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SHORT COMMUNICATION

Phylogenetic Relationships of Macaques (Cercopithecidae: *Macaca*) Inferred from Partial Mitochondrial DNA (mtDNA) Cytochrome Oxidase II (COII) gene

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ABSTRACT

The molecular phylogenetic relationships among ten species of macaques were assessed using mitochondrial DNA (mtDNA) cytochrome oxidase II (COII) gene. The 27 individuals comprising of ten species within genus *Macaca,* namely, *M. sylvanus, M. mulatta, M. cyclopis, M. arctoides, M. fascicularis, M. assamensis, M. thibetana, M. nemestrina, M. leonina and M. silenus* were used in this study. The phylogenetic trees were reconstructed using neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) methods. Based on our constructed tree, it is suggested that the results from phylogenetic analyses demonstrated four groups of macaques. In addition, the trees showed topology of *M. sylvanus* as a sister clade to all Asian macaques. The *silenus* group, which diverged first after *M. sylvanus* formed their own clade, consisted of macaque species *M. silenus, M. nemestrina* and *M. leonina*. Meanwhile, the *sinica* group consisted of *M. assamensis* and *M. thibetana*, and the *fascicularis* group comprised of *M. fascicularis*, *M. arctoides*, *M. mulatta* and *M. cyclopis*. Our ML tree also showed that *M. arctoides* is a member of *fascicularis* group. Our study, also indicated that our results neglect the classification based on outer appearances and supports the proposed molecular work view.

Keywords: mitochondrial DNA, cytochrome oxidase II gene, macaques, phylogenetic

There are approximately 67 genera and 376 species of primates in the world (Wilson & Reeder, 2005). The two major suborders of primates form the Strepsirhini and Haplorhini. Genus *Macaca* comes from the suborder of Haplorhini and infraorder of Simiiformes, which is then classified in the family of Cercopithecidae (Old World Monkey), and the subfamily of Cercopithecinae which is the omnivorous cheek pouch monkeys that possess basic stomachs.

The Asian representative Cercopithecine monkeys of genus *Macaca* has the widest distribution compared with other primates. Brandon-Jones *et al*. (2004) listed 19 species, and later, Wilson and Reeder (2005) stated that the genus has total number of 21 macaque species, a view supported by Perelman *et al.* (2011). The latest number of species updated by International Union for Conservation of Nature [IUCN (2013)] was 22, following the discovery of *M. munzala* as a new species (Sinha *et al*., 2005). According to Tosi *et al*. (2003), *Macaca* is the most successful of all the extant of nonhuman primate radiations and it manifests one of the most successful evolutionary radiations among primates (Hoelzer *et al*., 1992). They occupy a wide geographical range that varies from near desert to rainforest, and from sea level to snowy mountain tops. They are also widely distributed from East to Southeast Asia, Indian subcontinent, Afghanistan, surrounding islands, as well as limited areas in North Africa (Hayasaka *et al*., 1996), as the macaques are the only papionins with extant members outside of Africa, which also have the largest

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distribution of all non-human primates (Evans *et al*., 1999). The macaques are half terrestrial, with a short or long tail, cheek pouches for temporary storage of food and a simple stomach that adeptly of digesting only a limited amount of leafy materials (Payne *et al*., 1985).

Primitive characters are usually discriminated by contrast with an outgroup as some are less closely related species (Bryne, 2000). Fooden (1976) classified the macaques based on the male genitalia into four species groups which are the *silenus-sylvanus* group which includes *M. sylvanus, M. silenus, M. nemestrina* and Sulawesi macaques, the *fascicularis* group which includes *M. fascicularis, M. mulatta, M. fuscata,* and *M. cyclopis*, the *sinica* group which includes *M. sinica, M. radiata, M. assamensis* and *M. thibetana*, and the *arctoides* group which includes *M. arctoides*. Later, Delson (1980) provided a little customization in the study which removed *M. sylvanus* from *silenus-sylvanus* group and placed the *M. arctoides* in the *sinica* group. Grooves (2001) classified the genus into six species which puts the Sulawesi macaques and *M. mulatta* into their own species group. From the molecular study, Hayasaka *et al*. (1996) and Morales and Melnick (1998) both classified the macaque species into five species group. Tosi *et al*. (2000) conducted a study using Y chromosome and found largely congruent results with those of Fooden's (1976) and Delson's (1980) classifications where the genus formed four groups of macaques. Subsequently, Li and Zhang (2005) came up with a study of combined dataset of mitochondrial DNA sequences which classified the macaques into four species group. Li *et al.* (2009) provided congruent data with Delson's (1980) classification using the *Alu* elements which also produced four major species group. In another study of Y-chromosomal gene flow of *Macaca fascicularis* (Cercopithecidae) between the insular and mainland peninsula of Penang state, Rovie-Ryan *et al*. (2013) estimated that the *fascicular* is group shared common ancestors about 1.5 mya. The splitting of insular and continental lineages were about 0.7 mya and 0.4 mya respectively (Rovie-Ryan *et al*., 2013).

