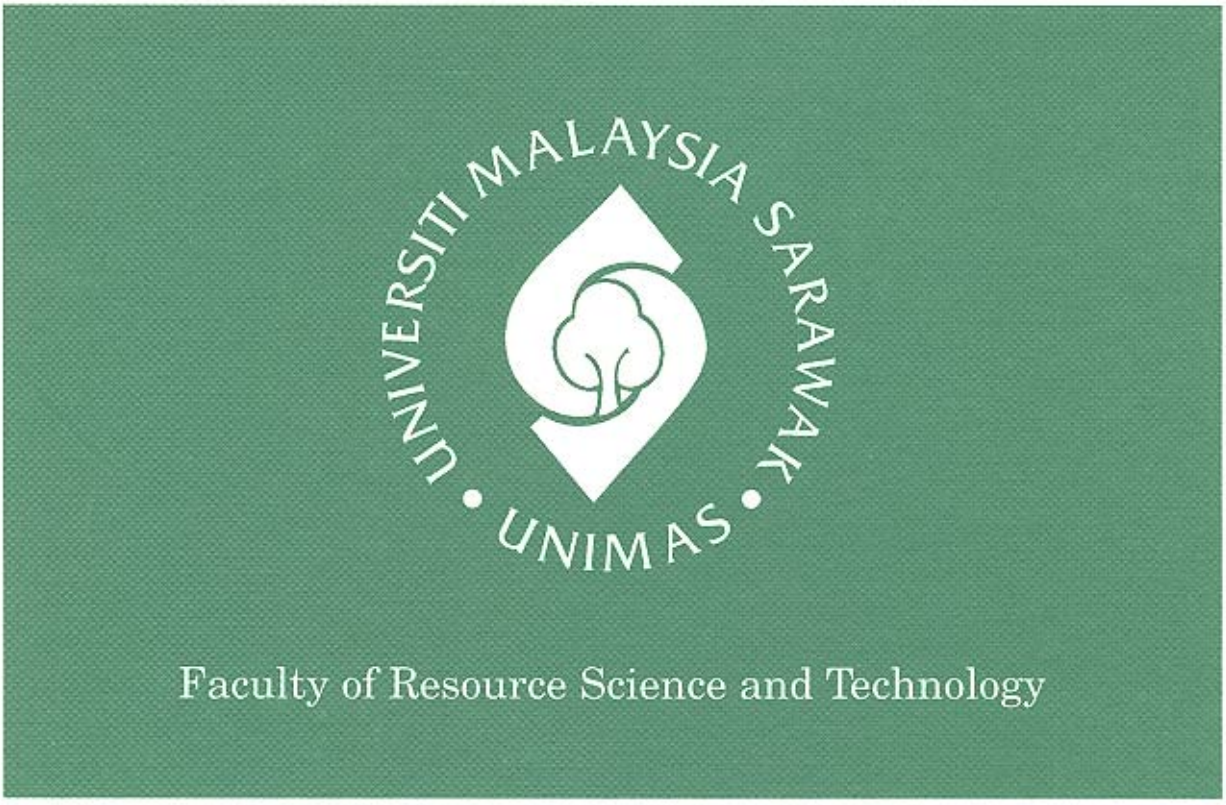

PHYLOGENETIC RELATIONSHIP AMONG SELECTED SPECIES OF BULBUL (FAMILY: PYCNONOTIDAE) BASED ON 16S rDNA

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Bachelor of Science with Honours
(Animal Resource Science and Management)
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Faculty of Resource Science and Technology

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ABSTRACT

Phylogenetic relationships among selected species of bulbuls from the family Pycnonotidae using 16S rDNA was examined with the aim of constructing phylogenetic tree. Samples were taken from six different locations in Sarawak and Sabah namely Wind Cave and Fairy Cave in Bau, Sebako in Lundu, Matang in Kubah National Park, Raven's Court in Lawas (Sarawak) and Balambangan Island (Sabah). A total of 28 blood samples were obtained from all locations. Of these 25 was successfully extracted for DNA and PCR products were obtained. Of the 25 PCR products, 20 produced good sequence. A neigbour-joining bootstrap 50% majority-rule consensus with Kimura-2 parameter trees was constructed. The nucleotide diversity among the selected species of bulbul ranged between 3.5% and 21.0% and within species was 0.6% and 32.6%. The rate of gene flow between Sebako and Matang Wildlife Centre population showed higher value (Nm=15.3) compared to Sebako and Bau (Nm=1.0). The difference between the population and existing landuse that has broken the wildlife corridor.

Key words: Pycnonotidae, Nucleotide diversity, Gene flow and Phylogenetic tree.

