PHYLOGENETIC ANALYSIS ON FAMILY CHIROPTERA USING CYTOCHROME B GENE SEQUENCES

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DECLARATION

I declare that this thesis entitled “title of the thesis”is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature : _____
Name : _____
Date : _____

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ABSTRACT

There are about 1,240 species of Chiroptera that have been divided into two suborders: the less specialized and largely fruit-eating megabats, or flying foxes, and the highly specialized and echolocating microbats. DNA sequences of 23 species subfamily Vespertilioninae of Chiroptera that used mitochondrial cytochrome b gene to evaluate phylogenetic relationships between genera was obtained from GenBank NCBI database and were successfully aligned. The analysis of phylogeny to obtain the confidency value of each model and to compare the differences between phylogenetic methods in estimating ancestry of samples of each model involved the method of Neighbor-Joining, Minimum Evolution, Maximum Parsimony, Maximum Likelihood and Bayesian Inference. All 63 partial cytochrome *b* sequence fragments with at least 1,000 bp linear DNA were run through five different methods of phylogeny analysis methods. Phylogeny tree were successfully constructed for each model and were summarised accordingly with its bootstrap value.

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ABSTRAK

Terdapat kira-kira 1,240 spesies Chiroptera yang telah dibahagikan kepada dua Suborder: kelawar besar yang kurang khusus dan sebahagian besarnya makan buah-buahan, atau keluang, dan gemalokasi kelawar kecil yang sangat khusus. Urutan DNA daripada 23 spesies pecahan keluarga Vespertilioninae daripada Chiroptera yang menggunakan genetik sitokrom mitokondria b untuk menilai hubungan filogenetik antara genera telah diperolehi dari pangkalan data GenBank NCBI dan telah berjaya dijajarkan. Analisis filogeni untuk mendapatkan nilai keyakinan bagi setiap model dan untuk membandingkan perbezaan antara kaedah filogenetik dalam menganggarkan keturunan sampel setiap model yang terlibat iaitu kaedah Cantuman-Jiran, Evolusi Minimum, Kekikiran Maksimum, Kebolehjadian Maksimum dan Kesimpulan Bayesian. Semua 63 separa serpihan sitokrom *b* urutan dengan sekurang-kurangnya 1,000 bp lelurus DNA telah dijalankan melalui lima kaedah yang berbeza kaedah analisis filogeni. Pokok filogeni telah berjaya dibina untuk setiap model dan diringkaskan sewajarnya dengan nilai bootstrap.

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LIST OF ABBREVIATIONS

Bp	base pair
BI	bayesian inference
Cyt b	cytochrome b
ME	minimum evolution
ML	maximum-likelihood
MP	maximum parsimony
NJ	neighbor-joining

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Chiroptera is a group of mammal that widely distributed in every continents, except cold area such as Antartica (Gunnel *et al.*, 2012). Bats are known as the only flying mammals in this world (Gunnel *et al.*, 2012). There are more than 1000 species of bats can be found (Gunnel *et al.*, 2012). Wilson & Reeder (2005) stated that Chiroptera is the second most diverse order of mammals. Chiroptera exhibits great numerical, functional, taxonomical, and diversity of ecology (Simmons & Conway 2003; Stevens & Willig 2002). The diversity of bats may greatly affected by the characteristic of their habitats such as association between bats and habitats, reflection of mobility and habitat mosaics, humans activities in particular habitats, and lastly recuperative powers of many ecosystems (Gunnel *et al.*, 2012).

Bats in South-East Asian region are classified into nine families, which can be distinguished by the characteristics of its ear shape, muzzle shape, the presence or absence of a noseleaf and the tail pattern (Gunnel *et al.*, 2012). Genera can be distinguished within each family by a variety of characters, such as noseleaf shape, dentition or even colour. Determining the genus characters at early stages can greatly ease the identification species of bats. General accounts of each family and genus are additionally given to the species account to assist with sorting out the very large number of bats in the region.

Research on bats have increased drastically by growing public interest in and care about their conservation in wild environment which lead to a phylogeny study on family species of Chiroptera more deeply on their genus in order to gain more knowledge about past evolutionary event, which contribute to understanding the process of Chiroptera evolution and ancestry history. The phylogenies are well known technique to describe the relationships between genera family (Maser *et al.*, 2001), histories of populations (Edwards, 2009), the evolutionary and epidemiological dynamics of pathogens (Marra *et al.*, 2003; Grenfell *et al.*, 2004) as well as a tools in comparing genome of species. Phylogenetic trees have been used in many studies to explain the relationships among species in systematics and taxonomy (Yang & Rannala, 2012). Phylogeny inference methods have arise parallel with rising of technology due to global modernization which resulted in new methods in analysing the relationship of genera among species. Several of those methods are Neighbor-Joining (NJ), Minimum Evolution (ME), Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI).

1.2 Problem Statement

Difference phylogenetic analysis may have discrepancies in estimating ancestry between samples due to different in algorithm. Thus, there is a need to determine which method is most reliable in determining ancestry history samples.

1.3 Objectives

The objective of this study is to compare the differences between phylogenetic methods estimating ancestry of samples

CHAPTER 2

LITERATURE REVIEW

2.1 Chiroptera

Chiroptera is one of the most successful order of mammals that widely distributed in every continents (Gunnel *et al.*, 2012). More than 20% of living mammalian diversity is bats which distributed throughout the globe except extreme latitude/extreme condition regime such as north and south poles of the world (Vaughan *et al.*, 2000). It is the only mammals that capable of flying due the unique physical characteristics built from their body where their wings are made of two thin layers of skin stretched over the digit of bats forelimb (Thewissen & Babcock, 1992). The metacarpal are very long compared to its body as it needed to provide a large surface area during the bat flight.

2.2 Diversity

Bats were originally divided into two subordinal groups which are Megachiroptera (included Old World family Pteropodidae) and Microchiroptera which consists of remaining 17 of bats families (Koopman, 1994; Simmons, 2005; Simmons & Geisler, 1998). The morphological and paleontological data which is mainly based on division group of bats, but also highlighted the difference in the dominant mode of sensory perception used by megabats (vision) and microbats (ultrasound) (Gunnel *et al.*, 2012). Microbats are capable of sophisticated laryngeal

echolocation unlike megabats which is mainly on vision and not ultrasound (Vaughan *et al.*, 2000). It was believe that the laryngeal echolocation was singly origin from the common ancestor of mirobats (Teeling *et al.*, 2000). The 17 families of microbats were regulated into two infraorders Yinochiroptera (craseonycterids, emballounurids, hipposiderids, megadermatids, nycterids rhinopomatids, rhinolophids) and Yangochiroptera (furipterids, molossids, mormoopids, mystacinids, myzopodid, natalids, noctilionids, phyllostomids, thyropterids, vespertilionids) as stated by Koopman (1994), Simmons & Geisler (1998), and Hutcheon & Kirsch (2006).

