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ANALYSIS OF MUTATIONS IN THE SUB - UNIT II CYT. OXIDASE GENE (*COX2*) OF *Tarsius tarsier* FROM BUTON ISLAND INDONESIA

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Abstract

Tarsius spreading over Sulawesi mainland and on the surrounding islands including Buton Island is still known as an interesting animal to be studied, especially related to gene mutation. The objective of this descriptive explorative research is to analyze mutations at partial sequence of subunit II *(COX2)* cyt. oxidase gene of *Tarsius tarsier* form Buton. The sequencing of PCR product produced a base sequence of 560 nt. The gene sequencing results were then aligned with the genes of *Tarsius dentatus* and *Carlito syrichta* taken from *Genebank* (*C. syrichta* is assumed as the Sulawesian tarsier ancestor). It can be seen that the total number of the invariable sites and variable sites were 432 and 128 respectively, but the total number of mutation was 130. Subtitution mutations were found in 128 sites; the total numbers of transition and transversion mutations were 89 and 16 respectively without calculating the double and single subtitution at the same site of transition. Most of subtitution mutations in *COX2* gene sequences occur in the third base of each codon.

INTRODUCTION

One of the endemic animals that inhabit the region of Sulawesi and the surrounding islands including the island of Buton is Tarsius. This genus belongs to the Tarsiidae family, the only surviving family of the Tarsiiformes order. Tarsius is known as the ghost animal classified as nocturnal animal. Its face looks like a small monkey having round-big-red eyes used for seeing at night [1]. Sulawesi has 11 species of Tarsius, namely *T. tarsier, T. fuscus, T. sangirensis, T. pumilus, T. dentatus, T. pelengensis, T. lariang, T. tumpara, T. wallacei* and 2 other types which are known to be different species, but they have not been named yet [2].

The illegal hunting and the damage of Tarsius natural habitat cause these animals to be endangered. This animal species has been protected since 1930 [3]. *International Union for Conservation of Nature and Natural Resourches* (IUCN) determines that Tarsius is in the category of *endangered* animal and is included *insufficiently known* species [4].

There were various non molecular studies on several Tarsius species ever conducted before. Those studies included studies in ecological and behavioural areas as well as in area of taxonomy etc. [5, 6, 7, 8, 9, 10]. There are only a few molecular studies on DNA of various Tarsius species related to nuclear genome, as well as on that of mitochondrial genome, ever conducted. In this connection any study on gene mutation in relation with certain genes of mitochondrial genome of some Tarsius species (including *T. tarsier* form Buton) was also very rarely conducted.

Information related to the gene mutations (including certain mitochondrial genes) will be useful in order to get any illustration about the events of gene mutation occurred at the group of Tarsius, other groups of Primata, as well as at the whole Primata and animal. Those study results will also be useful to uncover the event of organism evolution, related to any small group as well as whole organism in general.

This research used *COX2* genes as genetic markers. *COX2* encoding gene has a size of 684 bp, located between the tRNA ^{Asp} encoding genes (at the left or front) and the tRNA ^{Lys} encoding genes (at the right or behind) in the mt-DNA [11]. Furthermore it has been know the COX2 encoding gene of some animal species, had a greater mutation rate than the other encoding genes of the mitochondrial DNA [12].

Research involving *COX2* gene has previously been carried out [13,14]. Certain research was conducted to investigate the various sequences of amino acids and *COX2* nucleotides from 25 species of primates including two species of tarsius [13]. The research results found that there was a close kinship *(sister-group)* between tarsius and monkeys *(ape clade)*. The research did not focus on how close the kinship of the two tarsius used as the samples was. The other research showed that based on the nucleotide and amino acid sequences, the *COX2* gene could be used to distinguish tarsius of Lampung and tarsius of Sulawesi; but it could not be used to distinguish *Tarsius dianae* (Central Sulawesi) and *Tarsius spectrum* (North Sulawesi) [14].

