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Phylogenetic Position of North Sulawesi *Tarsius sp*. Based on Partial Cytochrome b Gene Sequences

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Abstract

Cyt b gene of North Sulawesi *Tarsius sp*., *T. tumpara*, *T. sangirensis* and *T. tarsier* (*T. spectrum*) had been partially sequenced. The homologous sequence of three groups had been compared to describe the phylogenetic position among them, as well as several other species accessed from the Genbank. Total DNA extracted from the muscular tissue had been obtained through tail cut sampling using the innuPREP DNA micro kit, and amplified using a pair of universal primer, L14841 and H15149. The size of the cyt b gene sequence amplified was 307 bp long. Sequence aligned using CLUSTAL-X program and diversity analysis were done using version 5.2.2. MEGA5 program. Genetic distance had been calculated by Tamura 3 parameter method and phylogenetic tree had been built using Neighbor-Joining and Maximum Likelihood methods. Genetic distance based on cyt b gene nucleotide was found from 0 to 0.240 with an average of 0.080. The phylogenetic tree constructed by Neighbor Joining and Maximum Likelihood methods indicated that *T. tarsier*, *T. sangirensis* and *T. tumpara* were closely related with *Tarsius tarsier*-complex, and distantly related with *Cephalopachus bancanus* and *Carlito syrichta*. The genetic distance and the phylogenetic tree had been constructed on the base of partial cyt b gene sequence of *T. tarsier*, *T. sangirensis*, *T. tumpara* and 5 other tarsier species and their accession. Those results are consistent with taxonomy based on morphology and vocal acoustic form.

Keywords

Molecular Phylogeny, *Tarsius sp*., Sulawesi, Cyt b Gene, Partial Sequence

1. Introduction

The main island of Sulawesi and its surrounding islands located at Wallacea zone, possess abundant biodiversity.

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In the past, Sulawesi Island did not join with any other land [1]. Isolated condition in long period of time seemed to push evolution of many species so that Sulawesi Island had a high endemism level [2]. Therefore, the main priority in the management of natural resources in Sulawesi is conserving a resource of genetic diversity, because species conserved in the Sulawesi are not found in other places [3]. Sulawesi has become "top hotspots" for biodiversity conservation [4]. Sulawesi has about 529 endemic vertebrates or approximately 1.9% of endemic vertebrates in the world [2], including 7 species of endemic monkeys [5] and 7 - 9 Tarsius species of *Tarsius tarsier*-complex [6]. Recently, they are threatened to distinct due to natural predators, illegal hunting, and habitat destructions by human intervention.

Tarsius taxonomy up to now remains a problem continuously debated. Originally, tarsier was a monotypic genus of *Tarsiidae* family [7]. Based on morphological characteristics, tarsier consists of two different groups, western tarsier and eastern tarsier [8]; but based on genetic analysis and vocal acoustic form, tarsier is divided into three groups, western tarsier living in great Sunda Islands (Borneo and Sumatera), eastern tarsier living in Sulawesi and Phillippine tarsiers living in southern Phillippine [6]. Nowadays, the genera of *Tarsiidae* family had been revised to be three separate genera, *Tarsius*, *Cephalopachus*, and *Carlito*, each of which is allopatrically distributed in three different biogeographic regions, Sulawesi, great Sunda Islands (Borneo and Sumatera) and Mindanao [6].

Sulawesi tarsier grouped in one group called *Tarsius tarsier*-complex, comprises of 9 species: *T. sangirensis*, *T. tumpara*, *T. wallacei*, *T. lariang*, *T. pumilus*, *T. fuscus*, *T. tarsier* (*T. spectrum*), *T. pelengensis* and *T. dentatus* [6] [9]. *Tarsius tarsier* complex is a vague taxonomic group due to consisting of closely related species, so that it is less distinct to recognize the interspecific diversity based on the morphological variations. The taxonomic status of Sulawesi tarsier, especially of low land tarsier, has been long disputed due to interspecific similarity. The low land tarsier species has no significant difference in body size or body proportion [9].