ABSTRAK

Kajian hubungan filogenetik antara spesies terpilih dari famili Pycnonotidae mengunakan 16S rDNA bertujuan untuk membina pokok filogenetik. Sampel telah diambil dari enam lokasi berbeza di Sarawak dan Sabah seperti di Gua Angin dan Gua Pari-pari di Bau, Sebako di Lundu, Matang di Taman Negara Kubah, Raven's Court di Lawas (Sarawak) dan Pulau Balambangan (Sabah). Sebanyak 28 sampel darah telah diperolehi dari semua lokasi ini. Dari kesemua sampel itu 25 DNA dan produk PCR telah berjaya dihasilkan. Sebanyak 20 produk PCR telah berjaya memperolehi turutan yang baik. Pokok Kimura-2 parameter bersama dengan "neihbour-joining bootstrap 50% majority-rule consensus" telah dibina. Kepelbagaian nukleotit diantara 3.5% ke 21.0% dan 0.6% ke 32.6% bagi spesies. Kadar aliran gen diantara populasi Sebako dan Pusat Hidupan Liar Matang menunujukkan nilai yang tinggi (Nm=15.3) dibandingkan dengan Sebako dan Bau (Nm=1.0). Perbezaan diantara populasi wujud dengan penerokaan tanah yang telah memutuskan koridor hidupan liar ini.

Kata kunci: Pycnonotidae, Kepelbagaian Nukleotit, Aliran gen dan Pokok Filogenetik.

Introduction

Birds are often cited as the best-known group of animals because almost all of living species have been discovered, described and named, but our understanding of their phylogeny and classification has been no better than for other groups of organism (Sibley and Alhquist 1995). Birds appear to be favourite pet to human beings due to its voices and beautiful color. In the wild birds play a very important role in the ecosystem as food chain, pollinator, and seed dispersal and balance the ecosystem. According to Wong (1982), in Malaysia birds that feed on insects are more than birds that feed on fruits and nectar. This shows that bird played important role in biological control of insect and at the same time controlling the population of insect. Birds are abundantly found in the tropical areas where about 85% of bird species are found in the tropic (Griscom, 1945).

In Borneo there are about 622 species of birds and 39 species are endemic to Borneo (Davison, 1999 and Pyne 1995). Sarawak has about 350 species of birds. According to Smythies (1981) there are about 24 species of bulbul found in Borneo and 14 species feed on fruits. Bulbuls are songster birds from the family of Pycnonotidae. Most of this species in this family can be found in primary and secondary forests, garden and montane resident (Smythies 1981). These birds are monomorphic with both male and female look similar. Monomorphic bird has similar color of the feathers and morphological characteristics between male and female. However according to Reduan (2002), most of the species are different in size between male and female. Morphology is an obvious source of evidence of genealogical relationship. We can expect related organism to be

similar in structure but morphological characters are prone to convergent evolution, which cause unrelated species look similar to the human eye because their body have been adapted to cope with the same environmental stresses (Sibley and Ahlquist 1995). Deoxyribonucleic acid (DNA) is the hereditary genetic material of most of the organisms on the Earth; except for certain viruses, which have ribonucleic acid (RNA) (Sibley and Ahlquist, 1995). Every gene has different rate of evolution. In this study 12S and 16S ribosomal DNA (rDNA) will be used as genetic markers. The rates of evolution for 12S and 16S rDNA restriction sites are slow and they are suitable for interspecific study. Indeed, complex organism such as human beings possess DNA sequences that are uniquely and specifically present only in particular individuals and these unique variation makes it possible to trace genetic material back to its origin, identifying with precision at least what species of organism it came from, and often which particular member of that species. DNA-DNA hybridization measures degrees of genealogical relationship among species to reveal the branching pattern of the phylogeny because the net effect of genetic evolution is sure to be divergence and this takes time. The degrees of differentiation among DNAs of living birds are relative to the length of time since their lineage diverged from their most recent common ancestor (Sibley and Alhquist, 1995).

According to Vrijenhoek (1998), molecular markers have been proven their usefulness in solving many difficult taxonomic problems with endangered species, in designing and also monitoring captive breeding Programme and understanding breeding systems, in detecting the geographical structure of genetic diversity, in managing gene flow and in understanding the factors contributing to fitness. Molecular systematic can also reveal the phylogeny of alleles within species, revealing previously inaccessible details of historical biogeography and population

process (Avise, 1994). Phylogenetic information provides a source of information for analysis of species and within species. According to Avise (1994) different types of molecular assay provide genetic information ideally suited to different subset of this hierarchy and a continuing challenge is to develop and utilize molecular methods appropriate for a particular biological problem at hand. Among these are the phylogenetic studies that may lead to the construction of the tree of life or phylogeny. From this information we can estimate the distances through the branching of the genetic changes from ancestral gene to ancestral species and species to subspecies. Molecular phylogenies may provide a perspective on population growth and connectivity over evolutionary time (Moritz, 1995). The molecular geneticist on the other hand, have focused on the structure of genes and on how genes work and are regulated (Paolella, 1998). Since the areas where some samples would be taken is the formation of limestone area and montane region with feature and vegetation that may have some influence on the species, so it is expected that the most recent speciation events within tropical avian group has predominantly occurred in montane regions (Roy, 1997).