The molecular analysis conducted in this research focused on mutations which occurred on the *COX2* gene sequences of *Tarsius tarsier* form Buton as well as of two species of tarsius from other areas in which the data were derived from *Genebank;* the other two species were *Tarsius dentatus* spreading in mainland of Sulawesi and *Carlito syrichta* spreading over several islands of the Philippines. The analysis of amino acid changes was supported by Clustal-W and Mega 7.0 *software*.

The mutation analysis of the *COX2* gene sequences of *Tarsius tarsier* form Buton had not been done before. Genebank does not have the data of the *COX2* gene sequences of *Tarsius tarsier* form Buton. *COX2* gene sequences are expected to be used as genetic markers of *Tarsius tarsier* form Buton species, as well as can be used for conservation purposes.

MATERIALS AND METHODS

Sample Collection

The data of *Tarsius tarsier* were obtained from the forest of Buton Island, consisting of two samples, while data of *Tarsius dentatus* and *Carlito syrichta* were obtained from *Genebank* with access codes of KC977310.1 and L22784.1.

DNA Isolation

The process of isolation and purification of DNA derived from limited muscle tissue of tail cut sampling, used *innuPrepDNA Micro Kit*. The tools used for measuring the purity of DNA was *Gene Quant Pro*, a tool for measuring the concentration of DNA using ultraviolet absorbance *spectrophotometer* with a wavelength of 260 nm and 280 nm.

Primer Design

The primer used was *COX2* gene primer designed before [14]. The Primer of *COX2* gene amplification of *Tarsius tarsier* can be seen in **Table 1.**

Table 1. Primer of *COX2* Gene Amplification of *Tarsius tarsier*

Target	R/F	Order of Bases	Number of base	Melting Temperature
660 bp	F	5' ACCCCTGTGTATTTTCATGGC 3'	21	58.59° C
	R	5' ACTAGTTCTAGGACGATGGGCA 3'	21	57.59° C

Amplifications of DNA Fragments by PCR

The tool used was a PCR Biometra T-Personal machine. The components and optimization of PCR conditions can be seen in **Table 2**.

Table 2. PCR Components

No	PCR Component	Consentration	Volume (µl)
1	Template DNA	-	4.0 – 5.0
2	ddH_2O	-	10 – 14
3	Buffer	5×	2.5
4	$MgCl_2$	25 mM	3 – 4
5	dNTP mix	1 mM	0.5
6	Forward primer	15 – 30 pmol μL^{-1}	0.5
7	Reverse primer	15 – 30 pmol μL^{-1}	0.5
8	Taq DNA polimerase	4 – 6 U μL^{-1}	0.3

DNA Sequencing

COX2 gene amplicons were then sent to the First BASE company, Laboratories Sdn, Bhd. Selangor, Malaysia for sequencing. The tool used was ABI PRISM 3730 × l Genetic Analyzer Biosystem USA.

The Analysis of the Sequencing Results

The results of *COX2* gene sequencing were then aligned with automatic alignment Clustal W [15], MEGA 7.0 *software* [16] and DNAsp 5:10 *software*. In this alignment process, *C. syrichta* was assumed as the ancestor of all Sulawesian tarsier.

RESULTS

The results of PCR visualization on agrose gel 1.5% can be seen in **Figure 1**. The number of base pairs (pb) of the results of COX2 gene amplification of Tarsius tarsier is 560 pb.

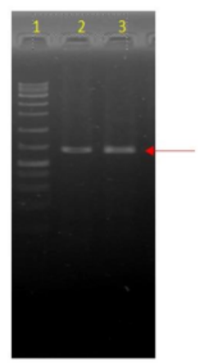

Figure 1. The results of *COX2* gene amplification by PCR: (1) DNA marker of 1 kb; (2) *Tarsius tarsier* from Buton, sample 1; (3) *T. tarsier* from Buton, sample 2

From partial *COX2* gene as long as 660 nucleotides (nt) after sequencing it has been produced 560 nt which can be analyzed. Those nucleotides are located in position 59 to position 619 of the 5 'end of the whole *COX2* gene **(Figure 2).**

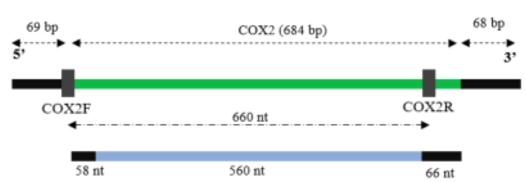

Figure 2. Primer attachment site and the sequence of the target gene amplified.