2.3 *Myotis*

Myotis or commonly known as mouse-eared bat is a diverse and widespread genus of bats within the family of vespertilionidae where it do belongs in the suborder of chiroptera, yangochiroptera (Koopman, 1994; Simmons & Geisler, 1998; Hutcheon & Kirsch, 2006) where it have been divided into three large subgenera which is *Leuconoe*, *Myotis*, and *Selysius*. However, the molecular data stated that these three subgenera are not natural groups, but more to an unnatural assemblages of convergently similar species.

2.4 Outgroup

Outgroup or was known as outside groups is a group of organism that serve as a reference group in determining evolutionary relationship and ancestral history between organisms. Schlee (1969) stated that, outside groups provide a basis for making strong inferences regarding the ancestral, or old, states. States common both to species inside and outside our group must be ancestral states, or they must of

convergent origin. Outgroup were chosen as reference group in phylogenetic analysis as the character of a particular group is likely to be present in the representatives of closely related group family (Crowson, 1970). The characteristic in choosing outgroup must be satisfy by two characteristics which the outgroup must not be members of the ingroup species and they must be related to the ingroup.

2.4.1 *Kerivoula*

Kerivoula is a genus within the family of Vespertilionidae, and in the subfamily of Kerivoulinae which act as a reference group in this phylogenetic analysis as *Kerivoula* is the closest outgroup to ingroup used in this analysis.

2.5 NJ

Study of molecular evolution and functional genomics have significantly increase with the contribution of inference of phylogenetic trees as they become more important in this field of study. The circumstances exist due to enormous increase in the size of data have lead to the extensive use of NJ methods, which quickly generates a final tree for large phylogenies under the principle of minimum evolution as stated by Saitou & Nei (1987). This provided a way for researches to overcome the hurdle in analysing bigger data sets of genes from diverse species as in the order of hundreds or thousands of sequences. The method of NJ in constructs phylogenetic trees is by clustering neighboring sequences in a stepwise manner. The sum of branch length was minimized in each step of sequence clustering, thus multiple topologies can be examined (Saitou & Nei, 1987).

2.6 ME

ME method is a phylogenetic inferences methods that based on estimated branch lengths, using ordinary least-squares methods (OLS), from distance matrices as stated by Rzhetsky & Nei (1992). Minimum Evolution method used an algorithm where the first step is by constructing a neighbor-joining (NJ) tree procedure (Saitou & Nei, 1987) and then compute the total sum (S) of branch lengths in order to construct ME tree. The S value mentioned above is a value obtained from computing each tree by examining each tree topologies that are close to the NJ tree criteria. The S value were then compared with each other, determining which tree had the smallest S value, thus be chosen as the final value which is usually the NJ tree (Rzhetsky & Nei, 1993).

2.7 MP

Phylogeny tree of Maximum Parsimony (MP) inference involved the identification of a tree topology that requires the smallest number of changes to explain the observed differences. Yang (1996) stated that, MP methods involved a very stringent assumptions, where the process of sequence evolution such as constancy of substitution rates between nucleotides, constancy of rates across nucleotide sites, and equal branch lengths in the tree took concern. The MP algorithm works by determining the amount character changed or tree length which is given by any tree, thus searching all possible tree topologies to produce a tree that can minimize this length. The best tree were chosen based on the shortest pathway, or the tree that have the most minimized tree length.

2.8 ML

Felsenstein (1981) stated that, Maximum Likelihood is a method that use an approach on an explicit and efficient sequence data to formulate a probabilistic model of evolution and to apply a known statistical methods. Maximum Likelihood estimation method used a readily applicable statistical inference data in which to find an evolutionary tree that yields the highest probability of evolving data. The likelihood of the tree produced is the probability of the data given it taken as a function of the hypothesis.

2.9 Bayesian Analysis

Bayesian inference is an analysis of phylogeny that based on the posterior probabilities of phylogenetic trees. Bayesian inference have several advantages compared to other methods of phylogenetic inference as stated by Larget & Simon (1999) such as, easy interpretation of results, the ability to incorporate prior information (if such information is available), and some computational advantages. The needs of MrBayes algorithm in bayesian analysis is crucial as the summation and integrals of the analysis cannot be evaluated analytically (Huelsenbeck & Ronquist, 2001) where the algorithm used Markov chain Monte Carlo (MCMC) to approximate the posterior probabilities of trees (Metropolis , 1953; Hastings, 1970; Green, 1995).

CHAPTER 3

MATERIALS AND METHOD

3.1 Materials

Usable laptop, Mega Evolutionary Genetic Analysis (MEGA 7.0), TOPALi v2.5, FigTree v1.4.3 and 63 partial cytochrome b sequence fragments with at least 1,000 bp linear DNA received from the NCBI GenBank database. The taxon of all genus are listed completely with the ascension number in table 3.1 shown below. The nucleotide compositions for each species were aligned and estimated by MEGA 7.0 software.

Table 3.1 Origin and GenBank accession number of sequenced specimens.

Taxon	GenBank	Localities
Chiroptera		
Family Vespertilionidae		
Genus *Myotis*		
Myotis welwitschii	AJ841954.1, AF376873.1, AF376874.1	Africa
Myotis formosus	AJ841950.1, AB106592.1, EF555234.1	Sumatra
Myotis nattereri	AB106606.1, JF412413.1, JF412411.1	Iran
Myotis altarium	FJ215677.1, EF553530.1, JX465366.1	Thailand
Myotis tricolor	AJ841953.1, AJ504409.1, AJ841952.1	Africa
Myotis auriculus	AM261884.1, JF489122.1, JX130482.1	Mexico
Myotis myotis	AM261883.1, AF376860.1, GU817388.1	Ukraine
Myotis blythii	AF376840.1, AF376842.1, AF376841.1	China

Subfamily Leuconoe		
Genus *Myotis*		
Myotis macrotarsus	AJ841960.1, AF376855.1, AF376856.1	Malaysia
Myotis montivagus	AM262333.1, AF376858.1, AF376857.1	Malaysia
Myotis ricketti	AJ504452.1, AB106608.1, EF517316.1	China
Myotis pruinosus	AB085737.1, AB855787.1, AB106607.1	Japan
Myotis daubentoni	AB106590.1, AB106589.1, AY665137.1	Japan
Myotis fimbriatus	EF555226.1, KP187858.1, KP187857.1	China
Myotis longipes	FJ215678.1, EF555231.1, EF555230.1	India