Most taxonomic groups were based on morphology and molecular study is another approach to produce a much clearer resolution on phylogenetic relationship between the species of bulbul. Since some species of bulbul are endangered, molecular data on this species would be most valuable information. The aim of this study was to determine the pattern of relationship among selected species of bulbul and genetic variation within selected species in the family of Pycnonotidae using 16S rDNA gene fragment.

Materials and Methods

A total of 28 samples representing nine species of bulbuls from family Pycnonotidae were collected from Wind Cave, Fairy Cave, Matang Wildlife Center, Sebako, Raven's Court (Sarawak) and Balambangan Island (Sabah). The bird was caught using mist-net that was put up in locations that are potential to catch them. Then the birds were identified by using Smythies (1981) and Lekagul (1991) and Mackinnon and Phillip (1993). Information regarding the measurement of the bird like the length of tarsus, beak, tail, body and body weight were recorded. Blood samples were then taken from the vein of the wing by piercing sterile syringe to the capillary vein. Blood was collected by using capillary tube (30µl) and transferred to an appendof tube containing lysis buffer (50 mM Tris buffer-pH 8.0, 0.1 M ETDA and 0.5% SDS). According to Sibley and Ahlquist (1995) EDTA chelates metallic ions, thus it prevents clotting and inhibit the activity of deoxyribunuclease (DNase), which degrades DNA by enzymatic cleavage of the nucleotide strands. Tissues from soft organs like liver, spleen, heart, lungs, kidneys and brain are good sources of DNA (Sibley and Ahlquist 1995).

Table 1: Samples description and locality of collection

Location	Species	No.of individual	Abbreviation
Fairy Cave	Red-eyed Bulbul (*Pycnonotus*	2	REBF
N01°22.390'	*brunneus)*		
E110°07.108'	Olive-winged Bulbul (*P. plumosus*)	3	OWBF
Wind Cave	Red-eyed Bulbul (*P. brunneus*)	2	REBW
N01°24.915'	Olive-winged Bulbul (*P. plumosus*)	1	OWBW
E110°08.109'	Spectacled Bulbul	1	SPBW
	(*P. erthropthalmos*)		
Matang Wildlife	Yellow-bellied Bulbul (*Criniger*	2	YBBM
Center	*phaeocephalus)*		
N01°36.664'	Puff-backed Bulbul (*P. eutilotus*)	1	PBBM
E110°09.503'			
Balambangan Island	Cream-vented Bulbul (*P. simplex*)	2	CVB
Sebako	Olive-winged Bulbul (*P. plumosus*)	4	OWBS
	Yellow-bellied Bulbul		
	(*C. phaeocephalus*)	6	YBBS
	Spectacled Bulbul		
	(*P. erthrothalmos*)	1	SPBS
	Hairy backed Bulbul		
	(*Hypsipetes iridescens*)	1	HBBS
	Ochraceous Bulbul (*C. ochraceus*)	1	OCHBS
Raven's Court	Yellow-vented Bulbul (*P. goiavier*)	1	YVBR
	Ochraceous Bulbul (*C. ochraceus*)	1	OCHBR

DNA Extraction

Genomic DNA was extracted from blood samples using commercial kits available from Genespin™ Blood DNA. DNA can be extracted from the nuclei of all cells, but nucleate red blood cells provide the best and most convenient sources that produce more cells per unit and long-chained DNA compare to tissues (Sibley and Alhquist 1995). The DNA containing cells are isolated and removed from within the nuclear and mitochondrial membranes. Elution buffer is used as reagent. Most reagents in lysis buffers have been noted for their ability to lyse cell and organelle membranes and other reagents are use in lysis buffer to maintain the pH, preserve the

DNA or interact with proteins. The process is to purify DNA from other cellular components and potential contaminants and to obtain a quantifiable amount of DNA clean enough to be amplified by PCR. The successful extraction of high quality DNA from plants and animal specimens is essential to the investigation of genetics at molecular level (Anon, 2002). The method of collection and preservation of tissue will greatly effects the longevity of the sample as well as the quality of the DNA. The DNA products were eluted in 200µl of preheated elution buffer and another 200µl elute to maximize the yield of genomic DNA. Successful DNA extractions were then visualized using electrophoresis technique in 1% agrose gels added with ethidium bromide.

Polymerase Chain Reaction (PCR)

PCR is an *in vitro* procedure for the enzymatic synthesis of DNA, using two oligonucleotide primers that hybridize to opposite strands flanking the region of interest in the target DNA (Anon, 2002). PCR process is a series of cycles involving template denaturation, primer annealing and the extension of the annealing primers by DNA polymerase. PCR was performed using DNA Thermal Cycler Model Gene® PCR System 2400 (Perkin Elmer). For PCR amplification a total volume of 50µl reaction constituting of 1-6µl of DNA, sterilized distilled water, Taq DNA Polymerase (Promega), 10x buffer (Promega), dNTP (10 mM), Magnesium chloride (25 mM) and Primer 16Sar (5' –CGC CTG TTT AAC AAA AAC AT-3') 16Sbr (5'-CCG GTC TAG ACT CAG ATC ACG T-3') were used. The initial denaturation was 96°C for 5 minutes, 35 second at 95°C denaturation, one minute at 51°C/52°C/55°C annealing, one minute at 72°C (30 cycles) for elongation and seven minutes at 72°C for final elongation. The amplified products were visualized on 1% agrose gel containing ethidium bromide and a standard marker of 1kb DNA ladder (GeneRuler™) was used.

