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The Phylogeny of *Tarsius tarsier* form Buton, Indonesia Based on MT-CO2 Gene

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ABSTRACT

MT-CO2 gene from two individuals of Tarsius tarsier form Buton was studied in this research. Tail cut sampling was used to obtain tissue samples of both individuals. The extract of the total DNA from tissue samples was done by using innuPREP DNA micro kit and MT-CO2 gene derived from the results of the extract was amplified with a pair of MT-CO2 primer, designed by Widayanti (2010). The sequencing stage was done at First BASE, Laboratory Sdn. Bhd., Selangor, Malaysia. The data obtained from the sequencing results and the data of comparison species obtained from Genbank were analyzed using MEGA 6.0 software. The phylogeny analysis used Maximum likelihood methods supported by MEGA 6.0 software. Based on the results of sequence similarity, genetic distance, and topology of phylogeny tree, it can be concluded that Tarsius species from the same area have a closer genetic relationship. Related to the position of T. tarsier form Buton, these species have a closer relationship with several Tarsius species of central Sulawesi than the Tarsius species of outside Sulawesi.

INTRODUCTION

Tarsius is one of the unique and endemic animals which are still alive today (Lumente et al., 2015). Its body length is only about 12 cm and its body weight is about 100 grams, making Tarsius as the world's smallest primate group (Merker et al., 2007; Lumente et al., 2015). These animals also have a nickname "living fossil" because of the morphological characteristics which are similar to the characteristics of primates which lived in the Eosen age (Wright et al., 2003). In the past, these small primates are widely spread, but nowadays Tarsius are only found in a few islands of Southeast Asia with a very small population (Wright et al., 2003; Merker, 2008).

One of Tarsius species which is known by scientists and still survive to this day is the *Tarsius tarsier (T. spectrum).* Similar to the Tarsius generally, *Tarsius tarsier* is nocturnal carnivorous primate, having big round eyes, small body size, and able to jump and flip 180^0 (Wright, 2003; Wirdateti & Dahrudin, 2006; Merker et al., 2007; Merker & Yustian 2008; Merker et al, 2008; Manori et al., 2014; Lumente, 2015). The distribution of the Tarsius is spread from North Sulawesi to South Sulawesi as well as some smaller surrounding islands (Gursky 2002; Wright et al., 2003; Merker et al, 2008; Wirdateti & Dahrudin, 2006; Shekelle et al., 2010; Lumente et al., 2015). Indonesian people recognize *Tarsius tarsier* in the name of tangkas (Manori, 2014; Lumente, 2015).

Tarsius tarsier which is spread in Sulawesi can be found in a variety of fields. Wirdateti and Dahrudin (2006) reported that *Tarsius tarsier* can be found either in the forest, plantation, bushes, or people's residence. Although it can be found in many lands, deforestation and land alteration are considered to be the main causes of the decline of the *Tarsius tarsier* population (Shekelle & Salim, 2008). This can be attributed to the fact that the nests of *T. tarsier* are in the form of holes in trees (Wirdateti & Dahrudin, 2006). In addition, based on the unique characteristics of this animal this primate is wanted by the people for the purpose of sale activity (Wirdateti & Dahrudin, 2006; Manori et al., 2014). Both of these conditions cause *T. tarsier* susceptible to extinction (Shekelle & Salim, 2008).

One group of Tarsius which are still alive today is the *Tarsius tarsier* form Buton. This species lives in Buton Island, satellite island on the southeast of Sulawesi. The existence of Tarsius in Buton has been reported in several reports, e.g. the reports by Shekelle & Leksono (2004), Gursky et al. (2008), Shekelle & Salim (2008), Nijman & Nekaris (2010), and

Shekelle et al. (2010). Nevertheless, the existence of Tarsius called as Tarsius form Buton, needs to be taken care of due to the large-scale deforestation occurring in this island region (Gursky et al., 2008).

Until now, a phylogeny research involving *T. tarsier* form Buton has never been done, based on reports collected until 2015 (Widayanti et al., 2004; Merker et al., 2009; Md-Zain et al., 2010; Shekelle et al., 2010; Kamagi et al., 2014; Driller et al., 2015). The unavailability of the molecular data of Tarsius from Buton Island in *Genbank* also reinforces the notion that the phylogeny of *T. tarsier* form Buton has never been investigated in previous studies. In this regard, a phylogeny research on *T. tarsier* form Buton with several other species of Tarsius needs to be done to provide information about the relationship between this species with the other species.