The partial sequence allignment of *COX2* gene of *Tarsius tarsier* form Buton resulted from amplification *forward* (COX2F) and *reverse* (COX2R) primers in both samples can be seen in **Table 3. Table 3** illustrates that the sequences amplified by the *forward* and *reverse* primers are derived from the two DNA strands acted as a template and complementary to each other. Thus, it can be ascertained that the amplified sequence is the *COX2* target gene with a length of 560 nt.

Table 3. Alignment of *COX2* Gene Sequences of *Tarsius tarsier*

Sample name	Sequence Length	Sequence	*Pairways Identify (%)*	*Identical Sites (%)*
*T. tarsier*_Buton1	560	2	100	100
*T. tarsier*_Buton2	560	2	100	100

The results of the multiple alignment analysis between the sample sequences that were successfully amplified and those data of the two species compared that were taken from *Genebank,* (*Tarsius dentatus* with an access code: KC977310.1 and *Carlito syrichta* with an access code: AB371090.1) show that the mutations occured in all the *COX2* gene sequences with the length of 560 bp are 130 points (**Table 4**). In this connection the rate of ts/tv (R) = 5,742; the ratio of the transition/transversion rate is k1 = 6.714 (purine) and k2 = 15.322 (pyrimidine). The ratio of transition/transversion (R) is = 5.742, where R = [A × G × k1 + T × C × k2] / (A + G) × (T + C)].

Table 4. The Analysis of *COX2* Gene partial Sequence

Parameter	Value
Frequency of invariable sites	77.14%
Frequency of variable sites	22.86%
Nucleotide diversity (Pi)	0.12440
Number of haplotypes	3
Total mutation	130
Polymorphic sites	128
Rate of ts / tv (k)	Purine = 6.714 and Pyrimidines = 15.322
Rate of ts / tv (R)	5,742
Discrete Gamma Distribution (Tamura-Nei Model)	3.74
Average evolutionary rate	0.40; 0.68; 0.91; 1.20; and 1.80 per situs substitution

The pattern and rate of subtitutions was analyzed using Tamura-Nei model (**Table 5**). The transition subtitution rate is shown by bold numbers and the transversion substitution is shown by italic number. The transition rates of purine base are A → G = 6.62, and G → A = 16.17; while those pyrimidine base are: T → C = 30.36, and C → T = 31.94. The transversion rates are: A → T = 2.08, A → C = 1.98, T → A = 2.41, T → G = 0.99, C → A = 2.41, C → G = 0.99, G → T = 2.08, and G → C = 1.98. Based on the data presented in the **Table 5**, it is also seen that transition mutation rate of T → C and C → T is far more higher compared to that of transition mutation of A → G and G → A. The transition mutation rate comparison clearly exhibits that transition mutation rate of T → C and C → is 2.73 times higher compared to that of A → G and G → A.

There is a difference related to the number of base types in the gene sequences analyzed. The comparison of the base type number of A, T, G, and C in the *COX2* gene partial sequences can be seen in **Table 6**. The proportion data of the base number found at the sequence of each sample showes that the highest proportion is related to base A as much as 33.4%, while the lowest proportion is related to base G as much as 12.1%. The highest and lowest proportion of base is found at the *COX2* gene partial sequence of *Carlito syrichta*. The average proportion of the overall bases analyzed (from the highest to the lowest frequency) is A = 32.4%, T = 27.6%, C = 26.7%, G = 13.0%.

