CHAPTER 3
MATERIALS AND METHODS

Study sites

Two study sites in northern Thailand were located in the Chiang Dao Wildlife Sanctuary near the Chiang Dao Wildlife Research Station (19°21’ N, 98°55’ E), and on Chiang Mai University campus (18°48' N, 98°57' E; Figure 1). These sites range in altitude between 300 and 700 m above sea level. These areas are typically lowland seasonal deciduous forest, most of which is secondary re-growth forest due to vast deforestation in the last several decades during the Teak (*Tectona grandis*) trade (Maxwell, 1992; Maxwell, 2007; Vaidhayakarn and Maxwell, 2010). The typical vegetation at Chiang Dao Wildlife Research Station is bamboo-deciduous, hardwood seasonal forest, which has many woody vines and abundant seedlings and saplings of both evergreen and deciduous tree species, as well as several species of bamboo (Maxwell, 1992; Ngoenjun and Sitasuwan, 2009). The forest undergoes dramatic changes within a year and is greatly influenced by the amount of rainfall present; the forest is largely leafless during the dry season between December and April, and then becomes green after the onset of rain in May and June. The Chiang Mai University campus is an urban area where the human activities are reasonably high. This urban site is on the base of the east side of Doi Sutep-Pui Mountain. The typical vegetation around this site is hardwood bamboo forest and deciduous, seasonal, hardwood forest (Vaidhayakarn and Maxwell, 2010), and also including open area. Recordings were collected throughout an area approximately 4 by 4 km in both study sites.

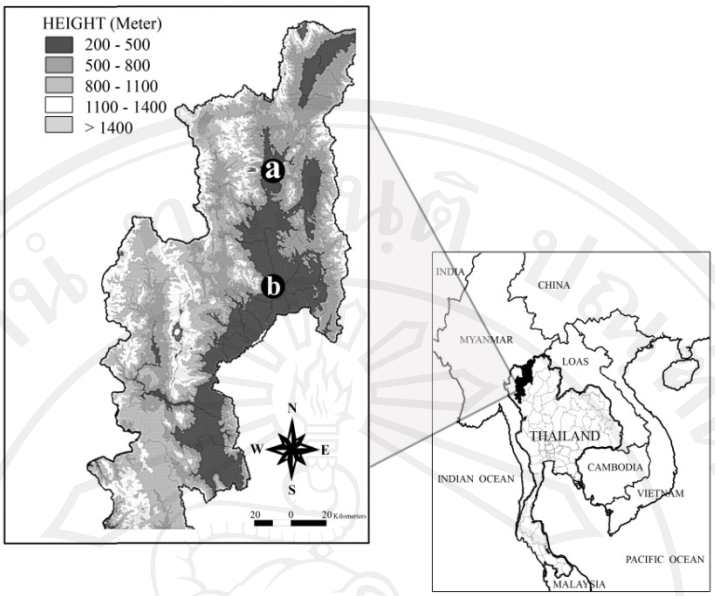

Figure 1. Illustrated map showing the two study sites in Chiang Mai, northern Thailand; a) Chiang Dao Wildlife Research station, and b) Chiang Mai University (adapted from Royal Thai Survey Department, 2002).

Climate of the Chiang Dao Wildlife Research Station

Climatic data were collected at weather station and provided by the department of National Parks, Wildlife and Plant Conservation, located at 550 m elevation. Weather data, such as rainfall and minimum/maximum temperatures, were recorded daily between 08:00 h and 09:00 h. Daily rainfall measurements were collected with a standard rain gauge (diameter of the collecting funnel = 20 cm). Temperatures were recorded with maximum/minimum thermometers. The weather conditions measured in each month were presented as the monthly accumulation of rainfall, and mean ± SE for maximum/minimum temperatures.