Study on genetic diversity and population biology had been carried out using mitochondrial DNA [10] [11]. Variation patterns of mtDNA can be used as species distinction as well as species investigation of those endangered species [12]. The mitochondrial DNA is often used as genetic marker for closely related interspecific and intraspecific genetic diversity study, since it evolves faster than nucleic genes so that it could give more variations to reconstruct the evolutionary history [13]. Mitochondrial DNA genes had been widely applied to estimate the phylogenetic relationship of primate [14]-[16]. These genes had also been used to restudy the phylogenetic relationship among closely related taxa [12]. The mitochondrial cyt b gene is known as a gene evolving faster, so it can have more variation and can be used for phylogenetic and biogeographic study [16]-[18].

Whole mitochondrial genome studies had been conducted on *T. syrichta* [19], *T. bancanus* [20], *T. wallacei* [21], *T. lariang* [22] and *T. dentatus* [23], so that these results can be used as references to study several genetic markers of encoding or noncoding genes in the mitochondria. The cyt b gene contain discrete character groups (base position at codon) representing the mutation rate, so that it can be used as a phylogenetic marker [24].

Partial sequencing of the cytochrome b gene had been used to uncover the phylogenetic position and the genetic relationship among several tarsier species and among other primates [25] [26]. The cyt b gene is one of the protein coding gene of the mtDNA. Its product is cytochrome b (cyt b) apoenzym identified as the central catalytic subunit of Q cycle. The cyt b gene of tarsier has a length of 1140 bp, known as coding region of the protein located at 14169 to 15308 mtDNA sequence, flanked by the tRNA-Glu gene and the tRNA-Thr gene [20].

Several universal olygonucleotide primers had been developed for amplificating and sequencing cyt b gene of different animals [27]. Partial sequence of the cyt b gene had been applied to uncover the genetic diversity among *T. sangirensis*, *T. tumpara* and *T. tarsier* (*T. spectrum* or Manado tarsier) and several other *Tarsius sp*. obtained from the GenBank.

2. Material and Method

2.1. Sample Collection and Treatment

Sample specimen used was muscular tissue obtained by *tail cut sampling*. It was then stored at –20˚C, in alcohol of 95%. Because of difficulty of collecting samples, each species consisted only 2 sample specimens. The sampling site and the specimen treatment is shown in **Table 1**.

The data sequence of *C. bancanus*, *C. syrichta*, *T. wallacei*, *T. dentatus*, *T. dentatus* × *lariang* and *T. lariang* had been obtained from the GenBank. Tarsier species and their accession numbers taken from GenBank are shown in **Table 2**.

Table 1. Sampling and specimen treatment.

Species	Location	Specimen treatment
T. tumpara	Kapeta village Southwest Siau district Sitaro Regency	• Wet (alcohol) 95% • Wet (formaldehyde) 20%
T. sangirensis	Ahapatung village South Southeast Tabukan district, Sangihe Regency	• Wet (alcohol) 95% • Wet (formaldehyde) 20%
T. tarsier	Matungkas village Dimembe district North Minahasa Regency	• Wet (alkohol) 95% • Wet(formaldehyde) 20%

Table 2. Tarsier species and accession numbers were taken from GenBank.

Species	Accession Number	Species	Accession Number
T. wallacei	HM115977.1	*T. lariang*	FJ614363.1
T. wallacei	HM115978.1	*T. lariang*	FJ614358.1
T. wallacei	HM115979.1	*T. lariang*	FJ614357.1
T. wallacei	HM115980.1	*T. lariang*	FJ614354.1
T. wallacei	HM115982.1	*T. lariang*	FJ614353.1
T. wallacei	HM115983.1	*T. lariang*	FJ614352.1
T. wallacei	HM115984.1	*T. dentatus* × *lariang*	FJ614361.1
T. dentatus	FJ614366.1	*T. dentatus* × *lariang*	FJ614359.1
T. dentatus	FJ614367.1	*T. dentatus* × *lariang*	FJ614356.1
T. dentatus	FJ614368.1	*C. bancanus* (*T. bancanus*)	AF348159.1
T. dentatus	FJ614369.1	*C. bancanus* (*T. bancanus*)	AB011077.1
T. dentatus	FJ614370.1	*C. syrichta* (*T. syrichta*)	AB371090.1
T. dentatus	FJ614371.1	*C. syrichta* (*T. syrichta*)	NC012774.1

The primer used was universal primer L14841 and H15149 [27]. The specific location of the primers, L14841 (33 bp) and H15149 (34 bp) in the cyt b gene region was from the 63st nucleotide sequence downstream related to the forward primer and from the 435st nucleotide sequence upstream related to the reverse primer.