Table 5. Patterns and Rates of partial Sequence Base Substitution of *COX2* Gene of Tarsius Species

	A	**T**	**C**	**G**
A	-	*2.08*	*1.98*	**6.62**
T	*2.41*	-	**30.36**	*0.99*
C	*2.41*	**31.94**	-	*0.99*
G	**16.17**	*2.08*	*1.98*	-

Table 6. Total Base Frequency (%) of *COX2* Gene partial Sequences of the all Tarsius Species

Species	Base Frequency (%)				
	T	**C**	**A**	**G**	**Total**
Tt _b1	28,2	26,3	31,8	13,8	560
Tt _b2	28,2	26,3	31,8	13,8	560
Cs	27,1	27,3	33,4	12,1	560
Td	28,2	26,4	32,1	13,2	560
Average	27,6	26,7	32,4	13,0	560

Note: Tt_b1 and Tt_b2 = *Tarsius tarsier* form Buton, Cs = *Carlito syrichta*, Td = *Tarsius dentatus*

COX2 gene as already known is a protein encoding gene. The frequency of base at each position in the codon encoded by the *COX2*, gene can be seen in **Table 7.** The base having highest frequency found at the first position of the codon is A, while the base having lowest frequency is T. The base having highest frequency at the second position is T, while the base having lowest frequency is G. At the third position, the base having highest frequency is A, and the lowest one is G. The average proportion of bases in the first position at codon is T = 19.7%, C = 25.5%, A = 30.5%, and G = 23.9%, the average proportion of bases in the second position is T = 36.5%, C = 24%, A = 26%, and G = 13%, and the average proportion of the base in the third position in the codon is T = 27%, C = 30%, A = 40.3% , G = 2.7%.

DISCUSSION

Based on the allignment of *COX2* gene sequences with a length of 560 nt, both related to the research samples (*Tarsius tarsier* form Buton and those taken from *Genebank, (Cmarlito syrichta* and *Tarsius dentatus),* there was a base difference in several sites indicating that there was a genetic diversity in gene sequences analyzed. On the other hand there is no difference between the two research samples, related to the COX_2 gene sequences alligned (100% identical). Furthermore related to the whole data analyzed, there are 128 different sites (variable). The results of the analysis also showed that the invariable sites (unchanged) were as much as 77.14% and variable sites (changed) were as much as 22, 86%.

The average proportion of the whole base analyzed from the highest to the lowest is base A = 32.4%, T = 27.6%, C = 26.7%, G = 13.0%. Base G is the lowest one among the other bases. This is

Table 7. The Base Frequency at each Position of the *COX2* Gene Sequence Codon

Species	Base Frequency (%)											
	First position				Second position				Third position			
	T	C	A	G	T	C	A	G	T	C	A	G
Tt _b1	21	24.1	30.5	24,1	36	24,6	25,7	13,4	27	30,1	39,2	3,8
Tt _b2	21	24.1	30.5	24,1	36	24,6	25,7	13,4	27	30,1	39,2	3,8
Cs	19	26.7	30.5	23,5	37	23,5	26,7	12,3	25	31,7	43	0,5
Td	18	27.3	30.5	24,1	37	23,5	26,2	12,8	29	28,5	39,8	2,7
Average	19.7	25.5	30.5	23,9	36,5	24,0	26,0	13	27	30	40,3	2,7

Note: Tt_b1 and Tt_b2 = *Tarsius tarsier* form Buton, Cs = *Carlito syrichta*, Td = *Tarsius dentatus*

in line with the research report before stating that characteristics of the protein-coding genes is to have a base composition of low G and high C [17]. This statement is supported by the research of Adkins and Honeycutt (1994) who found that the base frequency of G was low, particularly on the 2nd and 3rd base position of the codon in mammals.