The phylogenetic relationships among different species and grouping of species have been inconclusive and several issues remain to be adduced as the number of species group in the genus Macaca are not fixed, especially for the Asian macaques. For this macaques research the objective is to determine the phylogenetic relationships of Macaca and classify the group representative in order to update the taxonomic classification based on mitochondrial cytochrome oxidase II (COII) since, the number of species groups in the genus *Macaca*, especially for the Asian macaques is not constant and the phylogenetic relationship among the Asian species groups are still problematic (Li *et al*., 2009). Earlier Hayasaka *et al*. (1988) stated that among macaque species, the phylogenetic relationships induced from maternally inherited mtDNAs would not be interrupted by hybridization between different species of macaques the migration of male macaques between populations causing interspecific hybridization thus, the phylogenetic relationships inferred from mtDNA analyses may reflect the ancient process of speciation more accurately than those inferred from analyses of nuclear genes.

Meanwhile, COII exhibits one of the most heterogeneous rates of protein evolution between mammals and plays one of the most important roles in respiration, and in primates, the gene product shows changes in its physical interaction with cytochrome c. (Adkins *et al*., 1996). The phylogenetic inferences using COII gene in the Asian region remains less pronounced than in the Africa region according to Md-Zain *et al*. (2010). It was also stated that COII can be regarded as a good locus candidate to be used in representing the phylogenetic relationships and can act as a good gene candidate for portraying the phylogenetic relationships at the inter- and intra-subfamily levels.

Sample collection of blood, blood spots and tissues were obtained from two macaques species, from which five (LKW008, WPL135, WPL137, KG004 and KG005) were samples from *Macaca fascicularis* (from Langkawi and Labuan Island, Kuala Gula) and five (PRP0001, PRP0007, PRP0012, PRP0024, PRP0225 and PRP0226) were samples from *M. nemestrina* (from Matang Wildlife Center and Malacca Zoo). Even though these were samples obtained from captives, the individuals were known to come from local populations and originated from the wild before placed at

the zoo or wildlife center. *Trachypithecus obscurus* (Spectacled langur) and *Pongo pygmaeus* (Orangutan) were used in the phylogenetic tree as an outgroup to root the trees and to show distinct relationship within the Family Cercopithecidae. Ten partial sequences of COII mtDNA gene of macaques and two outgroups used from this study have been registered with the GenBank (submitted: 19th June 2014).

The DNA was extracted from blood samples using the QIAamp® DNA Mini and Blood Mini Handbook of DNA Purification from Blood or Body Fluids (Spin Protocol). Meanwhile, the blood spot preserved on the FTA (Flinders Technology Associates) cards was extracted using QIAamp® DNA Investigator Handbook of Isolation of Total DNA from FTA and Guthrie Cards and samples from the tissue were extracted using the Cetyl Trimethyl Ammonium Bromide (CTAB) method (Grewe et al., 1993). The product of extraction was visualised using 1% agarose gel electrophoresis ultraviolet (UV) transilluminator.

The amplification of DNA by Polymerase Chain Reaction (PCR) using primers cytochrome oxidase II (COII) gene 5'-AACCATTTCATAACTTTGTCAA-3' described by Adkins and Honeycutt (1994) was used. The amplifications were performed in a total volume of 25 µl. The parameters of PCR included a 94°C pre-denaturing phase for 3 minute and followed by 35 cycles of 94°C for 1 minute, with a range of 50-55°C for 1 minute, 72°C for 70 seconds and a 9 minute extension of 72°C. The annealing temperature obtained from the gradient experiment for *M. fascicularis* and *M. nemestrina* were 50°C and 52.7°C, respectively. The PCR product was visualised using 1% agarose gel electrophoresis under ultraviolet (UV) transilluminator. The PCR product was then purified using GeneJET Purification Kit (Fermentas Life Sciences) and was sent for sequencing to the First BASE (First BASE Laboratories Sdn Bhd).