2.2. DNA Extraction and Cyt b Gene Amplification

Total DNA had been extracted using InnuPREP DNA micro Kit. Purity measurement of total DNA isolated of the sample resulted in a concentration of 46 - 187 µg per gram sample with $\lambda 260/\lambda 280$ ratio.

The PCR component and condition were optimized so that the cyt b gene could be amplified (**Table 3** and **Table 4**).

2.3. Cyt b Gene Sequencing

The amplification product had been sent to *First BASE*, Laboratories Sdn. Bhd. Selangor, Malaysia to be sequenced. The equipment used was ABI PRISM 3730xl *Genetic Analyzer*. *Biosystem* USA.

2.4. Sequence Alignment and Data Analysis

The cyt b gene sequence data of the North Sulawesi *Tarsius sp*. and those accessed from the GenBank had been aligned using CLUSTAL-X programme [28]. Bayesian Information Criterion (BIC) had been used to consider the best substitution pattern. The genetic distance had been analyzed using the TN 93 + G (Tamura-Nei) and T92 + I (Tamura 3-parameter) methods. The phylogenetic tree had been constructed based on *Tarsius sp*. cyt b gene using two different approaches, Neighbor-Joining (NJ) [29] and Maximum Likelihood (ML) method [30] where *C. bancanus* and *C. syrichta* were treated as outgroup.

Table 3. Optimization component of PCR.

PCR Component	Concentration	Volume (µL)
DNA template	-	4.0 - 5.0
ddH_2O	-	10 - 14
Buffer	5 X	2.5
$MgCl_2$	25 mM	3 - 4
Mixture of dNTP	1 mM	0.5
Primer forward	15 - 30 pmol·µL^{-1}	0.5
Primer reverse	15 - 30 pmol·µL^{-1}	0.5
Tag DNA polymerase	4 - 6 U·µL^{-1}	0.3

Table 4. Condition optimation of PCR.

No. Cycles	Duration (min.)	Temperature ˚C	Phase
35	3	94	Initial denaturation
	1	94	Denaturation
	1	55	Annealing
	1	72	Elongation
	10	72	Post-elongation

Note: Before sequencing, the PCR product was purified and electrophorized in agarosa gel 1.5%.

3. Results

3.1. Extraction and Amplification of cyt b Gene

Total DNA of six samples of North Sulawesi *Tarsius sp*. had been isolated and amplified. The results of electrophoresis on 1.5% agarose gel showed that cyt b gene amplified was at about 400 bp (**Figure 1**).

3.2. Sequence Characteristic

Multiple alignment of cyt b gene sequence of 307 bp long derived from *T. sangirensis*, *T. tarsier*, *T. tumpara* and those from homologous cyt b gene sequence of several tarsier species taken from *GenBank* indicates that invariable sites character as much as 72.97%, informative parsymony sites as much as 27.03% and variable sites as much as 27.03% (**Table 5**).

Nucleotide composition of the partial gene cyt b sequence of each tarsier species exhibits variations indicated by frequency difference of each bases among species. Average base frequency of T = 32.80%, C = 22.67%, A = 28.63% dan G = 15.90%. Analysis of several parameters of partial cyt b gene sequence (**Table 6**) found that nucleotide diversity (Pi) = 0.0698, total mutation = 97, ts/tv ratio (R) = 4.978 and ts/tv (k) ratio between purine bases = 5.253 and between pyrimidine bases = 13.418, respectively.

3.3. Genetic Distance

Genetic distance measured using the Tamura 3 parameters indicate that the value varies from 0 to 0.240 (complete matrix data are not included). The genetic distance of 0 is shown by sample pairing of the same species. Genetic distance is shown by pairing of *C. bancanus* and other species, i.d. from 0.181 to 0.240, as well as pairing of *C. syrichta* and other tarsier species, with values from 0.181 to 0.200. Pairing among Sulawesi *Tarsius sp*. have the values 0 to 0.095. Overall mean distance is 0.080. These data indicate that those Sulawesi tarsiers are classified as closely related taxa and relatively distant related to the Borneo tarsier *C. bancanus* as well as to the Philippine tarsier *C. syrichta*.