One group of genes that are often used as molecular markers in a variety of molecular phylogeny studies is the gene group in the mitochondria. mtDNA has a number of specific characteristics that make it very suitable markers for analyzing molecular biodiversity. Galtier et al. (2009) mentioned some specific features, such as mtDNA is maternally inherited, evolution is more neutral (when compared with the genes of the nucleus), and the divergence level of mtDNA can describe the evolution hour of living things. In relation to Tarsius position as primates, mtDNA has also been extensively used for phylogenic analysis of various groups of primates (Md-Zain et al., 2010).

MT-CO2 gene *(Mitochondrially encoded cytochrome oxidase II)* is one of the mtDNA genes that can be used as molecular markers of various phylogeny molecular researches. MT-CO2 gene is one of three mitochondrial genes, which have been widely used in the phylogeny analysis (Russo et al., 1996). This gene is also not included as mitochondrial genes which are not bad in vertebrate phylogeny tree reconstruction (Zardoya & Meyer, 1996). The utilization of MT-CO2 as a phylogeny marker in primates has also been commonly used, such as in the research by Menezes, (2010) and Ruiz-Garcı'a et al. (2014). In addition, molecular studies involving MT-CO2 gene on Tarsius have also been conducted, such as the one conducted by Widayanti (2009). Therefore, this phylogeny research on *T. tarsier* Buton form based on MT-CO2 gene was conducted.

In this research, phylogeny analysis was done to reveal the relationship between the *T. tarsier* form Button and the Tarsius from the mainland region of Sulawesi and those from the other

islands or regions. Three species from central Sulawesi were selected in this research, namely *T. dentatus*, *T. wallacei*, and *T. lariang*, while the species from other islands or regions were *T. bancanus (Cephalopachus bancanus)* whose natural habitats were spread in Sumatra and Kalimantan, as well as *T. syrichta (Carlito syrichta)* found in several islands of the Philippines.

MATERIALS AND METHODS

Sample Collection and Treatment

This exploratory research was conducted by reconstructing the phylogeny tree based on the MT-CO2 gene sequence of *T. tarsier* form Buton along with that of *T. wallacei, T. lariang, T. dentatus, T.bancanus,* and *T. syrichta. Tail cut sampling* was the selected procedure in the sampling of the tissues of *T. tarsier* form Buton. The tissues at the end of the Tarsius tail was cut to a parallel position with the tail of about 1-2 cm with a width of 3-5 mm (Kamagi, 2014). After that, the incision wound was treated and the Tarsius was released back. The tissue samples were then stored at a cold temperature and soaked in a solution of 20% formalin, in order to be processed further. The data of MT-CO2 gene sequence of *T. tarsier* form Buton were obtained after the isolation stage until the DNA sequencing. The data of MT CO2 gene sequence of *T. wallacei, T. lariang, T. dentatus, T. bancanus,* and *T. syrichta* were obtained from NCBI *Genbank.* Table 1 presents the access numbers of each species in Genbank.

Table 1. Tarsius Species and Accession Numbers were taken from GenBank

Species	Accession Number
T. dentatus	KC977310.1
T. wallacei	KC977311.1
T. lariang	KC977309.1
T. bancanus	AF348159,1
T. bancanus	L22783.1
T. syrichta	L22784.1
T. syrichta	AB371090.1

Extraction and Amplification of MT-CO2 Gene, Sequence Alignment, and Data Analysis

The stage of DNA extraction and purification, DNA amplification and DNA amplicon test was conducted in November 2016 at the Molecular Biology Laboratory of the University of Brawijaya, Malang. Total DNA was extracted using InnuPRER DNA micro Kit. The primers used to amplify the MT-CO2 gene were a pair of primers that had been designed based on the sequence of the Tarsius mitochondrial genome by Widayanti (2010). The forward primer base sequence was 5 'ACCCCTGTGTATTTTCATGGC 3', while that of the reverse primer was 5 'ACTAGTTCTAGGACGATGGGCA 3'. The components and optimization of the required PCR conditions are shown in Table 2 and 3.