Nucleotide frequencies were analyzed based on Phylogenetic Analysis Using Parsimony, PAUP* (version 4) (Swofford, 2002). The highest frequencies of nucleotide found in mtDNA COII gene for all genus *Macaca* used sequences was indicated by the Adenine (A) with the record of 31.0%, ranging from 25.3 to 32.7%, while the lowest nucleotide frequency was shown by the Guanine (G) with the average of 14.0%, ranging from 11. 5 to 20.0%. Thymine (T) and Cytosine (C) were recorded with 25.2% and 29.8% nucleotide compositions respectively. The distance matrix between each individual was calculated using Kimura 2-parameter (PAUP 4.0b10) among ten species and total of 26 haplotypes with two outgroups were analyzed based on mtDNA COII gene. The highest genetic distance within the genus *Macaca* was found between the species *M. fascicularis and T. obscurus* (99.9%).

The Neighbor-joining tree of Figure 1 shows that it divided the sequences into four major groups of macaques. The first group consists of *M. sylvanus* with 96% bootstrap value, which divided the clade into three other groups. *Silenus* group formed monophyletic clade with the bootstrap value of 99% consisting of *M. silenus*, *M. leonina* and *M. nemestrina* as well as the *sinica* group with 100% bootstrap value that comprise of *M. assamensis* and *M. thibetana*. Meanwhile, the *M. fascicularis*, *M. arctoides*, *M. mulatta* and *M. cyclopis* formed paraphyletic clade of *fascicularis* group with a bootstrap value of 67%.

In Maximum Parsimony tree (Figure 2), the tree length was 872 with a consistency index (CI) of 0.6193 for the analyzed data, a retention index (RI) of 0.6616, and a rescaled consistency index (RCI) of 0.4097. Similar to NJ methods, the phylogenetic tree formed had four major clades with bootstrap values of 96%.

For the Maximum Likelihood (ML) in Figure 3, the estimates of tree topology were performed using the HKY+G model that was selected by Akaike Information Criterion (AIC). Parameters estimates from Modeltest 3.7 (Pasoda & Crandall, 2001) which Lset Base=(0.2930 0.3017 0.1623), Nst=6, Rmat=(0. 5158 4.006 0.4245 0. 5280 4.006), Rates=gamma, Shape=0.8191, Pinvar=0. The ML tree was similar to the MP tree in that they both showed high bootstrap values of 9% and formed four major groups.