Purification of PCR product

Products of PCR amplification were then purified before sending for sequencing. Only PCR products with clear band in the gel electrophoresis were the only product purified to remove the PCR buffer, excess primers and nucleotides left over from the PCR reaction mix and concentrate the DNA. PCR product were purified using Promega kit Wizard® SV Gel and PCR Clean-up system. About 47 µl of Mem bind solution were added to 47 µl of PCR product and transferred to column. The product was then left for one minute in the room temperature before it was centrifuged at 10,000 rpm for one minute. The solution that was trap in the collection tube was then discarded. About 700µl of membrane wash solution or isopropanol was added in the column and centrifuged at 10,000 rpm for one minute. The solution collected in the collection tube was discarded before another 500µl of the same solution was added. For this stages centrifuged were done for five minutes at 10,000 rpm and after discarding the solution in the collection tube another step of centrifuged was done to dry the column. The column was transferred to an appendof tube and 30µl of nuclease free water was added and left for one minute. After centrifuged for one minute the product was retained and stored in the freezer at -20°C.

DNA Sequencing

Purified PCR products were sequenced using ABI 377 automated DNA sequencer. The amount of purified product sent was 25 µl per sample with 5 µl of primer. The cycle sequencing was performed using Tpersonal Combi Thermocycler the 16Sar, 16Sbr primer and BigDye® Terminator v3.1 Cycle Sequencing Kit. The cycle sequencing reaction was done for 35 cycles of 96°C: 10 sec, 55°C : 5 sec, 60°C : 4 min, 4°C hold and proceed to Ethanol/Sodium Acetate

precipitation. Rapid themal ramp is 1°C/sec. BigDye® Terminator was used to sequence the reactions.

Sequence Analysis

DNA sequencing for PCR products were aligned using CLUSTAL X Version 1.8 program (Thompson *et al.* 1997). The phylogenetic Analysis Using Parsimony (PAUP*) program version 4.0b10 was used to examine phylogenetic relationship and Molecular Evolutionary Genetics Analysis (MEGA) version 2.1 (Kumar *et al.* 2001) was used for the phylogenetic tree reconstruction. CHROMAS (version 1.45) program was used to display fluorescence-based DNA sequence analysis results.

A few nucleotides were designated as noisy (N) and these may be due to not well-prepared sample of PCR products for sequencing especially during purification step (Kamarul, 2003). The cleaning process of N is by viewing the chromatograph and referring to the best peak to indicate the correct nucleotide. Then the alignment was done using CLUSTAL X (1.8) and 588 nucleotides site were used in this analysis. Population structuring, *Fst* and levels of gene flow, *Nm*, were computed using the software DNA Sequence Polymorphism (DNA SP) version 3.53 (Rozas & Rozas 1999).

Results

DNA Extraction

A total of 28 samples of blood were used for DNA extraction. The extractions of DNA from 28 sample of blood were successful even though some produce smearing in the gel electrophoresis. This happened to blood taken from Sebako only, whereas blood samples from Bau, Matang Wildlife Center and Balambangan Island, produced bright bands in the gel electrophoresis (Figure 1). Smearing was probably due to the purification process that might not separate other cellular components and potential contaminants from the DNAs.

DNA product that produced band during visualization will proceed to PCR amplification. In this study "smile effect" occurred in some of the DNAs product during the gel electrophoresis. According to Allen *et al* (1993), when drying at the edges of the gel occurs, the ionic strength at the outer edges of the gels is increased and the sample components move slower, causing the "smile effect".

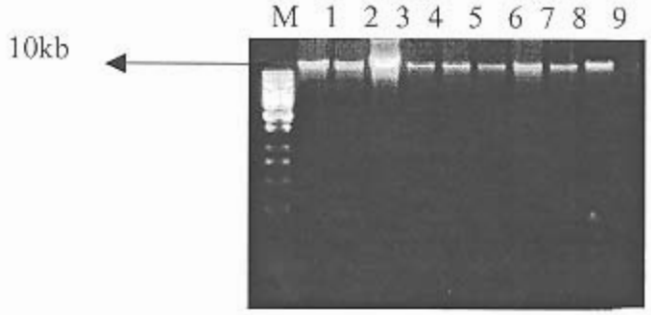

Figure 1. Gel electrophoresis of DNA extraction that produced bright bands with size of DNA approximately more than 10,000 base pairs. Sample 1. Cream-vented bulbul, 2.Cream-vented bulbul, 3.Yellow-bellied bulbul, 4.Yellow-bellied bulbul, 5.Puff-backed bulbul. Lane 6-9 represent second copy of the same samples. M represents GeneRuler™ 1 kb DNA Ladder (Fermentas) as a standard size marker.