Subfamily Selysius		
Genus *Myotis*		
Myotis siligorensis	FJ215679.1, KF312530.1, KF312528.1	Malaysia
Myotis brandtii	AM261886.1, AF376844.1, AY665139.1	German
Myotis muricola	AJ841957.1, AY665144.1, AY665143.1	Nepal
Myotis alcathoe	AJ841955.1, KF874511.1, JQ044687.1	Greece
Subfamily Chrysopteron		
Genus *Myotis*		
Myotis flavus	EU434932.1, KP187861.1, KP187860.1	China
Outgroups		
Subfamily Kerivoulinae		
Genus *Kerivoula*		
Kerivoula cf. papillosa	AJ841970.1	Malaysia
Kerivoula whiteheadi	EU188791.1	Malaysia
Kerivoula pellucida	EU188788.1	Malaysia

3.2 Phylogenetic Analysis

Each model of phylogenetic tree was inferred using different methods of analysis. Phylogenetic reconstructions were performed with the Neighbor Joining (NJ), Minimum Evolution (ME), Maximum Parsimony (MP) and Maximum Likelihood (ML) method implemented in MEGA 7 (Kumar *et al.*, 2016) and Bayesian inferences were carried out using TOPALi v2.5. The analysis involved 63 nucleotide sequences and the codon positions included were first +second +third + noncoding.

3.2.1 Neighbor-Joining Method

The Neighbor-Joining phylogeny test was run using bootstrap method. The number of bootstrap replications used for this test were 1000 replications. Kimura 2-parameter model was chosen as a method to compute the evolutionary distances (Kimura, 1980). Complete deletion were selected as a treatment for gaps/missing data treatment in data subsets to use option.

3.2.2 Minimum Evolution Method

The Minimum Evolution phylogeny test was run using bootstrap method. The number of bootstrap replications used for this test were 1000 replications. Kimura 2-parameter model was chosen as a method to compute the evolutionary distances (Kimura, 1980). Complete deletion were selected as a treatment for gaps/missing data treatment in data subsets to use option. The Close-Neighbor-Interchange (CNI) algorithm (Nei & Kumar, 2000) were used at a search level of 1 for the ME heuristic method.

3.2.3 Maximum Parsimony Method

The Maximum Parsimony phylogeny test was run using bootstrap method. The number of bootstrap replications used for this test were 1000 replications. Complete deletion were selected as a treatment for gaps/missing data treatment in data subsets to use option. The Tree-Bisection-Regrafting (TBR) algorithm (Nei & Kumar, 2000) were used at a search level of 1 for the MP search method.

3.2.4 Maximum Likelihood Method

The Maximum Likelihood phylogeny test was run using bootstrap method. The number of bootstrap replications used for this test were 1000 replications. Kimura 2-parameter model was chosen as a method to compute the evolutionary distances (Kimura, 1980). Complete deletion were selected as a treatment for gaps/missing data treatment in data subsets to use option. The Neighbor-Join and BioNJ algorithms (Gascuel, 1997) were applied for the ML heuristic method.

3.2.5 Bayesian Inference Method

The Bayesian Inference analysis was run using TOPALi v2.5 software. Generalised Time Reversible (GTR) were selected including Gamma and Invariable Sites as its run model selection. MrBayes algorithms were used for the BI search method (Huelsenbeck & Ronquist, 2001). The test were run 2 times with 1,000,000 number of generations with 10 sampling frequencies.

3.3 Phylogenetic Tree

Phylogenetic tree for Neighbor Joining (NJ), Minimum Evolution (ME), and Maximum Parsimony (MP) were exported from MEGA 7.0 as a Newick format and Maximum Likelihood (ML) and Bayesian inferences were exported from TOPALi v2.5 as a New Hampshire tree format or also known as Newick format. The exported file were then opened by using FigTree v.1.4.3 software and the phylogenetic tree for each tree were then designed accordingly.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Sequence Analysis

In this study, the gene partial sequences for length of 1,096 bp linear DNA comprising of cytochrome b gene from 63 sequences of different genus species of chiroptera obtained from GenBank as listed in Table 3.1 that used for phylogenetic analysis were successfully sequenced and aligned. There were a total of 1096 positions in the final dataset. All positions containing gaps and missing data were eliminated.

The evolutionary history of phylogenetic tree in Figure 4.1 was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 2.30194384 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site.