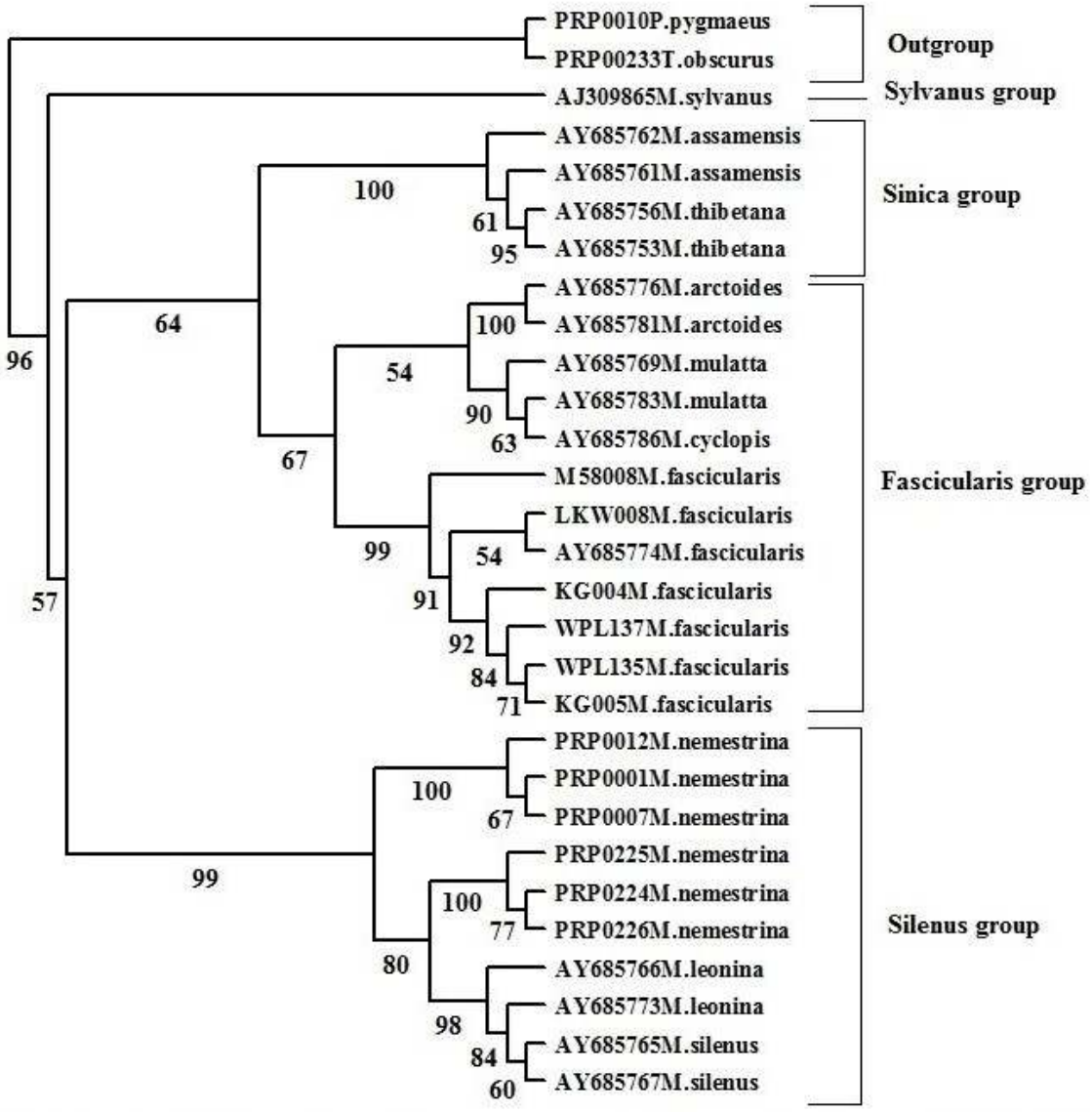

Figure 1. Neighbor-Joining (NJ) tree analysis of *Macaca* species inferred from partial cytochrome oxidase II mtDNA gene was constructed using PAUP4.0b10 with a *T. obscurus* and a *P. pygmaeus* outgroup. Only bootstrap value > 50% is shown. Bootstrap value with 1000 replicates.