Study species

Six sympatric tropical Bulbuls were studied including: Black-headed Bulbul (*Pycnonotus atriceps*), Black-crested Bulbul (*P. flaviventris*), Streak-eared Bulbul (*P. blanfordi*), Stripe-throated Bulbul (*P. finlaysoni*), Red-whiskered Bulbul (*P. jocosus*), and Sooty-headed Bulbul (*P. aurigaster*). All six species have been reported in the

forested habitat at lower elevations in northern Thailand (Lekagul and Round, 1991; Robson, 2000). Outside of the breeding season these birds usually forage within the same area, and all six species are sometimes found on the same flowering and fruiting tree (S. Kamtaeja, unpublished data). While breeding, birds spend most of their time in close proximity to their mate, and this is the only time of year when birds can be found outside of mixed foraging flocks. Previous studies indicate that bulbuls have small home ranges associated with the location of fruiting trees (Fukui, 1995; Sankamethawee *et al*., 2010).

Molecular data indicate these species are genetically related (Pasquest *et al*., 2001; Moyle and Mark, 2006). They are easily distinguished by plumage characters, as described in Lekagul and Round (1991), Robson (2000), and Fishpool and Tobias (2005). The six species can be separated into three morphological forms: the “Yellow bulbuls” have bright yellow body parts and glossy black heads, and include Black-headed and Black-crested Bulbuls; the “Plain bulbuls” are brownish-grey over the whole body and less elaborately ornamented than the other two groups, and they include Streak-eared and Stripe-throated Bulbuls; and the “Brown bulbuls” have a predominantly dull-brown body and a black head, and include Red-whiskered and Sooty-headed Bulbuls. These particular six species were selected for paired comparisons because they are similar in general behaviour and in foraging behaviour and diet (S. Kamtaeja, unpublished data), and because they form three pairs of species based on similar visual appearances (from a human perspective).

All six species are found in sympatry at low elevation in seasonal tropical forests in Northern Thailand. Some ideas tested in this study rest on the assumption that all six *Pycnonotus* bulbul species interact with each other in sympatry. Field observations confirm that this is the case. When collecting field recordings, numbers of species of the *Pycnonotus* foraging together within flock were routinely encountered in different compositions, confirming that they co-occur in mixed flocks. Field observations suggest there is some partial segregation by micro-habitat: Black-headed and Black-crested Bulbuls were regularly found together in mature forests where the vegetation is dense and comprised of multiple storeys; Streak-eared and Stripe-throated Bulbuls were often found together in the thicker under-storey of the deciduous forest and secondary forest close to human areas; although Stripe-throated

Bulbul were not found in urban areas, Red-whiskered Bulbuls and Sooty-headed Bulbuls were often found together in an urban area with occasional occurrences in mature forest habitat.

Sound recording

The song of each species were defined as the repeated series of stereotyped elements, produced by one bird, often while singing from a post at the top of a tree or bush. Songs were separated from other songs by silent interval of more than 0.5 s. Within songs, an "element" was defined as a continuous trace on a sound spectrogram (Thompson *et al*., 1994; Riebel and Slater, 2003; Cardoso and Price, 2010). Bird's singing behaviour observed during recording sessions indicated a significance of song of all six species in a context for species-specific communications. All species' song included in this study is particularly used within species and probably important for defending territory and resources (i.e. food). Other calls (i.e. alarm call, mobbing call, alert call) were not included in this study because call is rather used for wider situations (non specific) and, sometime, rather used for inter-specific communications (Goodale and Kotagama, 2005; Catchpole and Slater, 2008). Songs were the most conspicuous vocal signal in all six *Pycnonotus* species. For all six species, songs were common during the breeding period, and were less common, but still present, outside the breeding period. This matches what is known in two other related species that have been studied previously (Red-vented Bulbul, *P. cafer*; Kumar, 2004; Bare-faced Bulbul, *P. hualon*; Woxvold *et al*., 2009) and what is generally true across many tropical birds (Stutchbury and Morton, 2001). The sex of the recorded birds in this study was hardly to determine because all six species are sexually monochromatic. Although size dimorphism might be used to distinguish male and female Red-whiskered Bulbuls (females are slightly smaller; Amiot *et al*., 2007), differentiating between subtle size differences is difficult in the field.