The nucleotide diversity uncovered is due to the substitution mutations that occured in several sites. There are 130 substitution mutation in the *COX2* gene sequence with the length of 560 bp found in 128 polymorphic sites, with the number of transition mutations 89 and transversion mutations 16. The calculation of the substitution type is obtained by ignoring the double single-substitution at the same site. The substitution mutation causes the difference in the proportion of bases along the *COX2* gene sequences of Tarsius.

Transition substitution occurs when there is a change of purine base with another purine base (A↔G) or substitution of a pyrimidine base with another pyrimidine base (T↔C); or it may be referred to as a purine-pyrimidine base subtitution with other purine-pyrimidine. Transversion substitution occurs when there is a change of purine base by a pyrimidine base or vice versa (A↔T, A↔C, G↔T, and G↔C).

The greater frequency of transition substitution compared to that of transversion substitution (89/16) is in line the research report before [13], where the frequency of transition substitution is bigger than that of transversion substitution with the ratio of 10/1. In this connection there are several reports stating that based on the data related to the mutations occurred at all DNA sequences of each genome uncovered, the frequencies of transition mutation are higher compared to those of transversion mutation [18,19,20,21,23,]. Similiar phenomenon related to the research on cyt. b gene of North Sulawesian tarsiers has been reported [23]. Another research report saying that the modest transition bias found in mammalian nuclear DNA was up to 15 times higher compared to that of found in human mitochondrial DNA [24]. It was reported too that the phenomenon of transition bias was apparently found in broad scale, and the ts/tv ratio was always 1 or higher than 1; the ts/tv ratio found in amphibian group is as much as 2.4, but that of found in bird group was even up to 7.8 [25]. There is also report stating that ti/tv bias, as a general property of DNA sequence evolution, is found more frequently at the animal mtDNA compared to at that of nuclear DNA as well as chloroplast DNA [22]. It is said that this phenomenon is related to the deamination reaction frequently occurred from adenine to hypoxanthine (A → H, which leads to an A → G substitution), and from cytosine to thymine (C → T) [26].

The transition mutation rate of T → C and C → T which is 2.73 times higher than that of A → G and G → A is caused by the effect of CpGs site. It has been reported that the CpGs sites undergo mutation in a different way compared to other sites [27]. In this connection it has been uncovered that in mammals, most of the CpGs sites are hypermutable [28]. Furthermore it is also said that most of CpGs cytosine undergo methylation [29,30] and this condition will facilitates the deamination process of cytosine affecting the process of cytosine mutation.It has been even said that methyl-CpGs underwent mutation in the rate of 10 – 50 times higher than the cytosine mutation occurred at other sites [31].

Most substitution mutations in *COX2* gene sequences occur at the third base of each codon; those mutations according to Wobble rules usually do not alter yet the pair of any codon with its anticodon, so the amino acid related is not altered too. This third base of codon can form hydrogen bonds not only with its normal complementary base at the first position of anticodon, but also with different bases at that position [32]. Related to the Wobble rules the substitutions at first and second base of the codon is more at risk of altering the products of translation in the form of amino acid sequence. *Wobble* rule only applies to the third base of codon.

The substitutions occurring in the first and second base of codons are included as *nonsilent mutations,* where the substitution effect will be translated into amino acids. It has been stated too that all substitutions on the first base and the second base of each codon are included as *nonsilent* mutations (except the transition on the first base of the four leucine codons) [13].

CONCLUSIONS

The results of the alignment show that there are some base difference in several sites which indicate that there is a genetic diversity in the gene sequences analyzed, but especially related to the two research sample sequences of *Tarsius tarsier* form Buton, there is not any difference at all sites (100% identical). The number of invariable sites is 432, and that of variable sites is 128. The number of total mutation detected is 130. Substitution mutations are found in 128 sites where the transition mutations and transversion mutations are 85.09% and 14,92% respectively; and the rate ts/tv is 5.742. The transition mutation rate of pyrimidines (T → C and C → T) is 2.73 times higher than that of purines (A → G and G → A). Most of the subtitution mutations in the *COX2* gene sequences occured at the third base of each codon which is mostly included as *silent* mutations.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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