Polymerase Chain Reaction (PCR)

All 28 DNA products were amplified using primers 16Sar (Upstream) and 16Sbr (downstream). Some of the PCR products produce a bright single band (Figure 2). Purification of this products produce bright clean bands visualized under UV light and Polaroid film. The nonspecific product observed during visualization of PCR product under UV light like multiple bands was due to the unsuitable annealing temperature and the existence of primer dimer was observed below the PCR product band in (Figure 2). Primer-dimer formation is an amplification artifact caused when one primer is extended by the polymerase using the other primer or itself as a template, resulting in a short incorrect product (Newton *et al.* 1994). The sizes of PCR products were approximately between 500bp and 750bp. The single bands PCR product visualized under UV light are then purified to obtained clean DNA for sequencing.

Annealing temperature was gradually increased from time to time in order to obtain the PCR product. According to Kessing *et al.* (1989) if the melting temperature is too low or time too short the double stranded DNA may not denature between cycles, thereby reducing the efficiency of the reaction and on the other hand when temperature is too high, the *Taq* polymerase enzyme will die.

A total of eight PCR products were send for sequencing but result tend to be reaction failed. So the PCR amplification using primer 12S failed and primer assumed to be not suited the DNAs. Additional primer may be tried to achieve higher PCR specificity and in addition as with the asymmetric PCR system very often failed to work as a sequencing primer and new primer(s) may be required (Lau *et al.* 1994). The overall efficiency and specificity of PCR is determined by quality of DNA template the design of the primer and PCR condition such as MgCl2 concentration, *Taq* Polymerase and buffer condition (Newton & Graham, 1994).

The change of primer to 16S was only fruitful when the first PCR amplification produced bright band product during gel electrophoresis. The reducing of bubbles during the process of mixing the cocktail of PCR is one of the reasons of good results because bubbles with oxygen inhibit the *Taq* polymerase. In this study the annealing temperature could not be optimized since some temperature started from 50°c to 55°c sometime produced bright band and some multiple bands. According to Kessing *et al.* (1989) the trick is to lower temperature to a level that allows the primer to anneal to the complementary sequence and if the temperature in too low the primer will sit down (non-specifically) and if too high the primer will not sit down at all. Other problem that

might occur in PCR amplification is too much template and low yield DNA product. To avoid cross contamination during PCR reaction negative control had been used in the process. Reamplications and purification of PCR products were done to generate and obtained a clear and bright bands of PCR products (Paul, 2003). After purification of the PCR product and were send for DNA sequencing. Twenty-four PCR reactions were sent for DNA sequencing and 20 products are successful and having good signed generated shown in Figure 2.

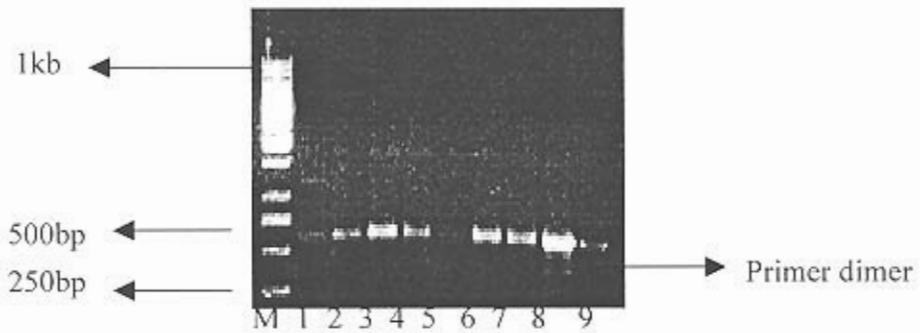

Figure 2. Gel electrophoresis showing PCR products. Lane 1 Red-eyed bulbul. Lane 2 Spectacled bulbul. Lane 3 Red-eyed bulbul. Lane 4 Olive-winged bulbul. Lane 5 Olive-winged bulbul. Lane 6 Olive-winged bulbul. Lane 7 Cream-vented bulbul. Lane 8 Cream-vented bulbul. Lane 9 Yellow-bellied bulbul. M represents GeneRuler™ 1 kb DNA Ladder (Fermentas) as a standard size marker.

Purification of PCR product

Twenty-five samples were purified before sending for DNA sequencing. The first batch consists of eight samples and second batch of 17 samples were sent for DNA sequencing.

DNA Sequencing

Appendix 1 shows the spectrophometric testing result of 24 DNA samples for purity and concentration using GeneQuest DNA/RNA UV Spectrophometer from Cecil, United Kingdom.

The results of DNA sequencing for the 24 samples were quite good where three samples obtained high background signal generated and the other were good signal generated (Appendix 2). Four samples failed probably due to poor preparation of samples for sequencing and purification.