Birds were recorded while they fed together on fruiting plants, often in large mixed-species flocks, between 6:00 and 18:00 hours during all months of the year (between January 2008 and April 2010). Both study sites were visited on at least 2 or 3 days in every month. Birds were recorded with digital recorders (Marantz PMD661 and EDIROL R-09), using the built-in microphone of each unit (frequency responses

of 20 Hz to 24,000 Hz and 20 Hz to 22,000 Hz, respectively). Files were recorded as 16-bit WAV files at a sampling frequency of 44.1 kHz. All birds were recorded at as close a distance as possible, with distances between the recorder and the bird ranging from 10 to 15 m.

To record as many individuals as possible, birds were recorded opportunistically by travelling around these sites. The recordings were conducted continuously from the time singing individuals or a flock was encountered, until birds stopped singing or moved to a different location. When recording birds in mixed flocks, one or two individuals of each species were recorded for as many species present as possible, distinguishing between individuals on the basis of their position in the vegetation. Certainly with based on their position, each recording represented a different individual, and recordings were excluded if it was unclear whether they came from unique individuals. Recordings were made 300 to 500 m, apart. In this study of unbanded birds, a separation distance of 300 to 500 m to be adequate for sampling different groups of bulbuls, based on previous studies that report that congeneric bulbul home range diameters are 200 to 300 m (e.g. 225 m in Olive-winged Bulbul, *P. plumosus*; 300 m in Cream-vented Bulbul, *P. simplex*, Peh and Ong, 2002; 311 m in Puff-throated Bulbul, *Alophoixus pallidus*, Sankamethawee *et al*., 2010).

All six species are abundant at the study sites with hundreds of birds of each species at both recording sites. Birds recorded at each location in each month were considered independent samples and it was assumed that each recording location contained unique individuals of each species. When recording in multiple months, we recorded from different localities inside the 4 x 4 km study sites, avoiding repeated recordings from the same species at the same location. In a few cases where multiple birds from similar locations were recorded (<500 m apart), an average value for that species at that location was calculated.

Owing to a lack of research, it is unclear what times of the year correspond to mixed species flocking behaviour or territorial behaviour in the six species studied. A recent study of Puff-throated Bulbuls demonstrated that birds live in pairs or social groups of 2-7 individuals and defend territories year-round; the average group size during the breeding season was 3.2 birds, and this increased to 4.1 birds during the

non-breeding season (Sankamethawee *et al*., 2010). In the *Pycnonotus* Bulbuls, it was found that birds were often found in pairs during what is assumed to be the breeding season (6 nests for 4 of the species were found), and in groups of up to >100 birds per flock in non-breeding season. Roosting sites of birds comprising 100-200 birds per group were found. Given the extremely different group sizes between the breeding and non-breeding season it is concluded that birds do not defend territories year-round.

All recordings were visualised using Syrinx-PC sound analysis software (J. Burt, Seattle, WA). Sound spectrograms of all field recordings were generated and the time and frequency cursors were used to select and annotate sounds of interest. The signal-to-noise ratio of each recording was estimated by assigning it a value between 1 and 3 (1: low quality signal containing high level of background noise that impaired parameter measurement; 2: moderate quality signal with some background noise but sufficient signal strength for structural analysis; 3: good quality recording with little background noise). The songs chosen for fine structural analysis were rated 2 or 3.

Song structure were analysed using SASLab Pro (v. 4.40; Avisoft Bioacoustics, Berlin). For each song and throughout this study, spectrograms were generated using same spectrogram parameter (512 points, 87.5% overlap, FlatTop window, time resolution 2.9 ms, frequency resolution 22 Hz). Nine fine structural features were measured: (1) song duration (the length of entire song; in seconds); (2) maximum frequency of the entire song (in Hz); (3) minimum frequency of the entire song (in Hz); (4) element duration (expressed as an average for all elements per song; in seconds); (5) maximum frequency of each element (average of all elements per song; in Hz); (6) minimum frequency of each element (average of all elements per song; in Hz); (7) duration of the longest element (in seconds); (8) average inter-element interval (in seconds); and (9) number of types of element within a song (elements types of a song were considered to be different types when structure visually differentiated based on shape, duration and frequency range). Nine variables for possible multicollinearity were examined for variance inflation factors. Variance inflation factors exceed 10 indicate strongly correlated variables (Chatterjee and Price, 1977); the variance inflation factors were between 1.99 and 7.25. All measurements were made using the automatic parameter measurements feature of SASLab to

minimise the influence of human subjectivity (hold time: 20 ms; amplitude relative to maximum: -20 dB). Measured song elements were checked by eye using the red cursors in SASLab to ensure accurate measurements of the target signal.