Table 2. PCR Component

PCR Component	Concentration	Volume (µL)
DNA template	-	4.0 - 5.0
ddH2O	-	10 – 14
Buffer	5 X	2.5
$MgCl_2$	25mM	3 – 4
Mixture of dNTP	1 mM	0.5
Forward primer	15 – 30 pmol·µL^{-1}	0.5
Reverse primer	15 – 30 pmol·µL^{-1}	0.5
Tag DNA polymerase	·4 – 6 µL^{-1}	0.3

Table 3. PCR Condition

Number of Cycle	Duration (min.)	Temperature (^{o}C)	Phase
35	3	94	initial denaturation
	1	94	denaturation
	1	55	annealing
	1	72	elongation
	10	72	post-elongation

The sequencing stage was done at First BASE, Laboratory Sdn. Bhd., Selangor, Malaysia. Data obtained from the sequencing results and Genbank were then analyzed using software MEGA 6.0. The phylogeny analysis was done by using MEGA 6.0 with Maximum likelihood method.

RESULTS AND DATA ANALYSIS

The Results of MT-CO2 Gene Amplification

The DNA from two samples of *T. tarsier* form Buton was isolated and amplified. The results of electrophoresis on agarose gel 1.5% show that the amplified MT-CO2 gene is about 600 bp (Figure 1).

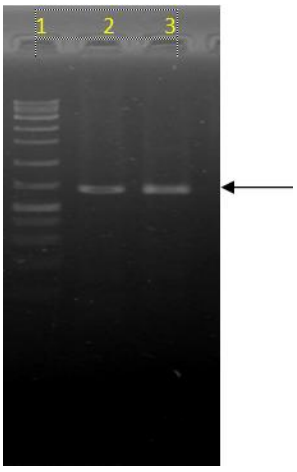

Figure 1. MT-CO2 gene amplification result: (1) DNA of 1 kb marker; (2) sample 1 of *T. tarsier* form Buton; and (3) sample 2 of *T. tarsier* form Buton

Characteristic of Sequences

Before the phylogeny analysis was done, the sequence of MT-CO2 gene of *T. tarsier* Buton was aligned with the sequence of MT-CO2 gene of the other Tarsius species compared. The alignment was done supported by automatic alignment with Clustal-X available in the MEGA 6 software. From the nine DNA sequences, which were aligned, it was known that the amount of the constant base was as much as 72.50%, parsimony 26.25%, and singleton 1.25%.

Table 4. Nitrogen Base Percentage Similarity of MT CO2 Gene from Nine Tarsius Individuals compared

	1	2	3	4	5	6	7	8	9
1									
2	100,00								
3	87,86	87,68							
4	87,86	87,68	96,43						
5	88,75	88,57	96,96	97,32					
6	82,68	82,50	83,57	83,21	82,50				
7	83,04	82,86	84,29	83,39	82,86	99,29			
8	85,18	85,18	85,71	85,18	86,25	85,18	85,18		
9	85,36	85,18	85,71	85,18	86,25	85,18	85,18	100,00	

Notes 1 = *T. tarsier* form Buton 1; 2 = *T. tarsier* form Buton 2; 3 = *T. wallacei;* 4 = *T. lariang;* 5 = *T. dentatus;* 6 = *T. syrichta* 1; 7 = *T. syrichta* 2; 8 = *T. bancanus* 1; 9 = *T. bancanus* 2

The general picture that can be seen from the alignment is that the closer the origin region of Tarsius individuals, the more identical the MT-CO2 gene sequences of individuals are. Further explanation about the results is described in the discussion. Based on the results of the alignment that had been done, the similarity percentage of nitrogenous bases of the nine species analyzed is also calculated. Table 4 presents the data from these calculations.

The average base composition of MT-CO2 gene of the nine Tarsius individual samples compared is presented in Table 5.

Table 5. The Average Base Composition of MT-CO2 Gene of nine Tarsius Individuals

Nucleotide Position	Base	Nitrogen Base	Frequency (%)
Position 1		T	20
		C	26,1
		A	30,3
		G	23,8
Position 2		T	37
		C	23,9
		A	26,2
		G	12,7
Position 3		T	28
		C	29,2
		A	40,4
		G	2,6
Total		T	28,3
		C	26,4
		A	32,3
		G	13,0

Table 5 shows that the composition of the nitrogenous bases of each base position is different. At the first base position and third base position, adenine has the highest frequency, while at the second base position, tyrosine has the highest frequency. Overall (at the three base positions), adenine has the highest frequency, while guanine has the lowest one.