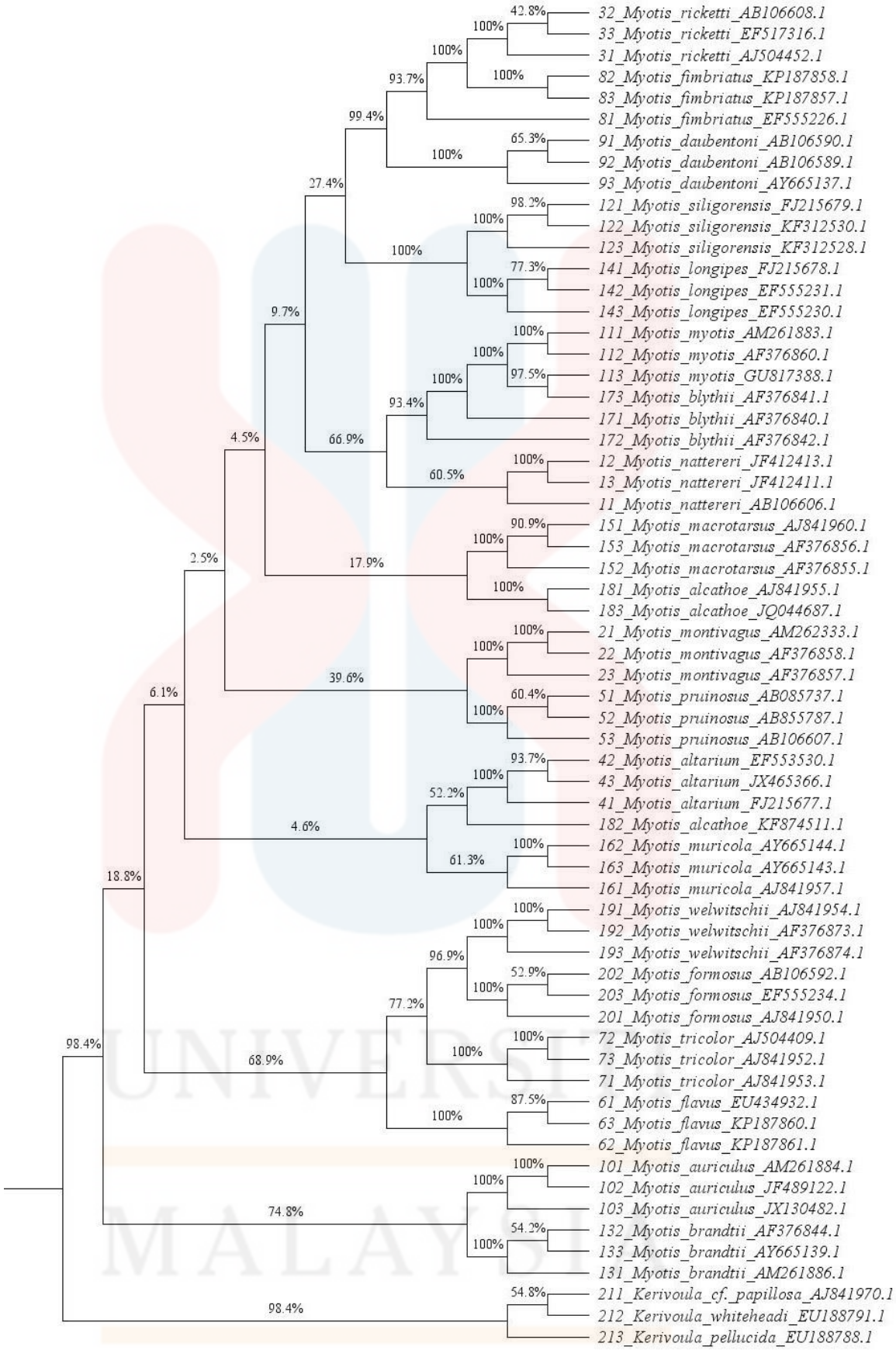

Figure 4.1 Rooted NJ tree generated using cyt b gene sequences of the *Myotis* species. Values on the branches represent NJ bootstrap estimates, based on 1000 replicates.

The evolutionary history of phylogenetic tree in Figure 4.2 was inferred using the Minimum Evolution method (Rzhetsky & Nei, 1992). The optimal tree with the sum of branch length = 2.30194384 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei & Kumar, 2000) at a search level of 1. The Neighbor-joining algorithm (Saitou & Nei, 1987) was used to generate the initial tree.

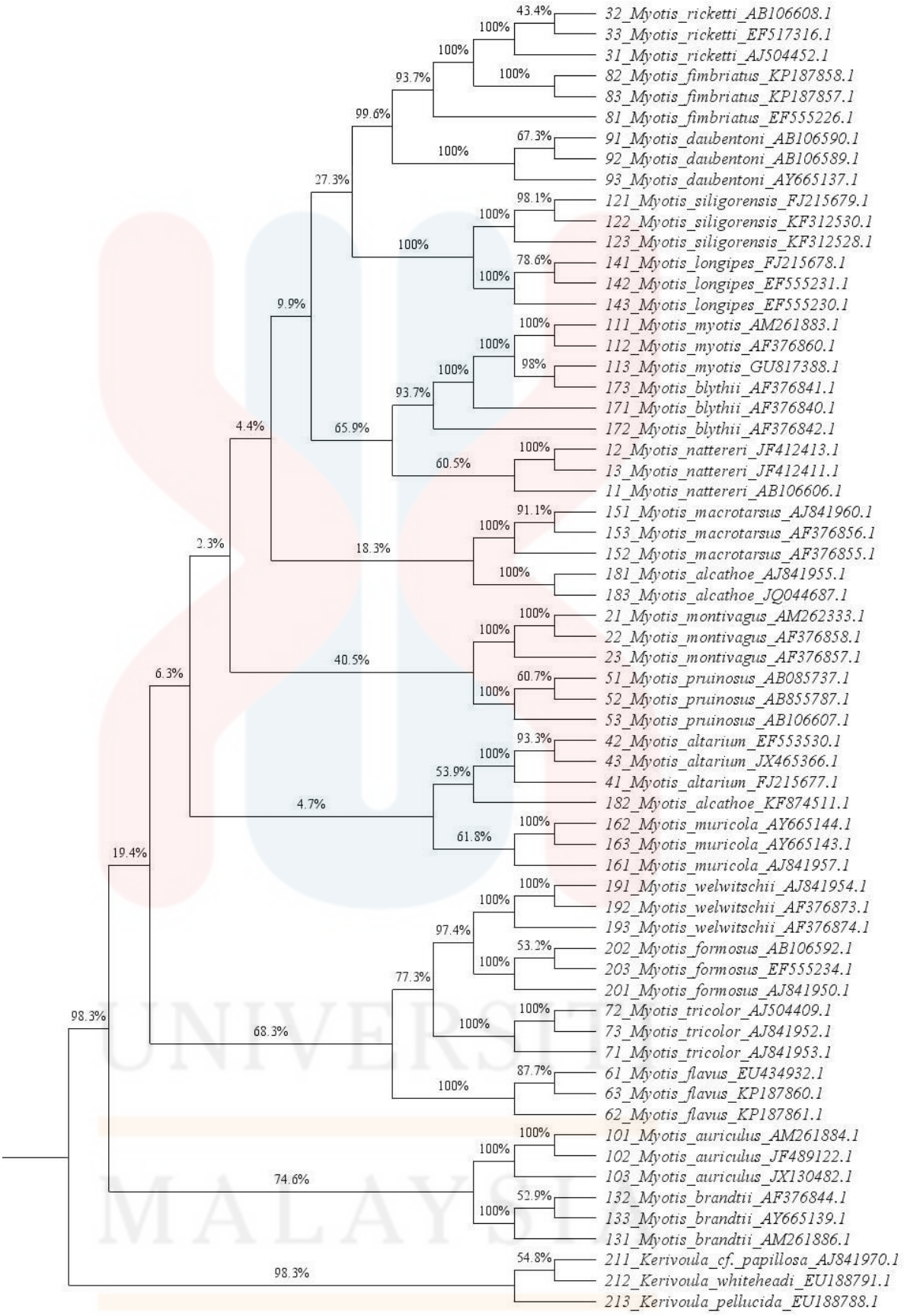

Figure 4.2 Rooted ME tree generated using cyt b gene sequences of the *Myotis* species. Values on the branches represent ME bootstrap estimates, based on 1000 replicates.

The evolutionary history of phylogenetic tree in Figure 4.3 was inferred using the Maximum Parsimony method. Tree #1 out of 3 most parsimonious trees (length = 2734) is shown. The consistency index is (0.247573), the retention index is (0.698624), and the composite index is 0.183727 (0.172960) for all sites and parsimony-informative sites. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The MP tree was obtained using the Tree-Bisection-Regrafting (TBR) algorithm (Nei & Kumar, 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates).