3.4. Phylogenetic Tree

Figure 2 and **Figure 3** are phylogenetic trees based on nucleotides of partial cyt b gene constructed by method

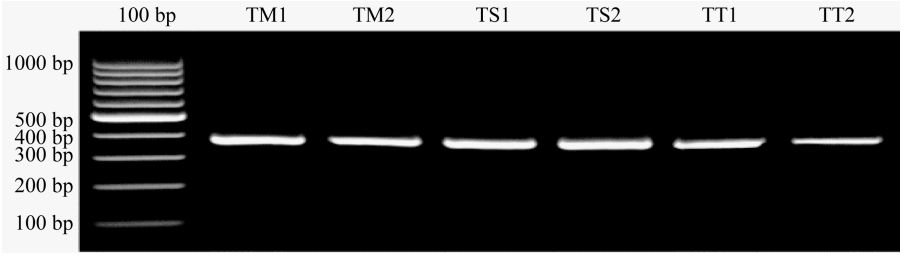

Figure 1. Cyt b gene amplification of North Sulawesi *Tarsius sp*. sample on 1.5% agarose gel. Lanes: 100 bp as a marker, TM1 and TM2 = *T. tarsier*, TS1 and TS2 = *T. sangirensis*, TT1 and TT2 = *T. tumpara*.

Table 5. Summary of cyt b gene sequence diversity.

Analysis	Amount	%
Tested character	307	-
Invariable sites	224	72.97
Variable sites	83	27.03
Informative Parsimony sites	83	27.03
Non-informative Parsimony sites	224	72.97

Table 6. Analysis results of cyt b gene partial sequencing.

Parameter	**Position at Codon**			
	First	**Second**	**Third**	**Total**
Thyrosine frequency	29.9%	42.2%	26.7%	306
Cytosine frequency	17.6%	21.6%	28.1%	306
Adenine frequency	25.8%	17.6%	42.7%	306
Guanine frequency	26.7%	18.6%	2.5%	306
Frequency of invariable sites		73%		
Frequency of parsimony informative sites		27%		
Nucleotide diversity (Pi)		0.069833		
Number of haplotipes		9		
Total number of mutations		97		
Polymorphic sites		83		
ts/tv ratio (k)		Purine = 5.253, Pyrimidine = 13.418		
ts/tv ratio (R)		4.978		
Gamma discrete distribution (Tamura-Nei Model)		0.1882		
Mean of evolutionary rate		0.00, 0.01, 0.10, 0.63, dan 4.26 substitution per site		

Note: Analysis of base frequency only involved 306 bp, adjusted to the reading frame encoding amino acids, started from second nucleotide of each sequence amplified.

based on distance of Neighbor-Joining (NJ) and Maximum Likelihood (ML). Both NJ and ML phylogenetic trees constructed based on the nucleotide sequences showed same tree topologies. Both NJ and ML phylogenetic tree put North Sulawesi tarsier, *T. sangirensis*, *T. tumpara*, and *T. tarsier* relatively at the same position at the tree.

In NJ phylogenetic tree, both *T. sangirensis* and *T. tumpara* form a monophyletic clade and separate from the others *Tarsius tarsier*-complex (99% BR). On the other hand, *T. tarsier* forms a monophyletic clade and occupies position in *Tarsius tarsier*-complex clade (94% BR). In ML phylogenetic tree both *T. sangirensis*, *T. tumpara* also form a clade separated from the other *T. tarsier*-complex (98% BR). *T. tarsier* is located on *Tarsius tarsier*-complex clade. In general, both the NJ and ML trees showed similarities in grouping populations, in which *T. sangirensis*, *T. tumpara*, *T. tarsier* and other species are clustered in a larger clade called *Tarsier tarsier*-complex.