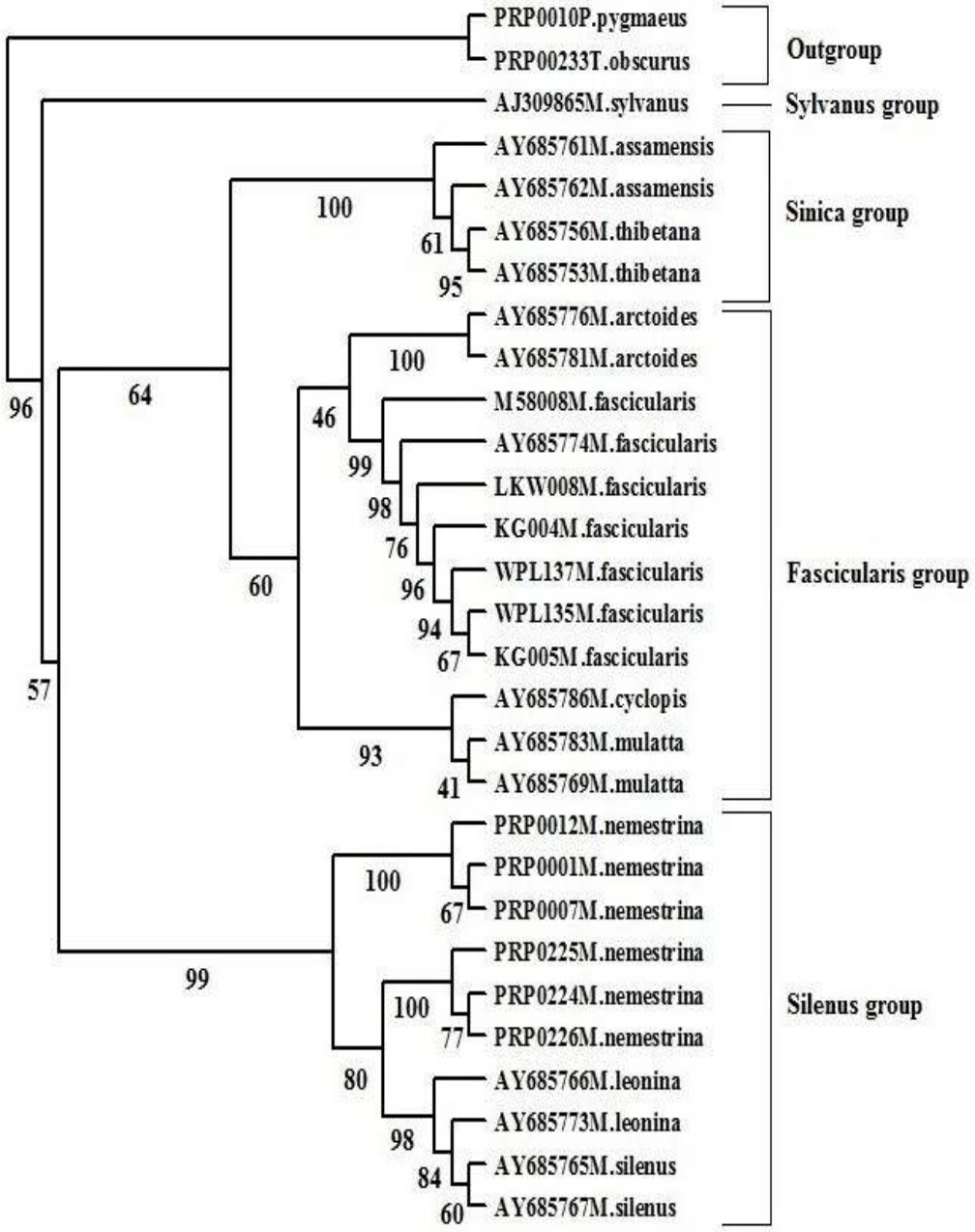

Figure 2. Maximum Parsimony (MP) tree analysis of *Macaca* species inferred from partial cytochrome oxidase II mtDNA gene was constructed using PAUP4.0b10 with a *T. obscurus* and a *P. pygmaeus* outgroup. Only bootstrap value > 50% is shown. Bootstrap value with 1000 replicates.