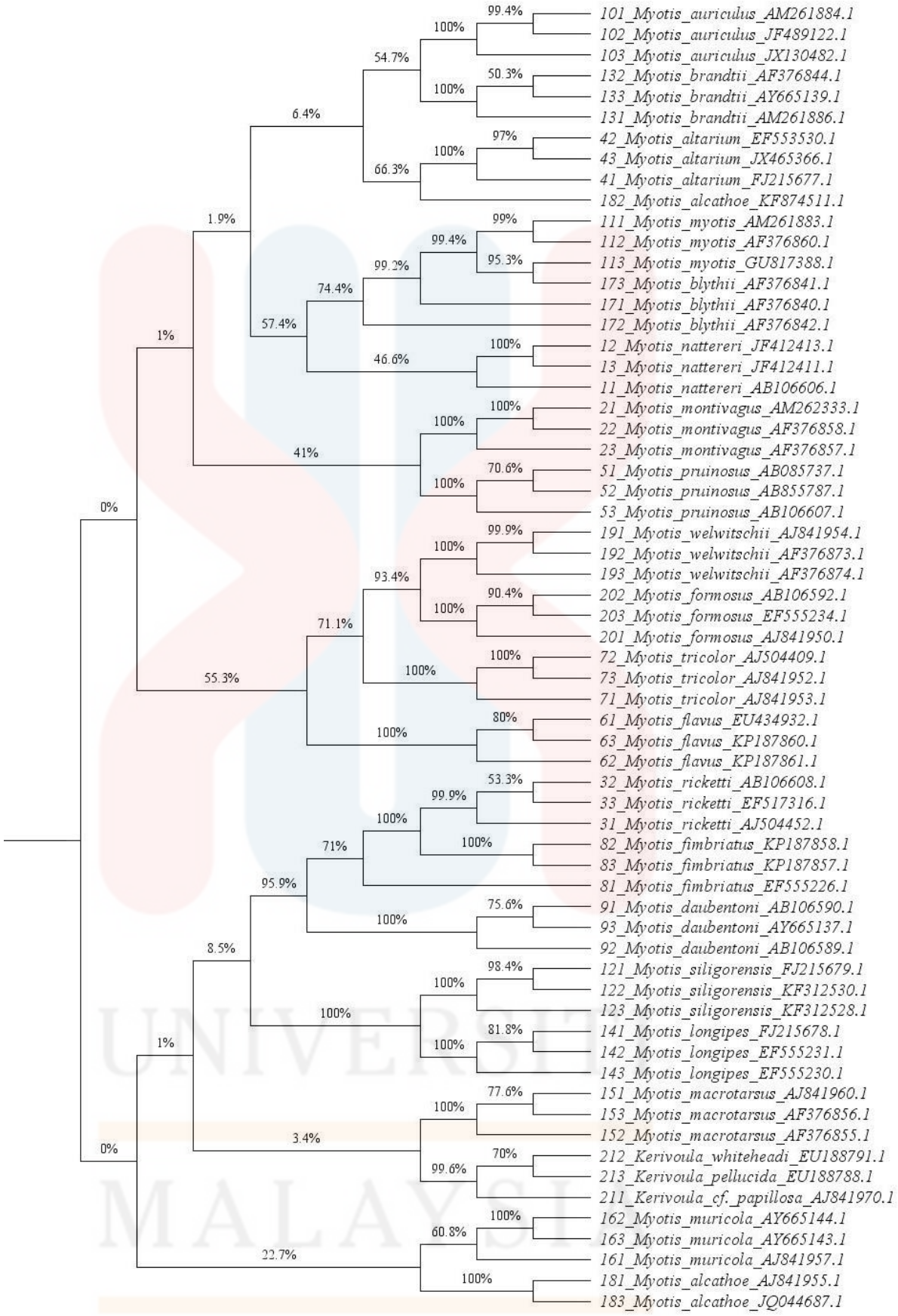

Figure 4.3 Rooted MP tree generated using cyt b gene sequences of the *Myotis* species. Values on the branches represent MP bootstrap estimates, based on 1000 replicates.

The evolutionary history of phylogenetic tree in Figure 4.4 was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-14426.8647) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