In addition to analyzing the base composition of MT-CO2 gene sequence, the variability percentage of nitrogenous bases at each base position of codon triplet among the nine gene sequences compared are also simultaneously calculated. Table 6 presents the results of these calculations.

Table 6. The Variability Percentage of Nitrogen Base of MT-CO2 Gene at each Base Position of Triplet Codons of nine Tarsius Individuals Compared

The Position of Base	The Comparison of the Number of Bases varied with the Total Base Number	The Percentage of Nitrogen Base Variability
Position 1	27/187	14,44%
Position 2	7/187	3,74%
Position 3	120/187	64,52%
Total	154/560	27,50%

Table 6 shows that the base at the third position of the codon triplet has the highest variability percentage compared with the other two base positions, while the base at the second position has the smallest percentage. In relation to the variability percentage, the third base position has the variability percentage four times bigger than that of the first base position and seventeen times bigger than that of the second base position.

Genetic Distance

The results of the of genetic distance analysis of each individual based on MT-CO2 gene are presented in Table 7. Based on the genetic distance analysis result presented in Table 7, the general picture that can be drawn is that the individuals originating from the same region or near regions have a relatively closer genetic distance than those originating from two distant areas.

Table 7. Genetic Distance of each Tarsius Individual based on MT-CO2 Gene

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1									
2	0,0								
3	68,0	68,0							
4	68,0	68,0	20,0						
5	63,0	63,0	17,0	15,0					
6	97,0	97,0	92,0	94,0	98,0				
7	95,0	95,0	88,0	93,0	96,0	4,0			
8	82,0	82,0	80,0	83,0	77,0	83,0	83,0		
9	82,0	82,0	80,0	83,0	77,0	83,0	83,0	0,0	

Note 1 = *T. tarsier* form Buton 1; 2 = *T. tarsier* form Buton 2; 3 = *T. wallacei;* 4 = *T. lariang;* 5 = *T. dentatus;* 6 = *T. syrichta* 1; 7 = *T. syrichta* 2; 8 = *T. bancanus* 1; 9 = *T. bancanus* 2

Phylogeny Trees

The results of the phylogeny tree reconstruction using *Maximum Likelihood* (ML) and *Neighbor Joining* (NJ) with *bootstrap* values 1000 are presented in Figure 2 and 3 respectively. Based on the two figures, it can be concluded that the topology of both

phylogeny trees is similar. The individuals of Tarsius originating from the same region are clustered in the same group.

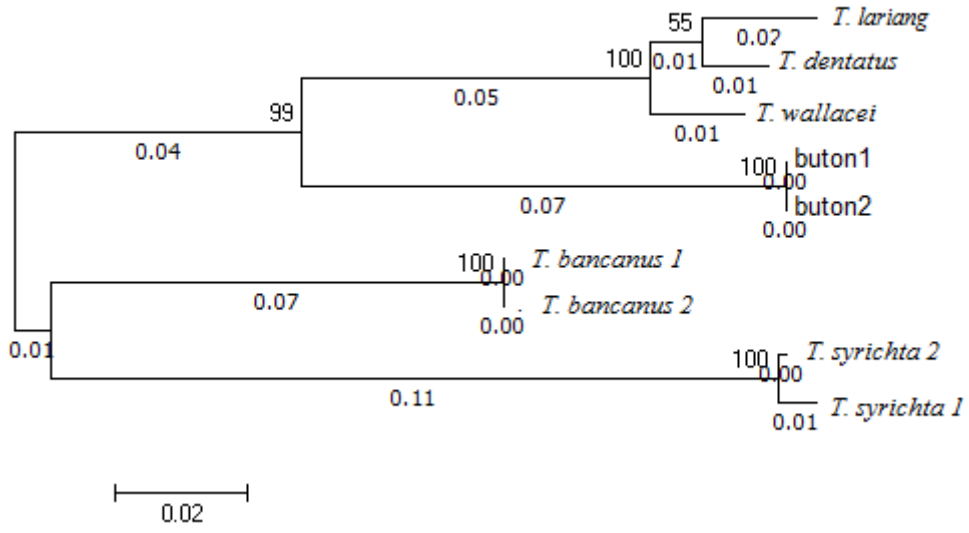

Figure 2. Phylogeny tree topology of *T. tarsier* form Buton with other Tarsius (*T. dentatus, T. wallacei, T. lariang, T. bancanus* and *T. syrichta*) based on MT-CO2 gene sequence using ML method

