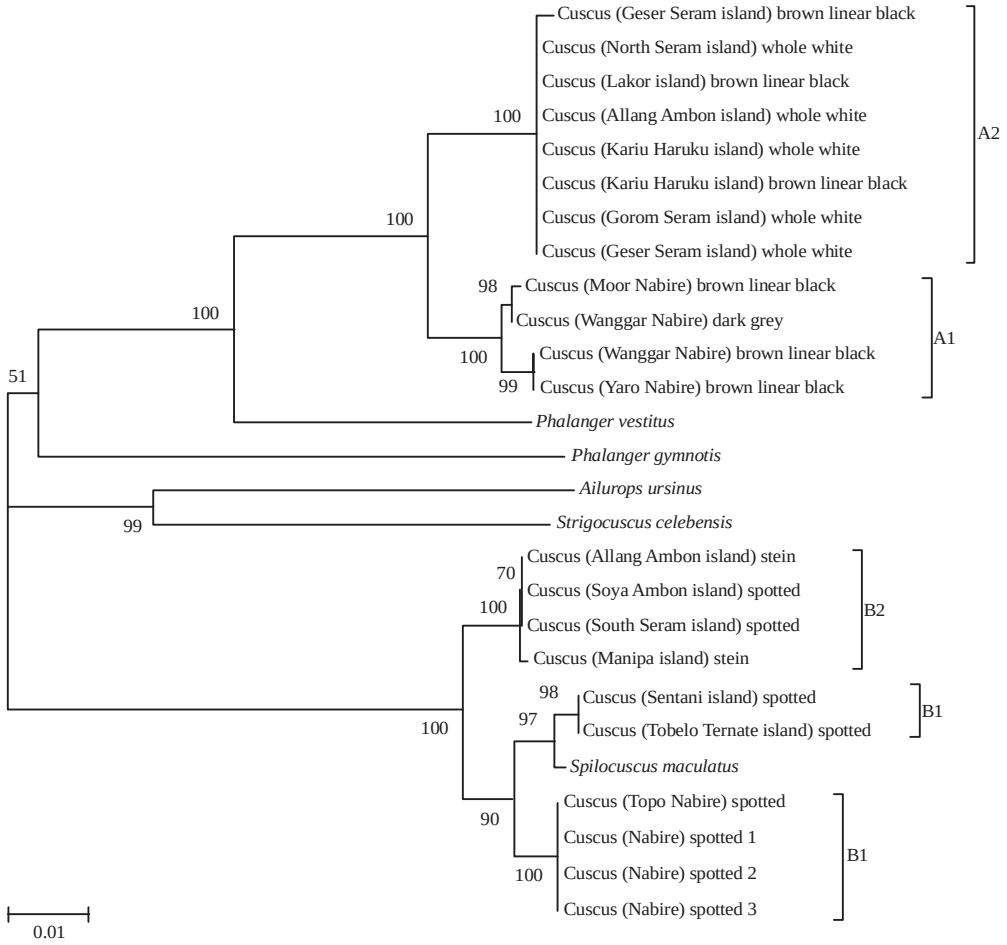

Fig. 2: Neighbour-joining tree (with bootstrap values, 1000 replicates) of ND1 gene sequences cuscuses from Papua and Maluku with five spesies of Phalangeridae from GeneBank

Table 4: Nucleotide diversity of Spilocuscus groups from Papua and Maluku that can be used as a marker

				1	1	1	1	2	2	2	2	3	4	4	5	5	6	7	7	8	8	8	8	9	9	9
		3	3	1	3	3	8	1	4	6	6	1	4	5	3	3	3	3	9	2	7	8	9	1	1	2
Origin		0	1	4	2	5	9	9	3	5	7	2	7	9	4	7	9	8	2	2	0	0	1	2	9	7
Papua	TNS	C	A	T	C	C	C	C	A	C	A	C	T	T	A	G	C	A	T	G	G	T	A	C	G	C
	NS1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	NS2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	NS3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	SiS	-	-	C	-	T	A	-	-	T	-	T	-	-	G	-	-	G	-	A	A	C	G	T	-	-
Maluku	TTiS	-	-	C	-	T	A	-	-	T	-	T	-	-	G	-	-	G	-	A	A	C	G	T	-	-
	SSiS	T	G	C	T	T	-	T	G	-	G	T	C	C	-	A	A	-	C	A	A	C	G	-	A	T
	MiSt	T	G	C	T	T	-	T	G	-	G	T	C	C	-	A	A	-	C	A	A	C	G	-	A	T
	AAiSt	T	G	C	T	T	-	T	G	-	G	T	C	C	-	A	A	-	C	A	A	C	G	-	A	T
	TNS	T	G	C	T	T	-	T	G	-	G	T	C	C	-	A	A	-	C	A	A	C	G	-	A	T