Phylogenetic Tree

The construction of Phylogenetic tree was constructed using Neighbour-joining method (Kimura-2 parameter) formed three clusters (Figure 3). The first cluster consists of species like the ochraceous bulbul, yellow-bellied bulbul and spectacled bulbul. The second cluster consists of olive-winged bulbul and red-eyed bulbul. The third cluster consists of red-eyed bulbul and puff-backed bulbul. The phylogenetic analysis revealed that the existence of genetic variation between the six species of bulbul even though some species were from the same population and from the same region.

Hence the data from bootstrap 50% majority-rule consensus tree of maximum parsimony analysis (Figure 4) support the result from neighbor-joining bootstrap analysis although levels of confidence are different in most of the branches. But still the level of confidence is equal at branches of yellow-bellied bulbul (YBB) and red-eyed bulbul (REBW2) and puff-backed bulbul (PBB) at value of 97% to 99%.

The bootstrap 50% majority-rule consensus of neighbour-joining are based on similarity of sequences which sequentially identifies neighbor pairs that minimize the total length of the tree. The neighbour-joining branches are scaled and their lengths are proportional to the number of changes (Li and Graur, 1991). The bootstrap 50% majority-rule consensus of maximum

parsimony identifies all the informative sites or dissimilarity among sequences. The parsimony method based on maximum number of substitution at each informative site.

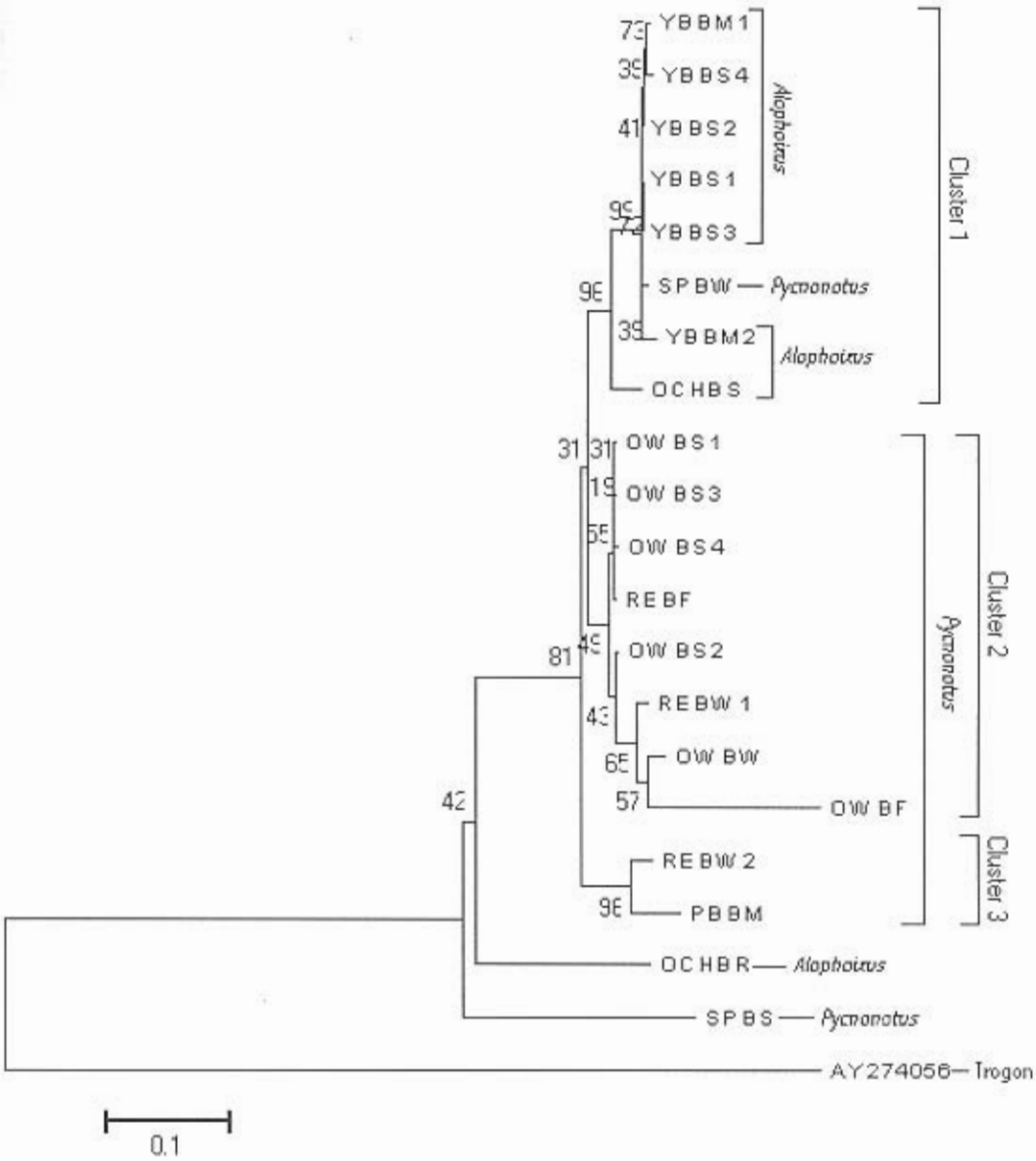

Figure 3: Neighbor-joining tree (original tree) bootstrap 50% majority–rule with Kimura-2 parameter, 1000 replications (MEGA) were used.
YBB=Yellow-bellied bulbul. OCHB=Ochraceus bulbul. OWB=Olive-winged Bulbul. SPB=Spectacled Bulbul. REB=Red-eyed Bulbul. PBB=Puff-backed Bulbul
M=Matang. S=Sebako. W=Wind Cave. F=Fairy Cave. R=Raven's Court