Morphometric analyses

Birds were caught using mist nets during for 78 days between March 2008 and March 2010; the overall effort amounted to 624 net hours or 4-5 days per each month. Birds were caught between 7:00 and 16:00 hours, except in hot seasons; nets were closed when temperatures exceeded 40 ^{o}C. Two different sizes of 3 channel mist nets were used: 9 m long nets with dimensions of 1.5 x 1.5 cm, and 12 m long nets with dimensions of 2.0 x 2.0 cm for catching bulbuls or other small birds. The nets were positioned between bushy areas close to fruit trees where mixed-species flocks of bulbuls aggregated to forage together in the same trees. Standard morphometric measurements were obtained for all captured birds, including: (1) bill length (mm), (2) tarsus length (mm), (3) wing length (mm), (4) tail length (mm), and (5) weight (g). Birds were weighed to the nearest 0.1 g using a digital balance (model: A&D Scales HL-2000), bill length and tarsus length were measured using vernier calipers to the nearest 0.1 mm, and wing length and tail length were measured using specified rulers to the nearest 1.0 mm. Five variables for multicollinearity were examined for variance inflation factors which were between 2.2 and 4.6. All measurements were made by one researcher, to minimise human error.

To distinguish between adult and juvenile birds, several characteristics were used due to similar plumage characteristics. Unlike juvenile plumage, the rectrices feathers of adults are usually distinctively worn and heavily damaged from rubbing against vegetation, and their remiges are moderately worn at the tips (Pierce, 2009). Individuals were examined for the presence of down feathers, usually on the head or throat, a yellow patch on the base of the beak gape; these characteristics are typical of juvenile plumage. In addition, growing crests were used to determine juveniles that usually have shorter crests and growing crest feathers, especially in Black-crested Bulbul, Red-whisker Bulbul, and Sooty-headed Bulbul. Based on these characteristics, juveniles were excluded from this study.

Blood samples were collected from caught birds using the leg bleeding technique. Blood was spotted on filter paper (Whatman®), and kept at room temperature until molecular and sex identification and genetic analyses. Subjects were measured and blood samples taken, then immediately released in the same locations where they were caught.

The *Pycnonotus* Bulbuls are sexually monochromatic, and using plumage color for sex identification is impossible. Molecular technique was therefore used for sex determination. Genomic DNA was extracted with proteinase K and phenol-chloroform extraction (Kocher *et al*., 1989; Wu *et al*., 2007). Polymerase chain reactions (PCR) using primers P8 (5´-CTC CCA AGG ATG AGR AAY TG-3´) and P2 (5´-TCT GCA TCG CTA AAT CCT TT-3´) (Griffiths and Korn, 1997) was conducted. The reactions were run a 25 µl volume, and included 1 µl of DNA template, 1.5 µl of 10X PCR buffer (Bioscience), 1 µl of 10 µM of forward and reverse primers, 1.5 µl of 10 µM dNTP, 1.5 µl of 25 mM $MgCl_2$, 1 µl of *Taq* DNA polymerase (vivantis), and 14.5 µl of diH_2O. The PCR protocol was as follows: 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and a final extension step of 5 min at 72 °C. The amplified PCR products were visualized on 2% agarose gel stained with ethidium bromide using electrophoresis. Sizes of all PCR products were estimated by comparing with standard 100bp DNA Ladder, 100bp - 1500bp (O'RangeRuler™, ©2011 Thermo Fisher Scientific Inc., Fermantas). Sex was determined by assessing allele sizes: double bands females and single band for males. Corrected identification of both fragments were confirmed with sexual dimorphic species, references species included: Blue-winged Leafbirds (*Chloropsis cochinchinensis*), White-rumped Shama (*Copsychus malabaricus*), and Black-naped Monarch (*Hypothymis azurea*).