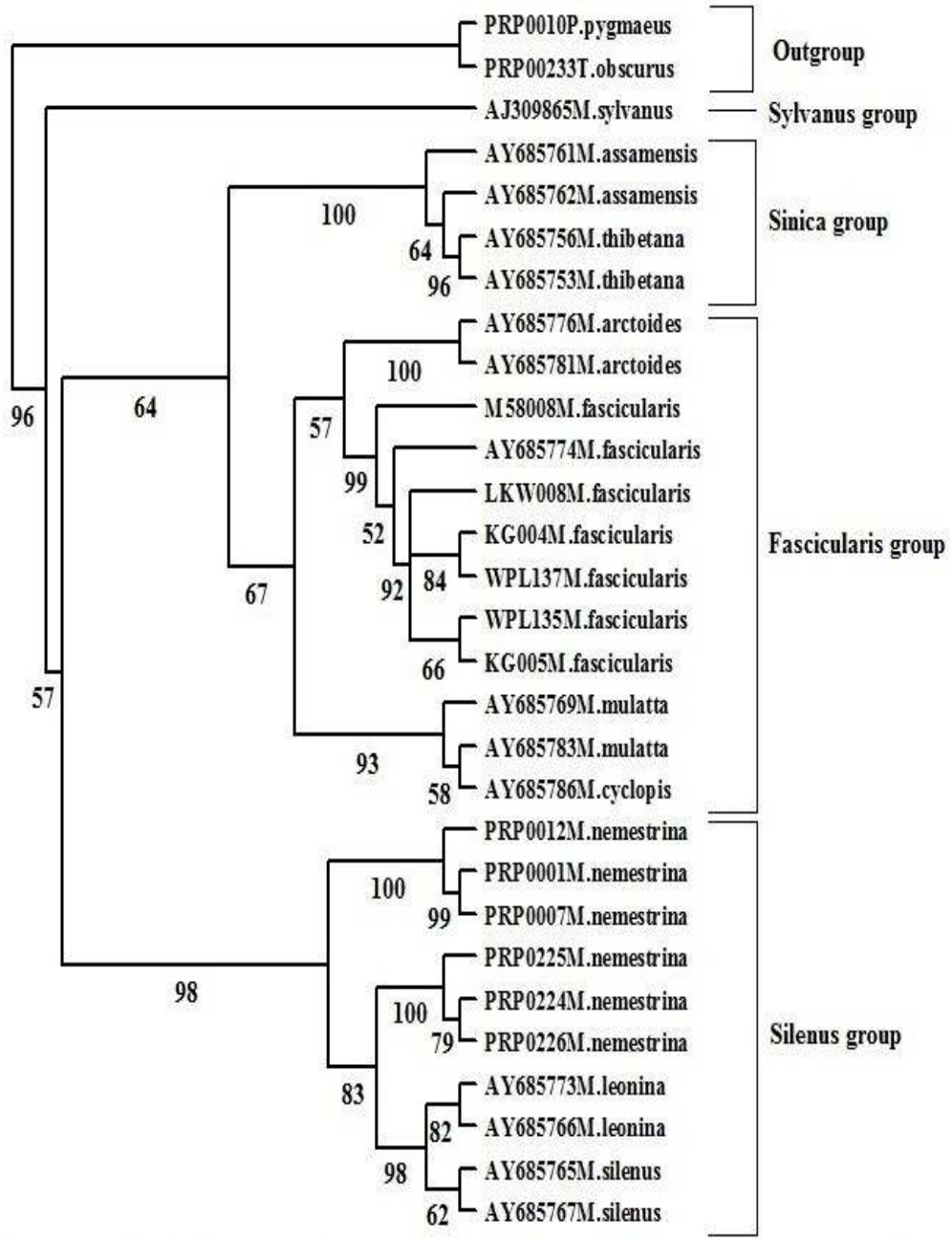

Figure 3. Maximum Likelihood (ML) tree analysis of *Macaca* species inferred from partial cytochrome oxidase II mtDNA gene was constructed using PAUP4.0b10 with a *T. obscurus* and a *P. pygmaeus* outgroup. Only bootstrap value > 50% is shown. Bootstrap value with 1000 replicates.

In this study, Neighbor-joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) were used to reconstruct the phylogenetic trees. The three methods have their own approaches in constructing trees. NJ uses the evolutionary distances to construct trees by the distance methods. Meanwhile, the ML methods used a model for sequence evolution to create a tree that gives the highest likelihood of occurring with the given data. In ML, the optimum criterion is determined by Modeltest to establish the model of DNA evolution that best fits the data. According to Posada and Crandall (2001), the usage of different model selection strategies is to lead to the selection of different models of evolution. The analysis on MP tree showed that only 215 characters (43.52%) resulted in the parsimony informative sites. From the analysis, it can be concluded that COII gene is a conserved gene because it showed a greater informative site at only 494 bp. Nonetheless, only partial COII gene was used in this study. Therefore, not much of genetic information could be analysed in the sequence. Genebank sequences that have 'AY', 'AJ' and 'M' initials which were followed by accession number as shown in the diagram were also used in this phylogenetic analysis to infer the relationship within this genus more clearly.

The Old World Monkey in the genus Macaca demonstrates one of the most successful evolutionary radiations among primates (Hoelzer *et al*., 1992) and based on a study conducted by Hayasaka *et al*. (1988) the rate of nucleotide substitution for the sequenced region of mtDNAs was found to be higher in macaques, while the rate of nucleotide substitution was estimated to be (1.12-2.24) x 10^{-8}/site/year/lineage between Barbary (*M. sylvanus*) and Asian macaques. In the nucleotide composition, the Adenine (A) was the highest with an average of 31.0% while the lowest was Guanine (G) with an average of 14.0%. Since mtDNA is clonally inherited through the maternal lineage, the phylogenetic relationships inferred would not be interrupted by other genes from different macaque species.