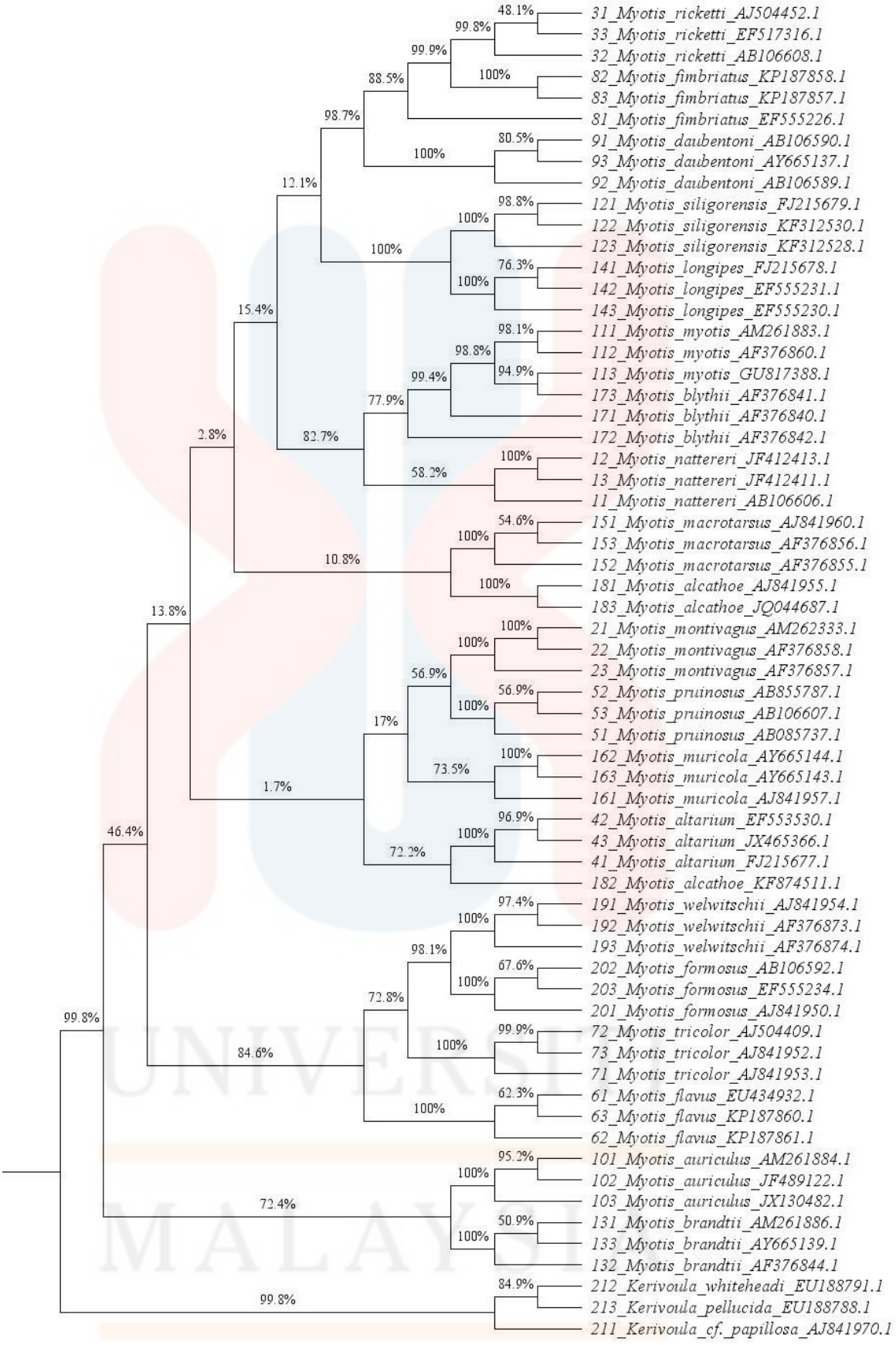

Figure 4.4 Rooted ML tree generated using cyt b gene sequences of the *Myotis* species. Values on the branches represent ML bootstrap estimates based on 1000 replicates.

The evolutionary history of phylogenetic tree in Figure 4.5 was inferred using the Bayesian Inference analysis method (Huelsenbeck & Ronquist, 2001). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The BI tree was obtained using the MrBayes algorithm (Huelsenbeck & Ronquist, 2001). There were a total of 1096 positions in the final dataset. Evolutionary analyses were conducted in TOPALi v2.5 (James Berger, 2006**).** MrBayes output at 95% credit interval were shown in table 4.1 below.

Table 4.1 MrBayes output at 95% credit interval

Parameter	Mean	Variance	Lower	Upper	Median	PSRF*
TL	5.721034	0.129144	5.086000	0.516096	0.48673	1.000
r(A<->C	0.024704	0.000011	0.018589	0.031657	0.02462	1.002
r(A<->G	0.398108	0.001545	0.324254	0.480580	0.39620	1.012
r(A<->T	0.021118	0.000010	0.015295	0.027449	0.02106	1.001
r(A<->G	0.004339	0.000009	0.000211	0.011363	0.00384	1.001
r(A<->T	0.531711	0.001446	0.453194	0.602959	0.53342	1.012
r(A<->T	0.020020	0.000031	0.010381	0.031933	0.01963	1.000
pi(A)	0.336551	0.000152	0.312259	0.361031	0.33623	1.001
pi(C)	0.303478	0.000081	0.285775	0.321130	0.30360	1.005
pi(G)	0.079019	0.000063	0.064341	0.095681	0.07892	1.007
pi(T)	0.280952	0.000074	0.264257	0.297918	0.28118	1.002
alpha	1.391355	0.026349	1.097284	1.725609	1.38244	1.001
pinvar	0.486693	0.000228	0.456685	0.516096	0.48673	1.000

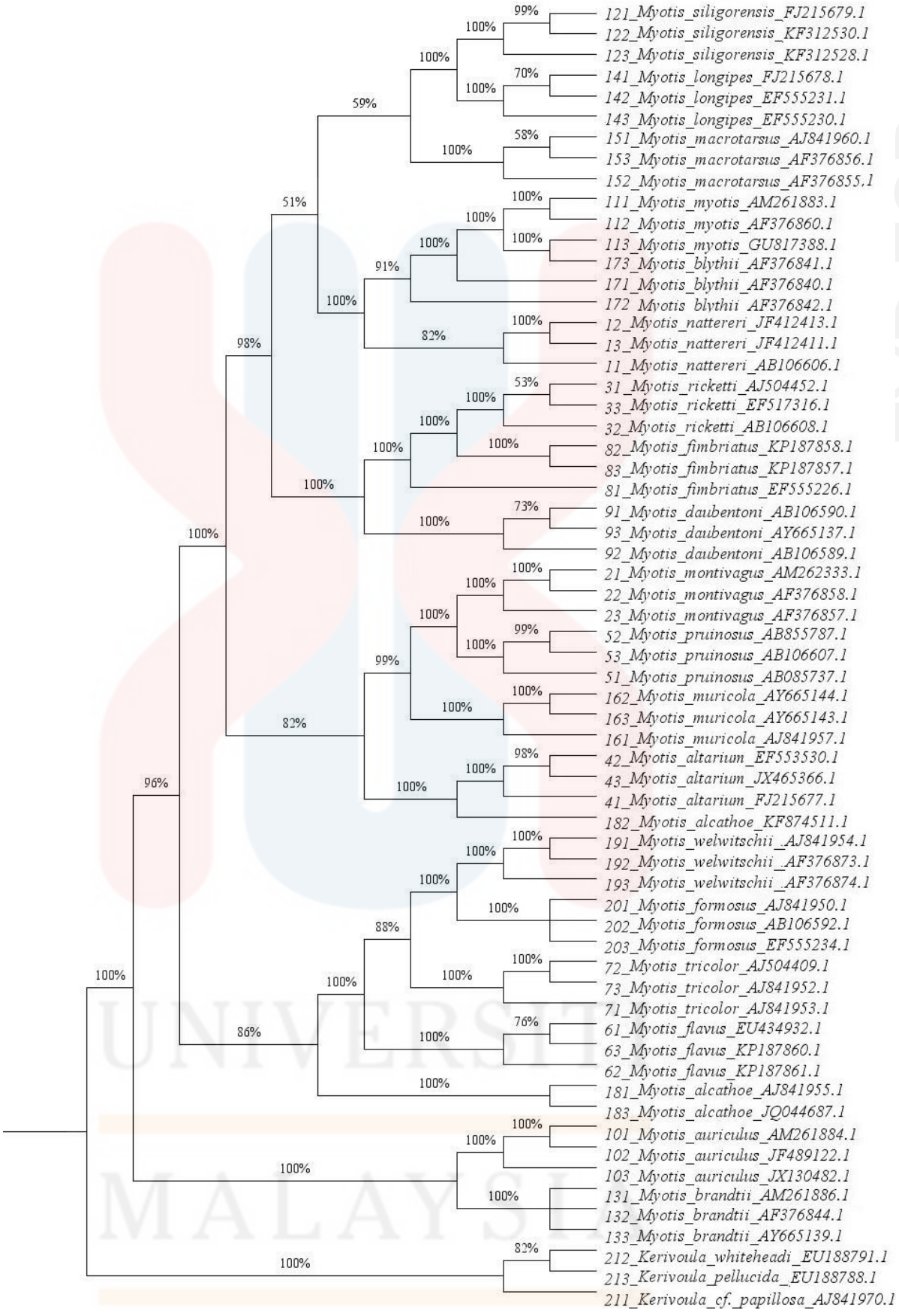

Figure 4.5 Rooted Bayesian tree generated using cyt b gene sequences of the *Myotis* species. Values on the branches represent BI bootstrap estimates, based on 500 replicates.