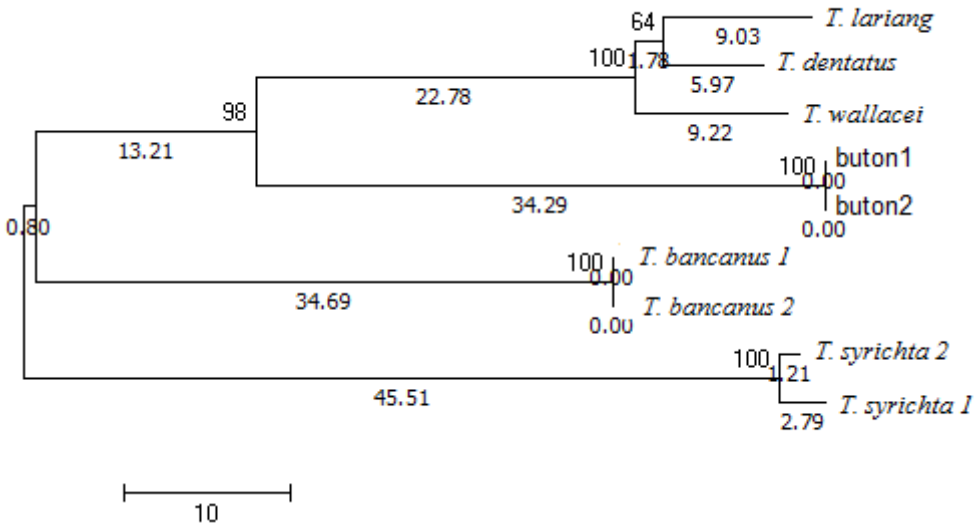

Figure 3. Phylogeny tree topology of *T. tarsier* form Buton with other Tarsius (*T. dentatus, T. wallacei, T. lariang, T. bancanus* and *T. syrichta*) based on MT-CO2 gene sequence using NJ method

DISCUSSION

In this research, MT-CO2 gene has been selected as the molecular marker in the phylogeny analysis between *T. tarsier* form Buton with *T. dentatus, T. wallacei, T. lariang, T. bancanus* and *T. syrichta.* The reason for using MT-CO2 as the molecular marker in phylogeny research was due to the fact that MT-CO2 gene had been proven as a good marker in the study of primate phylogeny (Ruvolo et al., 1991; Adkins & Honeycutt, 1994). Furthermore, the utilization of MT-CO2 gene as the molecular marker, especially in the phylogeny study of primates, is in line with several previous studies. Chatterjee et al. (2009) utilized this gene as a molecular marker to assess the phylogeny involving various primate genus. Some other researchers, such as Menezes et al. (2012) and Ruiz-Garcia et al. (2014) also used this gene in the phylogeny analysis of primates in their research.

Based on the similarity analysis of MT-CO2 gene sequence, both samples of *T. tarsier* form Buton have identical DNA sequences with each other. Similarly, in both species of *T. bancanus (C. bancanus),* they also have identical sequences. On the other hand, related to the *T. syrichta (C. syrichta),* the sequences that have been aligned have nearly identical sequences (In both sequences, base variations only occurred at four positions, namely at the positions of 79, 171, 333, and 450).

Referring to the similarity percentage of DNA sequences in Table 4, the information that can be revealed is that the Tarsius originating from the same area have a relatively close relationship than the Tarsius originating from other areas. The general picture obtained from the similarity analysis of MT-CO2 gene sequence is also strengthened by the results of genetic distance calculations. The genetic distance of two Tarsius from the adjacent area has a value of ≤ 20, while the genetic distance of two Tarsius from distant regions has a value of ≥ 60. The low value of the genetic distance indicates a close relationship between the two Tarsius (Dewoody et al., 2010; Gil & Brumm, 2014; Read, 2017). Thus, it is seen that the geographical factors have effects on the relationship among the Tarsius groups examined in this research.