-: Homologous with TNS sequences

20 sites, the amino acid: 4 sites) within the genus between cuscus from Papua and Maluku can be used as markers of genetic inter Phalanger from Papua and Maluku and also as a marker of genetic inter Spilocuscus from Papua and Maluku(Nucleotide: 20 sites, amino acids: 2 sites). Especially for Spilocuscus from Sentani island and Spilocuscus from Tobelo (Ternate island) have a unique nucleotide sequence, which is solely owned by the cuscus (Nucleotide sites 189, 265, 534,

738 and 912th) so that it can be used as a genetic marker to distinguish the Spilocuscus origin from Maluku and Papua other (Table 5). However, if compared from amino acid composition, Spilocuscus from Sentani island and Spilocuscus from Tobelo (Ternate island) were similar to Spilocuscus from Papua. Geographically, Ternate island located far from Papua but both Spilocuscus has a very close relationship.

Because of the limited comparative data from GenBank, so that the data presented have not been able to give instructions definite species of cuscus partially derived from the Maluku and Papua. In the group Phalanger seem that Papua and Maluku cuscus from the two most closely related to *P. vestitus* but it is possible that the both are not a part of *P. vestitus* when seen fairly large genetic distance is 7.7% (Table 6) when compared with the genetic distance between *S. maculatus* and Spilocuscus from Papua and Maluku island were only 1.0-1.7%. It is the same to Widayanti *et al.*[10] research by using gene sequences 12S rRNA.

Figure 1 shows that the colors and patterns of the hair could not be used as a basis for determining taxonomic cuscus. Cuscuses from clade A (A1 and A2) are very varied from the colors and patterns of hair (brown linear black, dark gray and white whole) but the views of the nucleotides and amino acid sequences showed that all of them are the same species (*Phalanger* sp.) The same thing happened to group B (B1 and B2) there are variations in colors and patterns of hair (spotted and stein) but it is genetically the same species.

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 to wards the predominantly white color while *P. orientalis* remains brown. Sexual dimorphism is clearly visible on spotted cuscus[22]. Isaac and Johnson[23], 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. The results of this study is different from the conventional taxonomy by Fatem and Sawen[21] and Petocz[2].

Table 5: The amino acids diversity of *Spilocuscus* sp., group from Papua and Maluku that can be used as a marker

Origin		11	307
Papua	TNS	I	V
	NS1	-	-
	NS2	-	-
	NS3	-	-
	SiS	-	-
Maluku	TTiS	-	-
	SSiS	V	I
	MiSt	V	I
	AAiSt	V	I
	TNS	V	I

-: Homologous with TNS sequences

Table 6: Information sampling for 22 individual cuscuses from Papua and Maluku with the characteristics of each hair colors

Samples	Abbreviation	Origin	Characteristic hair color
Cuscus 1	MNBLB	Moor, Nabire, Papua	Brown linear black
Cuscus 2	WNDG	Wanggar, Nabire, Papua	Dark grey
Cuscus 3	WNBLB	Wanggar, Nabire, Papua	Brown linear black
Cuscus 4	YNBLB	Yaro, Nabire, Papua	Brown linear black
Cuscus 5	GeSiBLB	Geser, Seram island	Brown linear black
Cuscus 6	GoSiWW	Gorom, Seram island	Whole white
Cuscus 7	KHiBLB	Kariu, Haruku island	Brown linear black
Cuscus 8	KHiWW	Kariu, Haruku island	Whole white
Cuscus 9	AAiWW	Allang, Ambon island	Whole white
Cuscus 10	LAiBLB	Larike, Ambon island	Brown linear black
Cuscus 11	LiBLB	Lakor island	Brown linear black
Cuscus 12	NMiWW	North Seram island	Whole white
Cuscus 13	GeSiWW	Geser, Seram island	Whole white
Cuscus 14	TNS	Topo, Nabire, Papua	spotted
Cuscus 15	NS1	Nabire	Spotted 1
Cuscus 16	NS2	Nabire	Spotted 2
Cuscus 17	NS3	Nabire	Spotted 3
Cuscus 18	SiS	Sentani islnad, Papua	Spotted
Cuscus 19	TTiS	Tobelo, Ternate island	Spotted
Cuscus 20	SSiS	South, Seram island	Spotted
Cuscus 21	MiSt	Manipa island	Stein
Cuscus 22	AAiSt	Allang, Ambon island	Stein