Mitochondrial DNA sequencing

DNA samples of each species for genetic analysis were randomly selected. PCR amplified the 16s mitochondrial gene. The primers used for polymerase PCR were; 5’-CCG ACT GTT TAC CAA AAA CAT-3’ and 5’-CCG GAT CCC CGG CCG GTC TGA ACT CAG ATC ACG-3’. Double-stranded PCRs were run in a 25 µl volume, including 1 µl of DNA template, 1.5 µl of 10X PCR buffer (Bioscience), 1 µl

of 10 µM of forward and reversed primers, 1.5 of 10 µM of dNTP, 1.5 µl of 25 mM $MgCl_2$, 1 µl of *Taq* DNA polymerase (vivantis) and 14.5 µl of diH_2O. The PCR protocol was as follows: 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 - 64 °C for 45 s, extension at 72 °C for 30 s, and a final extension step of 5 min at 72 °C. The amplified PCR products were approximately 550bp. PCR products were confirmed approximated length by visualizing on 1% agarose gel stained with ethidium bromide using electrophoresis. The PCR products (~20 µl) were transported to Macrogen Laboratory, Korea (info@macrogen.com) to obtain DNA sequencing.

All sequences were checked for corrected sequence identity using Blast (NCBI database) and were higher than 98%. All sequences were analysed using ClustalX sequence alignment software. Genetic distances between species were analyzed using maximum composite likelihood (MCL) based on 23 mtDNA sequences; 5 from Black-headed Bulbuls, 5 from Black-crested Bulbuls, 3 from Streak-eared Bulbuls, 5 from Stripe-throated Bulbuls, 3 from Sooty-headed Bulbuls and 2 from Red-whiskered Bulbuls. The phylogenetic tree was constructed using the neighbor-joining methods with 1000 replications bootstrapped analysis. All genetic analyses were conducted in MEGA4 (Tamura *et al*., 2007).

Ethical note

This study complied with the guidelines for wildlife research and study contributed by the Department of National Parks, Wildlife and Plant (Department of National Parks, Wildlife and Plant, 1999), and was approved by the University committee.

Statistical analyses

Song analyses

To evaluate whether song structure could distinguish among the recorded species of *Pycnonotus* bulbuls, a discriminant function analysis (DFA) with cross-validation was conducted. Recordings from each location (i.e. each flock) were used as the unit of replication. A total of 1,149 songs were recorded including 93 from

Black-headed Bulbuls, 318 from Black-crested Bulbuls, 85 from Streak-eared Bulbuls, 122 from Stripe-throated Bulbuls, 265 from Red-whiskered Bulbuls, and 266 from Sooty-headed Bulbuls. The average value for each recording location was calculated, resulting in a total of 186 average measurements: 16 Black-headed Bulbuls, 48 Black-crested Bulbuls, 20 Streak-eared Bulbuls, 29 Stripe-throated Bulbuls, 41 Red-whiskered Bulbuls, and 32 Sooty-headed Bulbuls. The DFA using a randomly-selected subset of 91.9% of the data (minimum of 16 songs, maximum of 38 songs representing each species) was constructed describing three canonical axes based on their correlation coefficients with the original nine variables in this subset of the data. DFA with cross-validation was performed to determine whether this analysis could predict the correct species based on fine structural features; the ability to correctly identify species using the remaining 8.9% of the data was tested. The accuracy of the DFA was reported as the percentage of songs assigned to the correct species for the 8.9% of songs. In addition to DFA based on the songs of all six species, three additional DFAs on pairs of species with highly similar plumage was conducted using a randomly-selected 80% of the data, and cross-validated the analysis with the remaining 20% of the data.