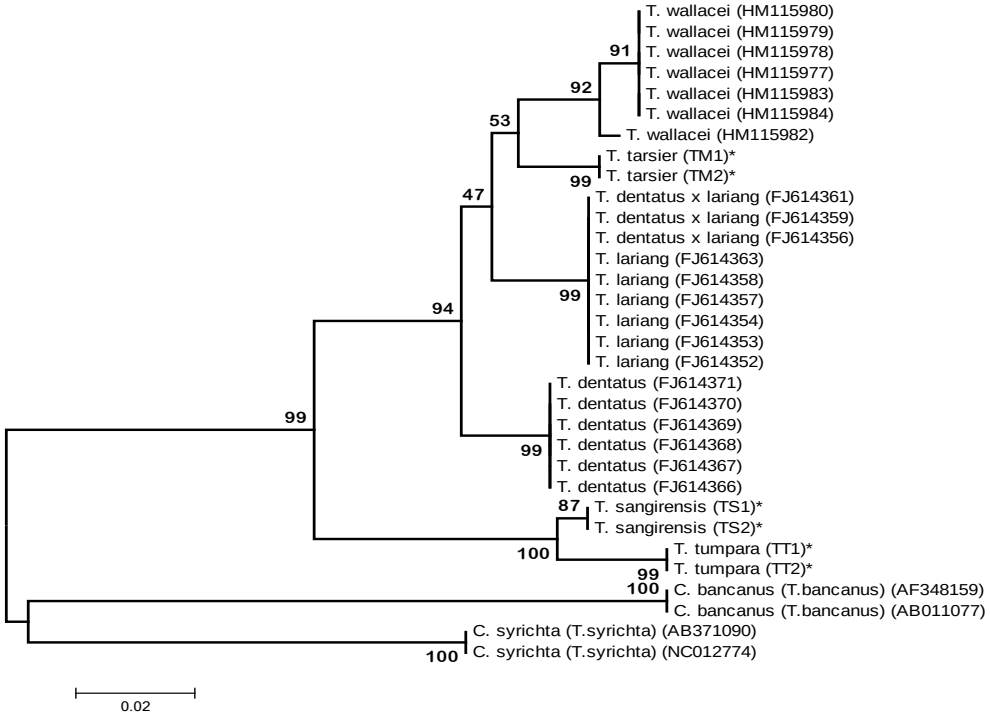

Figure 2. Phylogenetic tree based on nucleotide sequences had been constructed by Neighbor-Joining method. NJ-1000 BR, substitution models: Tamura-Nei model, (TN93 + G). Numbers at the branches are bootstrap values. Note: species marked with an asterisk are species sampled.

4. Discussion

Prior classification of tarsier consisted of *C. bancanus* (*T. bancanus*), *C. syrichta* (*T. syrichta*) and *T. tarsier* (*T. spectrum*) [7]. These three species are closely related [31]. Recently, genera and species of the *Tarsiidae* family originally known as a monotypic genus, right now this family consists of three genera [9]. These three genera are *Chepalopachus*, inhabiting the biogeographic region of Borneo and Sumatra, *Carlito* known as the Philippine tarsier and *Tarsius* known as east tarsier or Sulawesi tarsier or *Tarsius tarsier*-complex. Striking morphological characteristic differences of all three genera are mainly in the form of teeth, long legs and arms, tail end hair (tail-tuft) and mammary gland. Most *Tarsius* forms social group and has a duet song; *Carlito* does not form social groups in the wild but could socialize in captivity, as well as has no duet song, and *Chepalophacus* does not form social groups in the wild, as well as can not socialize even in captivity and has no duet song [9].

Some species of *Tarsius tarsier* complex had separated from the other Sulawesi tarsier, because these species inhabit separate biogeographic region of Sulawesi mainland (See **Figure 4**). Both species of *T. sangirensis* and *T. tumpara* inhabit the archipelago biogeographic region known as the Sangihe archipelago. *T. tumpara* inhabits Siau island but *T. sangirensis* inhabits Sangihe island. Two islands are located at a distance of ± 60 km and restricted by the deep sea. *T. sangirensis* and *T. tumpara* are not allied with the Philippine tarsier *C. syrichta*, although their biogeographic regions are close to each other. In other hand *C. syrichta* is allied with *C. bancanus*, although their biogeographic regions are relatevely far apart. *T. tumpara* is subtly different from *T. sangirensis*, but both are significantly different from other tarsier of the *Tarsius tarsier* complex. Morphological features related to rare tail hair of *T. sangirensis* and *T. tumpara* resemble the features of Philippine tarsier *C. syrichta*.