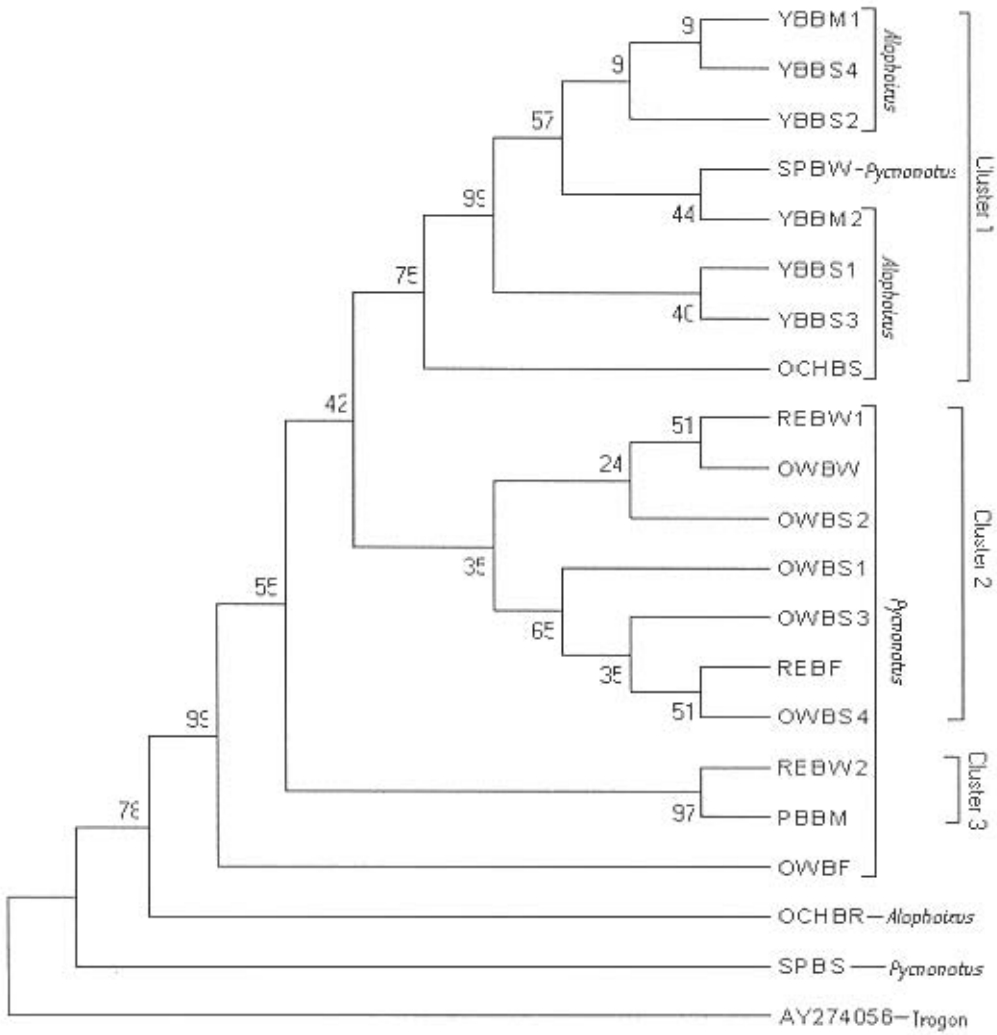

Figure 4: Bootstrap 50% majority-rule consensus maximum parsimony analysis with Kimura-2 parameter, 1000 replications (MEGA)

Nucleotide diversity

Levels of nucleotide diversity (Jukes and Cantor) among individual within species ranged from 0.6% and 32.6% (Table 2). However the nucleotide diversity within the species showed that spectacled bulbul was highest (32.6%) and yellow-bellied bulbul was the lowest (6.4%).

Table 2: Nucleotide diversity (Jukes and Cantor) within species of bulbul.

Species	No. Of species	Percentage %
Spectacled Bulbul	2	32.6
Yellow Bellied Bulbul	6	0.77
Ochraceous Bulbul	2	23.9
Olive-winged Bulbul	6	10.2
Red-eyed Bulbul	3	6.4
Puff-backed Bulbul	1	0.000

Haplotype and Nucleotide diversity within species

Both haplotype and nucleotide diversity within species were high in four species (Table 3). The values of haplotype diversity were between 0.933 and 1.000. Two individuals of yellow-bellied bulbuls share the same haplotype. The values of nucleotide diversity were between 0.00785 and 0.32611. The lowest was within yellow-bellied bulbul and the highest was in spectacled bulbul.