In the NJ tree, the macaques were clearly divided into four groups which placed the *M. sylvanus* on its monophyletic clade, *M. nemestrina, M. silenus* and *M. leonine* in the *silenus* group; *M. assamensis* and *M. thibetana* in the *sinica* group; and *M. arctoides*, *M. mulatta*, *M. cyclopis* and *M. fascicularis* in the *fascicularis* group. Both MP and ML analyses using COII nucleotide sequences data produced the same tree topology which formed three major groups. In the classification of macaques based on a previous study by Li and Zhang (2005), the tree produced from MP and ML supported molecular data which were congruent in the formation of four macaque species, namely, the *sylvanus* group, the *fascicularis* group, the *sinica* group and the *silenus* group. The *sylvanus* group formed a monophyletic clade in which the *M. sylvanus* diverged first from other Asian macaques as its distribution is restricted to Africa and north of the Sahara desert, and it is the only native species of primate to exist in Europe (IUCN, 2013). The *M. sylvanus* is among the first macaques that diverged first from the lineage before it split into the other group. In the *silenus* group, comprising *M. silenus*, *M. leonina* and *M. nemestrina* there are no overlaps in their zoogeographic distribution that can be related with their clustering. In Morales and Melnick's (1998) study, other species of macaques were included, all of which were Sulawesi macaques that can be clustered into this *silenus* group. The *fascicularis* group gave paraphyletic clade that comprised *M. fascicularis* itself and *M. arctoides* which showed different branching when both NJ and MP methods were applied. In the NJ tree, *M. fascicularis* formed their own monophyletic group while in the MP tree, *M. arctoides* was included in the clustering which made the topology more concordant with previous studies of Morales and Melnick (1998) and Li and Zhang (2005).

Based on the results, the classification grouping seemed to be neglected based on physical appearances, for instance, characteristics of tail-length and fur coloration. Hence, this study gave an overall support for the molecular work in viewing the classifications of macaque groups. Table 1 shows the systematics of macaques proposed by different authors based on molecular works that also includes the results from this study.

Table 1. Systematics of macaques proposed by different authors.

Authors	Hayasaka *et al*. (1996)	Morales & Melnick (1998)	Tosi *et al*. (2000)	Li & Zhang (2005)	Li *et al.* (2009)	This study
Locus	mtDNA of 896 bp region NADH4, $tRNA^{His}$, $tRNA^{Ser}$, $tRNA^{Leu}$, ND5.	mtDNA of 780 bp ribosomal region (12S, 16S)	3.1 kb of region TSPY, SRY	2322 bp of region RNA (12S, $tRNA^{Glu}$ - CY), COI, COII, COIII	*Alu* elements of 358 loci	mtDNA of 494 bp region COII gene
Classification group	**five species groups** 1. the *sylvanus* group • *M. sylvanus* 2. the Sulawesi group • *M. nigra* • *M. tonkeana* 3. the *fascicularis* group • *M. fascicularis* • *M. fuscata* • *M. cyclopis* • *M. mulatta* • *M. arctoides* 4. the *sinica* group • *M. sinica* • *M. nemestrina* • *M. silenus* 5. the radiata • *M. radiata* • *M. assamensis* • *M. thibetana*	**five species groups** 1. *sylvanus* group • *M. sylvanus* 2. *silenus* group • *M. nemestrina* • *M. silenus* • Sulawesi macaques group 3. *fascicularis* group • *M. fascicularis* • *M. arctoides* 4. *sinica* group • *M. radiata* • *M. assamensis* • *M. thibetana* • *M. sinica* 5. *mulatta* group • *M. fuscata* • *M. cyclopis* • *M. mulatta*	**four species groups** 1. *sylvanus* group • *M. sylvanus* 2. *silenus* group • *M. silenus* • *M. nemestrina* • Sulawesi macaques group 3. *fascicularis* group • *M. fascicularis* • *M. mulatta* • *M. cyclopis* • *M. fuscata* 4. *sinica* group • *M. arctoides* • *M. thibetana* • *M. radiata* • *M. assamensis* • *M. sinica*	**four species groups** 1. *sylvanus* group • *M. sylvanus* 2. *silenus* group • *M. silenus* • *M. leonina* 3. *fascicularis* group • *M. fascicularis* • *M. arctoides* • *M. cyclopis* • *M. mulatta* 4. *sinica* group • *M. assamensis* • *M. thibetana*	**four species groups** 1. *sylvanus* group • *M. sylvanus* 2. *silenus* group • *M. silenus* • *M. nigra* • *M. nemestrina* 3. *fascicularis* group • *M. fascicularis* • *M. mulatta* • *M. fuscata* 4. *sinica* group • *M. radiata* • *M. thibetana* • *M. arctoides*	**four species groups** 1. *sylvanus* group • *M. sylvanus* 2. *silenus* group • *M. nemestrina* • *M. silenus* • *M. leonina* 3. *fascicularis* group • *M. fascicularis* • *M. arctoides* • *M. mulatta* • *M. cyclopis* 4. *sinica* group • *M. assamensis* • *M. thibetana*

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