The findings presented based on the similarity percentage and genetic distance values can also be supported by the phylogeny tree topology constructed. Based on the reconstruction of the phylogeny tree with *Maximum Likelihood* method with *bootstrap* values of 1000, *T. dentatus, T. lariang and T. wallacei* clustered in one *clade.* All three species are the Tarsius

groups that can be found in the region of central Sulawesi (Merker & Groves, 2006; Gursky et al., 2008; Merker et al., 2010). The two samples of *T. tarsier* form Buton taken from the island of Buton are also within one *clade.* Then, the two Tarsius samples of *T. bancanus or C. bancanus* (outside Sulawesi) are also within one *clade.* Similarly, the two samples of *T. syrichta or C. syrichta* are also within one *clade.*

In relation to the position of *T. tarsier* form Buton, the Tarsius found on the island of Buton become *sister group* of the *clade* of the Tarsius of central Sulawesi. On the other hand, *T. bancanus (C. bancanus)* and *T. syrichta (C. syrichta)* are grouped in different clusters with *T. tarsier* form Buton. It shows that *T. tarsier* form Buton have a closer relationship with the Tarsius from central Sulawesi, rather than with the Tarsius species from outside of *Sulawesi. T. bancanus* is a Tarsius species found in Borneo and Sumatra, while *T. syrichta* is found in Philippine (Wright et al., 2003; Yustian, 2007). Both areas are located in the areas which are farther than Buton Island, compared with the area of central Sulawesi.

Furthermore, similarity analysis, genetic distance calculation, as well as the reconstruction of the phylogeny tree also show that among the three Sulawesian Tarsius species compared, *T. wallacei* is a species that has the closest relationship with *T. tarsier* form Buton. This is maybe interesting to be studied more in depth because based on the distribution of habitat and location in which it is found; *T. wallacei* is the Tarsius whose habitat is the farthest from the island of Buton compared with the other two Tarsius species compared from other central Sulawesi regions (Merker & Groves, 2005; Merker et al., 2010).

Related to the topology of phylogeny tree formed based on *Neighbour Joining* method (NJ), the positions of Tarsius represented on the phylogeny tree are similar to the reconstruction of phylogeny tree on ML methods. In addition, both phylogeny reconstructions on the basis of molecular markers in this research are also in line with the Tarsius taxonomy revised by Groves & Shekelle (2010) based on various aspects. Furthermore, in both the phylogeny trees, Tarsius clumped in accordance with the distribution of geographical areas where Tarsius are found. These findings indicate that biogeography has a strong role in the evolution process of Tarsius.

The analysis of Tarsius biogeography has not been undertaken yet, but some phylogeny analyses on other organisms support the indication of a correlation between phylogeny and biogeography of Tarsius in this research. Among several studies before, those conducted by

Amin (2003), Amin et al. (2015) and Korhonen et al. (2016) have examined the phylogeny and connected it with biogeography study of Peking duck, *Bubalus bubalis, and Trichinella* complex respectively. Biogeographic patterns that appear in this Tarsius phylogeny study and in some previous studies mentioned show that the geographic isolation between one population and the other population due to the existence of ecological barriers gradually accumulate diversity of each species detected on their DNA sequences. The populations, which initially can interact with each other, are finally separated by the emergence of the ecological barriers due to the earth changes, so that speciation starts to occur. The speciation mentioned in the explanation is allopatric speciation (Salomon, 2001).

Another phenomenon that can be informed based on the findings of this research is that the grouping of the Tarsius species regarding the phylogeny analysis in this research is consistent with previous research utilizing *cyt b* gene as the molecular marker (Kamagi et al., 2014). The similarities of the phylogeny analysis between MT-CO2 and *cyt b* gene are in line with the explanation by Switzer et al. (2005). They explained that the phylogeny analysis based on the MT-CO2 gene sequence has been proven to be able to produce a phylogeny tree which was similar to the analysis results based on the other genes. Right now, the phylogeny analysis to determine the position of *T. tarsier* form Buton based on *cyt b* gene is also going to be conducted by another team.

CONCLUSION

Based on the results of sequence similarity, genetic distance, and topology of phylogeny tree, it can be concluded that Tarsius species from the same area have a closer genetic relationship. Related to the position of *T. tarsier* form Buton, these species have a closer relationship with several Tarsius species of central Sulawesi than those of outside Sulawesi.

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