Table 3: Haplotpye diversity and Nucleotide diversity within species.

Species	Haplotype diversity	Nucleotide diversity
Yellow-bellied Bulbul (n = 6)	0.933	0.00785
Olive winged Bulbul (n = 6)	1.000	0.10150
Red eyed Bulbul (n = 3)	1.000	0.06407
Spectacled Bulbul (n = 2)	1.000	0.32611
Ochraceous Bulbul (n = 2)	1.000	0.23878

Estimation of Gene flow

Due to the limited sample of red-eyed bulbul, ochraceous bulbul, puff-backed bulbul and spectacled bulbul, hence only yellow-bellied bulbul and olive-winged bulbul were selected for gene flow analysis using *Fst* approach. Estimation of gene flow is given in Table 4.

Table 4: Estimation of gene flow (*Fst*) .

Species	Localities/Sample size	Distance	*Fst* and *Nm* value
Yellow bellied bulbul	Sebako = 4	45km	*Fst* = 0.00778
	Matang = 2		*Nm* = 15.25
Olive-winged bulbul	Sebako = 4	60km	*Fst* = 0.08925
	Bau = 2		*Nm* = 1.00

Yellow-bellied bulbul show the highest gene flow between Sebako and Matang Wildlife Center at estimation value of *Nm* 15.26 and *Fst* is 0.0078. However, olive-winged bulbul shows the lowest level of gene flow at a value of *Nm* 1.00 and *Fst* is at 0.089. This analysis indicates that yellow-bellied bulbul from Sebako and Matang Wildlife Center has a high number of migrants per generation compare to olive-winged bulbul from Sebako and Fairy Cave and Wind Cave.

Discussion

Phylogenetic analysis.

The CLUSTAL X (1.81) program was used in analysis of multiple sequence alignment for forward reactions of 16S gene sequence and species *Trogon curucui* (AY274056) from Genbank was used as an outgroup. The phylogenetic tree has shown some differentiation within species, which was probably due to the geographic isolation. Genetic drifts probably have been a major source of genetic variation between some population (Halliday, 1993).

Phylogenetic analysis showed the separation of genera *Alophoixus* and *Pycnonotus* into two clusters. From this analysis, one species of spectacled bulbul from the genus *Pycnonotus* was found to be separated, one individual was placed between genus *Alophoixus*. Apparently, the two individual were from different populations, Wind Cave (Bau) and Sabako (Lundu). The genetic variation within this species could have been due to the distance of the two populations, which is approximately 60 km as results of isolated population, adaptation to a different environment and condition.

The ochraceous bulbul (genus: *Alophoixus*) and spectacled bulbul (genus: *Pycnonotus*) were placed at the bottom of the tree could also be due to the geographic distance between these two individuals from the rest of the individuals in the species. The two species were sampled from Raven's Court (Lawas) and Sebako (Lundu). In birds, regional trends of size variation can

change gradually due to topographic features and the mechanism that maintain such clines of character variation. It is traditionally assumed to be natural selection of polygenic traits that represent adaptations to local conditions (James, 1983).

Morphologically, some species are almost look alike, for example spectacled bulbul, red-eyed bulbul and olive-winged bulbul. They share morphological characteristic such as red-eyed. Only orange yellow eyelid in spectacled bulbul and green wash on wings in olive-winged bulbul can differentiate the three species. However, genetic variation was found between them. Individual who are members of the same species shares many morphological, physiological and behavioral characters, but no two individuals are exactly the same (Mclannahan, 1993).

The separation within this two species in the phylogenetic tree revealed that geographic variation due to distance separation will happened within species. The barrier between the two locations such as mountains, fragmentation of forest cause by existing land use and land development have broken the wildlife corridor. The barrier to gene flow between populations may have caused genetic divergence within species of different populations (Pond, 1993).

Haplotype and Nucleotide diversity within species

Comparisons of haplotype and nucleotide diversity within species are high in all the four species (Table 3) except in yellow-bellied bulbul. This could be due to individuals sampled from different populations. The value of haplotype diversity was 0.933 to 1.000. Two yellow-bellied bulbuls share the same haplotype. The low rate of haplotype diversity in yellow-bellied bulbul was probably due to individuals sampled from the same population. In general, geographic

distance and barrier between two populations can contribute to the high rate of nucleotide and haplotype diversity.

Estimation of Gene flow

The amount of gene flow between Sebako and Matang Wildlife Center was high. However, the physiography between these two populations does not agree with the results. It was expected that gene flow between these two population would not occurs due to the presence of physical barrier such as mountain, existing land use and human development. The small number of sample size could also influence the result.

Conclusion

The phylogenetic analysis revealed that among the selected species of bulbul there was not much variation genetically. However, the results show that some individual within species were separated when the phylogenetic tree was constructed. Besides variation due to distance separation and physical barriers between populations, the way the results of this study turned out could have been due to errors in identification, cross-contamination during experiment and small sampling size.

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