4.2 Phylogenetic Tree Analysis

Phylogenetic trees constructed from NJ, ME, MP, ML, and BI methods were summarised in Figure 4.1, 4.2, 4.3, 4.4 and 4.5 respectively with its bootstrap value. Topologies construction of the phylogenetic tree obtained from NJ, ME, ML and BI shown a minor differences in genera grouping. The bootsraps value shown within the genera relationship *Myotis* and *Kerivoula* in these four trees show a high confidence values (with boostrap value of 90% and above). Bootstrap value within inter genera relationship in NJ, ME, and ML are variable (with 2% < bootsrap value > 90%) while BI shown a high confidence value within inter genera relationship (with 50% bootstrap value). As for Maximum Parsimony method, the topologies constructed from the phylogenetic tree shown a major differences in genera grouping when compared to other four method.

NJ, ME, ML, and BI method produced tree a comprising of two major clades as shown in Figure 4.1, 4.2, 4.3, and 4.4. The clades of each tree were similar as the first clade was formed by genera *Myotis* (with 98% bootstrap value) and the second clade was formed by genera *Kerivoula* (with 98% bootstrap value).

As for MP methods, all characters were weighted equally, the tree length was 2734 with consistency index (CI) of 0.247573, the retention index is (0.698624), and the composite index is 0.183727 (0.172960) for all sites and parsimony-informative sites. The tree topology consists of 2 major clades with a 0% bootstrap value (Figure 4.3). The first clade consists of *Myotis* inter genera species (with 1% to 55% bootstrap value) and second clade consist of six group of *Myotis* and one group of *Kerivoula (M. ricketti, M. fimbriatus, M. daubentoni, M. siligorensis, M. longipes, M. macrotarsus,*

K. whiteheadi, K. pellucida and *K. cf. papillosa*) with a 1% bootstrap value and two groups of *Myotis* (*M. muricola* and *M. alcathoe*) with a 22% bootstrap value.

The analysis using partial cytochrome b gene sequences among the 63 species of Chiroptera provided a proof of their phylogenetic relationship. The methods used on the nucleotide sequences data employing construct phylogenetic trees (Neighbor-Joining, Minimum Evolution, Maximum Parsimony, Maximum Likelihood and Bayesian Inferences) with bootstrap method analysis have helped with clarifying the relationship in this study (Guan, 2006).

It can be observed that the phylogenetic trees produced from all five methods are reliable as the relationship within Chiroptera species can be deducted from the branches. There are no separated branches shown in all five phylogeny tree which indicated that there is a relationship in evolutionary history between genera *Myotis* and *Kerivoula.* The bootstrap value shown in BI phylogeny tree in figure 4.5 indicate a high confidence value with bootstrap value of 51% to 100% compared to other trees. Based on the result made from the approach method, bayesian analysis methods have shown the highest confidence value compared to other four methods as the bayesian methods have more advantages compared to other four methods (Larget & Simon, 1999).

CHAPTER 5

CONCLUSION

5.1 Conclusion

It can be concluded that, the results produced from all the approached methods proves the ancestry evolution history between genera *Myotis* and *Kerivoula*. Bayesian methods give the best possible value of minimum sum of branch (confidency) lengths with a differences in comparison of topologies in each tree shown in Figure 4.1, 4.2, 4.3, 4.4 and 4.5. The phylogenetic methods used in this study were able to show the differences of estimation in ancestry of samples and comparison can be made. Thus, the correct tree with highest probabilities is the Bayesian inferences phylogeny tree.

5.2 Recommendation

There are few recommendation that can be done to improve the results of phylogeny tree produced. The gaps or missing data present during the sequencing and aligning DNA sequences phase can be treated by eliminating the gaps and replacing the missing data that present in any sequences with other partial cytochrome b genes sequence of that particular species. The improvement also can be done by using the best outgroups species that meet the characteristic for it to undergo phylogenetic analysis with genus *Myotis.*

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APPENDICES

Table 1 Nucleotide composition for each species used in this study

No	Species	Nucleotide Composition			
		T(U)	C	A	G
1	*Myotis nattereri*	29.4	27.1	30.8	12.7
2	*Myotis nattereri*	32.3	24.6	31.3	11.8
3	*Myotis nattereri*	31.6	25.3	30.9	12.3
4	*Myotis montivagus*	31.6	25.8	29.5	13.1
5	*Myotis montivagus*	31.6	25.8	29.5	13.1
6	*Myotis montivagus*	32.1	25.5	29.2	13.2
7	*Myotis ricketti*	30.5	26.5	30.5	12.6
8	*Myotis ricketti*	30.4	26.6	30.7	12.4
9	*Myotis ricketti*	30.5	26.4	30.6	12.5
10	*Myotis altarium*	31.1	25.6	30.7	12.6
11	*Myotis altarium*	31.5	25.2	30.5	12.8
12	*Myotis altarium*	31.2	25.4	30.4	12.9
13	*Myotis pruinosus*	32.1	25.6	30.4	11.9
14	*Myotis pruinosus*	32.3	25.4	30.3	12.0
15	*Myotis pruinosus*	32.1	25.6	30.2	12.1
16	*Myotis flavus*	31.7	25.4	30.9	12.0
17	*Myotis flavus*	31.8	25.3	30.9	12.1
18	*Myotis flavus*	31.6	25.5	30.9	12.0
19	*Myotis tricolor*	31.0	26.2	29.5	13.3
20	*Myotis tricolor*	30.7	26.0	30.1	13.2
21	*Myotis tricolor*	30.7	26.0	30.1	13.2
22	*Myotis fimbriatus*	31.5	25.5	30.4	12.5
23	*Myotis fimbriatus*	31.2	25.7	30.6	12.5
24	*Myotis fimbriatus*	31.2	25.7	30.4	12.7
25	*Myotis daubentoni*	30.6	26.1	30.7	12.5
26	*Myotis daubentoni*	30.5	26.1	30.8	12.5
27	*Myotis daubentoni*	30.6	26.1	30.4	12.9
28	*Myotis auriculus*	30.6	26.3	29.6	13.5
29	*Myotis auriculus*	30.5	26.5	29.6	13.4
30	*Myotis auriculus*	30.8	26.3	29.5	13.4
31	*Myotis myotis*	31.7	25.4	30.1	12.8
32	*Myotis myotis*	31.6	25.6	30.1	12.7
33	*Myotis myotis*	31.8	25.2	30.3	12.7
34	*Myotis siligorensis*	29.6	27.9	29.3	13.2
35	*Myotis siligorensis*	29.7	27.9	29.1	13.4
36	*Myotis siligorensis*	29.8	27.8	29.1	13.4
37	*Myotis brandtii*	30.1	26.8	29.9	13.2
38	*Myotis brandtii*	30.1	26.8	29.7	13.3
39	*Myotis brandtii*	30.2	26.8	29.7	13.3
40	*Myotis longipes*	30.2	27.4	29.1	13.3
41	*Myotis longipes*	30.2	27.4	29.1	13.3
42	*Myotis longipes*	30.4	27.2	29.1	13.3
43	*Myotis macrotarsus*	29.6	27.8	29.9	12.6
44	*Myotis macrotarsus*	29.8	27.7	29.4	13.1