George[24,25] used the generic names Spilocuscus and Strigocuscus to split species groups from the Phalanger genera. George[25] asserted *Strigocuscus celebensis* was separated at the generic level from *Phalanger gymnotis*. Flannery *et al*.[26] revised the systematics of the Phalangeridae based analysis of 35 morphological characters as grouped *P. gymnotis* with Trichosurini. Springer *et al*.[27] supported the grouping of *P. gymnotis* with *P. orientalis* and *P. vestitus*, to the exclusion of Spilocuscus genera. Halmilton and Springer[28], 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 Halmilton and Springer[28], asserted there was also strong support (93%), *P. gymnotis* including monophyly of Spilocuscus genera.

Widayanti *et al*.[10] 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. Widayanti *et al*.[10] 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*.[10] asserted cuscus from Manipa island have genetic relationship with *Spilocuscus maculatus* (94%) than Phalanger members.

Ailurops is found on Sulawesi and the nearby islands of Togian, Peleng, Muna, Buton and Lirong, while Strigocuscus is found only on Peleng and Taliabu just East of Sulawesi. The range of Spilocuscus includes Northern Australia, New Guinea, Buru, Seram, Banda and Selayar, whereas the range of Phalanger extends from Northern Australia to New Guinea[3,29].

Reconstructing the taxonomy based on biogeographical history of the Phalangeridae is complicated by the very intricate and poorly understood geological history of Southeast Asia[30]. As a result, the interpretation of the biogeographic history of Phalangerids that was provide can only be considered tentative. The molecular phylogenies presented in this study are considered more reflective of the different evolutionary relationships of Phalangeridae than previous morphological analysis, as the molecular phylogenies show high levels of congruence between them versus what has been seen in the morphologically based phylogenies.

Kirsch and Wolman[29] and Ruedas and Morales[9], asserted the molecular divergence between Phalangerinae and Ailuropinae has been estimated to have occurred 16-23.3 mya. The movement of crustal fragments in Southeast Asia can possibly explain the divergence of the Phalangerinae from the Ailuropinae. The Philippine or Muluccas sea plate had been carrying the bird's head microcontinent Westward for the past 50 million years[30,31]. The Buton-tukang besi block separated from the bird's head microcontinent 17-15 mya, accreting onto the Sulawesi Southeastern Peninsula approximately 11 mya[32] and possibly transporting an Ailuropinae ancestor. It is also a possibility that the ancestral Ailuropinae dispersed Westward island to island, eventually ending up in the Sulawesi region.

Furthermore, the radiation of Phalanger and Spilocuscus can be explained by the rising of the New Guinean highlands approximately 15 mya[25,33]. The presence of Phalanger and Spilocuscus in Cape York (mainland Australia) are only recent arrivals from their Northerly distribution during one of the more recent lowering of sea level separating mainland Australia from New Guinea and its adjacent islands[29].

CONCLUSION

Present result revealed that ND1 gene grouping cuscus from Papua and Maluku become two genus Phalanger and Spilocuscus and classified into *Phalanger* sp. and *Spilocuscus maculatus* species. We found 20 nucleotides and 4 amino acid distinguished from ND1 gene that can be used as genetic marker within *Phalanger* sp. while *S. maculatus* from Papua and Maluku have 20 nucleotides and 2 amino acid differences. Spilocuscus members from Ternate and Sentani have 5 nucleotides differences, at the site of 189, 265, 534, 738 and 912, which distinguish the cuscus with cuscus from Papua and Maluku.

ACKNOWLEDGMENT

The authors would like to give a thank you remark to the Directorate General of Higher Education (DGHE) Indonesia No. 227/LPPM/2015, March 02, 2015 that has given financial support to this research.

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