Related to the phylogenetic posisition of *T. sangirensis*, *T. tumpara* and *T. tarsier* this research result supports the hypothesis that *T. sangirensis*, *T. tumpara* are the sister takson and allied with *Tarsius tarsier*-complex [32]. Grouping *T. sangirensis*, *T. tumpara* together and grouping of *T. tarsier* to the large clusters of *T. wallacei*, *T. dentatus* and *T. lariang* are in accordance with distribution of Sulawesi tarsiers.

Related to the distribution of Sulawesi tarsiers, there were some distribution forms like Manado form, Libou form, Sejoli form, Tinombo form, Kamamora form, and Togian form [2]. The distribution of Sulawesi tarsiers had occured in part by events of Pleistocene vikarian and partly had been influenced by tectonic activities

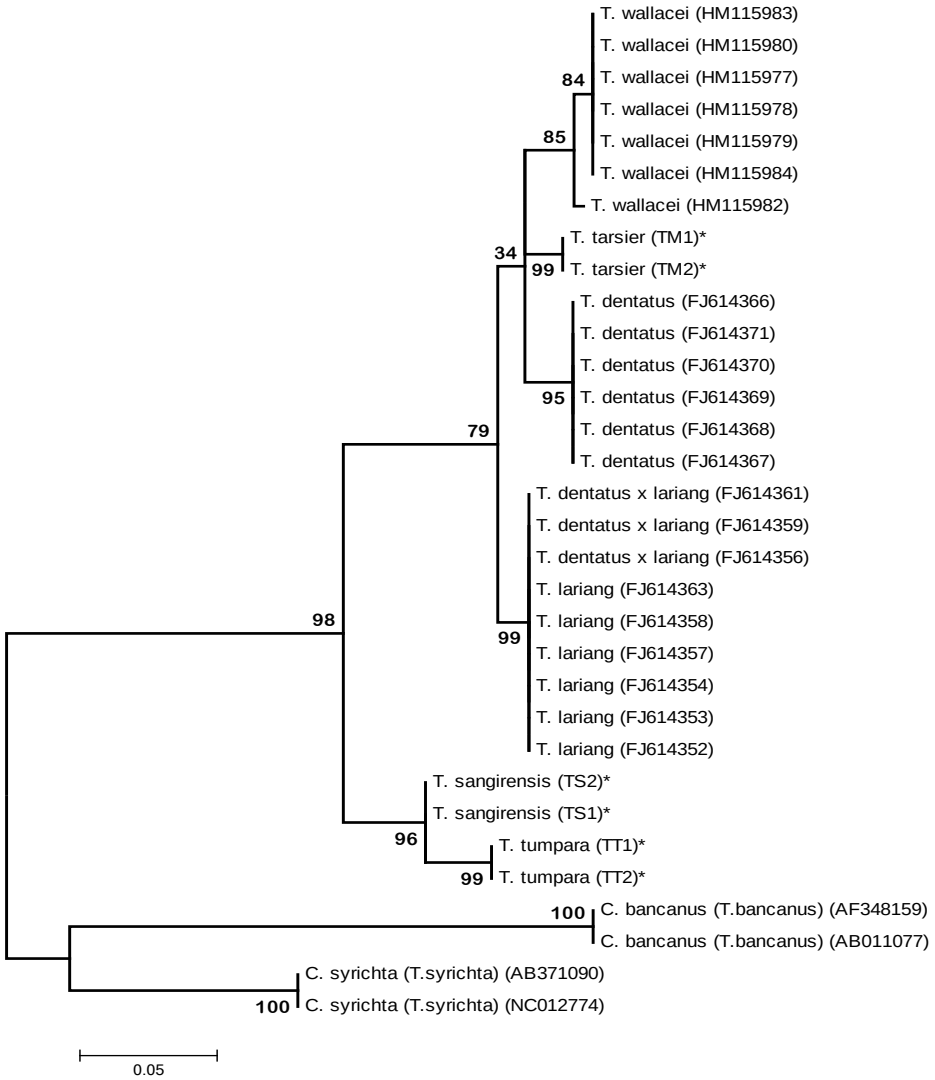

Figure 3. Phylogenetic tree based on nucleotide sequence had been reconstructed by Maximum Likelihood (ML), Bootstrap 1000. Substitution Model: Tamura 3-parameter model (T92 + I). Branch Swap Filter: Very Strong. Numbers at the branches are bootstrap values. Note: species marked with an asterisk are species sampled.

occured before the Pleistocene era [3].

Distribution of Sulawesi tarsier is closely related to that of vocalization forms (duet song). Distribution of vocalization forms of tarsiers of the northern and central parts of Sulawesi are in accordance with each species locality [33]. There was a presupposition saying that the vocal forms of tarsiers were different among the tarsier population in the south and southeast Sulawesi, as well as in offshore islands of Selayar, Buton and Kabane region [34]. The striking difference is related to their acoustic features; tarsier populations of south, southeast, and offshore islands of Sulawesi are classified as different species. Tarsier species having different acoustic duet song features are *Tarsius dianae* (*T. dentatus*), *T. lariang*, Togian tarsier, *T. pelengensis*.