Continued Table 1

No	Species	Nucleotide Composition			
		T(U)	C	A	G
45	*Myotis macrotarsus*	30.0	27.5	29.9	12.6
46	*Myotis muricola*	29.2	28.6	29.3	12.9
47	*Myotis muricola*	32.0	25.3	28.9	13.8
48	*Myotis muricola*	32.7	24.6	29.0	13.7
49	*Myotis blythii*	31.6	25.7	30.3	12.5
50	*Myotis blythii*	32.2	24.5	30.6	12.6
51	*Myotis blythii*	32.6	24.5	30.2	12.8
52	*Myotis alcathoe*	32.6	23.9	30.3	13.2
53	*Myotis alcathoe*	32.7	24.4	29.9	13.0
54	*Myotis alcathoe*	32.7	23.9	30.2	13.2
55	*Myotis welwitschii*	33.9	22.6	31.1	12.4
56	*Myotis welwitschii*	33.7	22.8	31.0	12.5
57	*Myotis welwitschii*	33.3	23.4	30.9	12.5
58	*Myotis formosus*	30.9	26.5	29.3	13.3
59	*Myotis formosus*	30.8	26.3	29.8	13.1
60	*Myotis formosus*	30.6	26.5	29.8	13.1
61	*Kerivoula cf. papillosa*	26.1	29.7	31.7	12.5
62	*Kerivoula whiteheadi*	28.6	27.5	31.6	12.3
63	*Kerivoula pellucida*	29.2	29.1	28.1	13.6
	Avgerage (Outgroups Exluded)	31.1	25.9	30.1	12.8
	Avgerage (Outgroups Included)	31.0	26.0	30.1	12.8

Table 2 Pairwise distances in percentage among 23 species of family Chiroptera analysed based on the partial cyt *b* gene. The distances were calculated using Kimura's two-parameter model of nucleotide substitution.

No	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	*Myotis nattereri*	-																						
2	*Myotis montivagus*	16.5	-																					
3	*Myotis ricketti*	15.6	16.8	-																				
4	*Myotis altarium*	18.0	16.1	16.7	-																			
5	*Myotis pruinosus*	15.8	15.8	14.6	18.6	-																		
6	*Myotis flavus*	18.6	17.6	15.1	17.2	19.3	-																	
7	*Myotis tricolor*	17.8	18.4	16.3	16.1	18.5	14.9	-																
8	*Myotis fimbriatus*	17.9	16.6	10.3	16.3	16.5	17.4	17.5	-															
9	*Myotis daubentoni*	15.2	16.4	11.3	15.9	16.0	16.3	18.0	10.8	-														
10	*Myotis auriculus*	19.3	18.9	16.8	16.5	17.5	17.1	17.8	18.1	16.3	-													
11	*Myotis myotis*	14.2	17.6	14.7	15.9	15.5	17.1	16.4	15.0	13.8	15.4	-												
12	*Myotis siligorensis*	16.9	17.1	15.4	16.4	17.2	18.8	19.3	16.4	13.7	18.4	14.2	-											
13	*Myotis brandtii*	17.3	18.7	16.3	17.9	17.1	17.9	19.3	19.1	16.6	15.9	16.7	16.0	-										

Continued Table 2

No	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
14	*Myotis longipes*	16.7	17.9	15.3	18.1	15.9	17.9	19.3	16.6	15.1	16.9	15.0	8.5	16.4	-									
15	*Myotis macrotarsus*	15.3	16.1	16.6	16.7	17.6	17.7	17.3	16.1	15.5	17.6	16.5	16.3	18.5	17.1	-								
16	*Myotis muricola*	20.2	18.8	18.0	18.4	17.5	20.3	16.6	17.5	17.7	18.3	17.8	17.7	19.3	18.7	19.0	-							
17	*Myotis blythii*	14.3	17.1	14.3	16.1	16.0	18.7	17.5	15.0	14.7	16.0	5.4	15.6	16.4	15.8	15.6	18.6	-						
18	*Myotis alcathoe*	17.4	19.8	16.8	17.6	16.8	19.3	18.1	19.0	16.9	19.0	18.5	18.0	19.5	17.3	17.3	18.3	18.7	-					
19	*Myotis welwitschii*	19.2	17.5	18.8	18.1	19.9	18.7	16.7	18.0	17.6	20.3	18.9	20.3	19.2	19.4	19.3	19.6	19.0	19.6	-				
20	*Myotis formosus*	20.2	18.1	19.5	20.4	18.9	18.7	14.8	20.9	19.6	20.7	18.2	20.3	20.3	20.5	19.2	20.0	19.4	18.6	13.4	-			
21	*Kerivoula cf. papillosa*	17.5	20.3	16.8	18.7	18.9	20.1	19.5	17.5	17.8	18.9	18.4	18.6	17.4	17.7	18.8	17.2	18.5	20.6	21.0	20.3	-		
22	*Kerivoula whiteheadi*	20.2	19.6	20.2	20.1	19.4	20.1	21.2	19.7	18.8	20.6	21.7	19.9	20.9	19.9	19.4	23.7	20.9	21.1	22.7	22.8	15.4	-	
23	*Kerivoula pellucida*	24.2	23.4	21.4	21.7	22.4	23.9	21.1	21.6	21.5	22.7	22.0	23.3	22.7	22.9	23.9	21.3	22.4	22.1	24.1	23.7	18.5	19.8	-