Morphometric analyses

The morphometric data measured from live birds was based on a total of 134 adult birds, including 10 Black-headed Bulbuls (female = 5, male = 5), 40 Black-crested Bulbuls (female = 19, male = 21), 27 Streak-eared Bulbuls (female = 12, male = 15), 31 Stripe-throated Bulbuls (female = 18, male = 13), 10 Sooty-headed Bulbuls (female = 5, male = 5), and 16 Red-whiskered Bulbuls (female = 5, male = 11). Sexual dimorphism within species was evaluated using *t-test*. Based on the morphometric data, there is evidence of sexual differences, although testing of the significance using the *t-test* revealed little evidence of size differences between males and females.

To evaluate whether body size could distinguish species among the captured bulbuls, DFAs were conducted based on morphometric data measured from each sex of each species, cross-validated DFAs of males and females were calculated, independently.

To evaluate whether morphometric differences between female *Pycnonotus* bulbuls, a DFA using a randomly-selected subset of 85.7% of the data, and described the three resulting canonical axes based on their correlation coefficients with the original five variables in this subset of the data was constructed. Cross-validation was performed to determine whether this analysis could predict the correct species based on morphometric features; the ability to correctly identify species using the remaining 14.3% of the data was also tested. The accuracy of the DFA is reported as the percentage of songs assigned to the correct species for the 14.3% of morphometric traits.

To evaluate whether morphometric differences between males *Pycnonotus* bulbuls, a DFA using a randomly-selected subset of 88.6% of the data was used, and describe the three resulting canonical axes based on their correlation coefficients with the original five variables in this subset of the data. Cross-validation to determine whether this analysis could predict the correct species based on morphometric features was performed and tested using the remaining 11.4% of the data. The accuracy of the DFA is reported as the percentage of songs assigned to the correct species for this 11.4% of morphometric traits.

Genetic analyses

Pair-wise composition distances of 16s mtDNA were generated using the maximum composite likelihood (MCL distance) that takes into account the evolutionary distance between each species. Composition distance is a measurement of the difference in nucleotide composition for a given pair of sequences which based on a total of 23 mtDNA sequences included; 5 from Black-headed Bulbuls, 5 from Black-crested Bulbuls, 3 from Streak-eared Bulbuls, 5 from Stripe-throated Bulbuls, 3 from Sooty-headed Bulbuls, and 2 from Red-whiskered Bulbuls.

Test for acoustics distance correlated with morphometric and genetic distance

Euclidean distances between pair-wise species song of all six *Pycnonotus* were calculated using the variables of functions at group centroids. Functions at group centroids are derived results from acoustic discriminant analysis based on correlation

of nine acoustics variables which generated five functions for each species and are explained by the maximum discriminant function (100% of variance explained). Pair-wise Euclidean distance was measured using cluster analysis on SPSS which generated a 6 x 6 matrix presenting acoustic distances between pair-species of all six species of *Pycnonotus* Bulbuls (clustering method: between-groups linkage; standardize: range -1 to 1).

To calculate the Euclidean distance of morphometric features, the variables from function group centroids were used, which are based on five morphometric features. Euclidean distance of male and females were calculated separately. DFA generated five functions at group centroids for the morphometric analysis of both males and females explaining 100% of the variance. Pair-wise Euclidean distance as measured using cluster analysis on SPSS which generated a 6 x 6 matrix presenting morphometric distances between pair-species of each sex of all six species (clustering method: between-groups linkage; standardize: range -1 to 1). Euclidean distance generated from morphometrics of females and males are not significantly difference (Paired-samples *t-test* = -1.724, *df* = 14, *p* = 0.107).

The evolutionary distance between species sequences were measured by comparing number of nucleotide substitutions occurring between them. All results are based on the pair wise analysis of 23 sequences. Analyses were conducted using the maximum composite likelihood method. All positions containing alignment gaps and missing data were eliminated only in pair wise sequence comparisons. There were a total of 487bp in the final dataset. Nucleotide substitution patterns of 16s mtDNA indicate that six species of the *Pycnonotus* bulbuls indicated relatively similar genetic relationships with short genetic distances between species; genetic distances of all six species were between 0.023 and 0.063.

Whether acoustic distance of all six species is predictable based on morphometric and genetic difference was tested using the Mantel tests for matrices comparisons to predict the correlation between acoustic and morphometric distances, as well as between acoustic and genetic distances.