This research result reinforces the fact that *T. tarsier*, *T. sangirensis* and *T. tumpara* are three distinctive species genetically, bioacoustically, morphologically as well as they have different distribution. This explanation is consistent with the hypothesis that speciation of tarsiers had occured as a result of the spread of proto-Sulawesi island, followed by various subsequent fragmentations [23]. The phylogenetic positions of the North Sulawesi tarsiers uncovered by this research are based only on 307 nt sequence of the cyt b gene, but these results are in accordance with the several publications reported [9] [33] [34]. To obtain more reliable results, the results of this

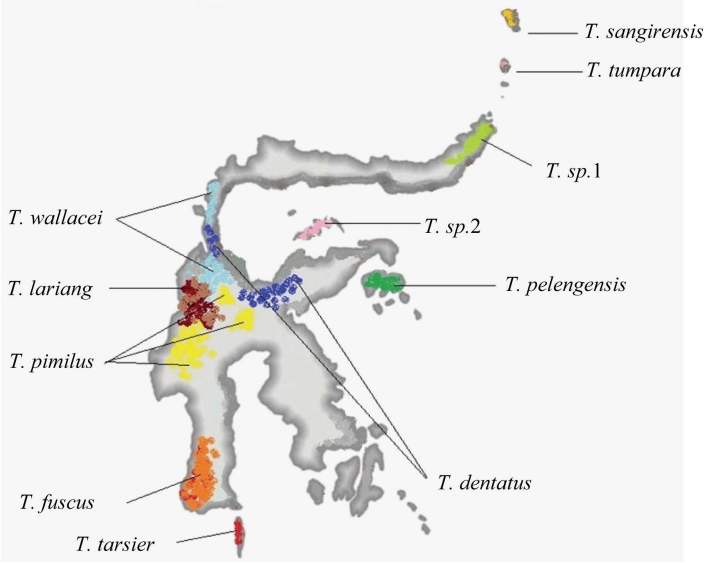

Figure 4. Distribution Map of Tarsier Found in Sulawesi. This figure modified from Groves and Shekelle, 2010.

study need to be examined again by cyt b gene sequence analysis as a whole, or to be examined using other applications of genetic markers.

5. Conclusions

Based on the cyt b gene partial sequence of North Sulawesi *Tarsius sp*., this result of research uncovers that *T. tarsier*, *T. sangirensis* and *T. tumpara* are closely related to *Tarsius tarsier*—complex, and relatively open related to *C. bancanus* and *C. syrichta*. This was supported by the genetic distance of each species uncouvered at NJ phylogenetic tree as well as at ML phylogenetic tree.

The positions of North Sulawesi tarsiers at the phylogenetic trees constructed based on the partial sequence of cyt b gene are in accordance with the classification based on morphology, distribution according to biogeographic region, as well as on distribution according to vocalization forms.

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