CHAPTER 5
DISCUSSION

Bioacoustic analyses of the songs of six sympatric species of *Pycnonotus* bulbuls presented in this study are the first quantitative description of each species' vocalization. This study shows a twofold significance of the songs of the six sympatric species. Firstly, the vocal distinctiveness was analysed and revealed an increase in song difference between the plumage similar species (from a human visual perspective). These results are evidence that vocalizations are important as species isolation and recognition mechanisms between closely-related and sympatric *Pycnonotus* bulbuls. Second, a congruent relationship of a species' acoustic features is correlated with their morphological features and genetic features. Acoustic distance of all six species has a positive correlation with morphometric distance and genetic distance, which indicates that speciation by morphological divergences and genetic drift may influence on vocal behaviour among all six species. This evidence suggests that species-specific vocalizations of all six species have experienced stochastic selective pressures as well as species isolation and recognition mechanisms, in particular between closely-related, sympatric *Pycnonotus* bulbuls.

Vocal distinctiveness a tool for species recognition in six sympatric Pycnonotus Bulbuls

Detailed acoustics analyses reveal that each species produces distinctive vocalizations that provide acoustic cues of species identity. These features may be the basis for species recognition signals, which may be particularly important in the context of mixed-species flocks (Barker and Boylan, 1999; Slabbekoorn and Smith, 2002; Päckert *et al*., 2003; Cadena and Cuervo, 2010). Although all six species sing songs in overlapping parts of the frequency spectrum (1.2 to 5.1 kHz), results of cross-validated discriminant analysis reveal significant acoustic differences for the six bulbuls. Pair-wise analyses are consistent with the idea that sympatric species with similar plumage features show pronounced acoustic differences.

Quantitative acoustic measurements of the voices of most tropical birds are lacking, in spite of the high biodiversity that characterizes the tropics and the conservation pressures that imperil the future of many tropical ecosystems (Gaston, 2000; Stutchbury and Morton, 2001). Our study provides quantitative descriptions of the songs of six species of *Pycnonotus* bulbul (Table 1). Black-headed and Streak-eared Bulbuls both sing trilled songs composed of one type of element sung in repetition; the former sings narrow-bandwidth, down-slurred elements at a slower pace, whereas the latter sings broad-bandwidth, up-slurred elements at a faster pace. Consequently, the two species that sing trilled songs can be readily distinguished from each other based on these differences. These trilled songs match the published description of the trilled song of another congener, the Bare-faced Bulbul (songs of 3 to 15 elements; frequency range of 2 to 5 kHz; Woxvold *et al*., 2009). In contrast to these trilled songs, the remaining four species that we studied produce songs composed of various frequency-modulated tones. Red-whiskered Bulbuls have the most complex songs, with many elements featuring rapid frequency modulations. Their complex songs are one of the reasons that Red-whiskered Bulbuls are of conservation concern in Thailand; although this species has been introduced in many parts of the world, and even become a pest in some regions, they continue to face conservation pressure in their native range due to the pet trade (Clergeau and Mandon-Dalger, 2006), including trade for song competitions (Anderson, 2005). Sooty-headed Bulbuls have the simplest songs of those described here, with few elements that descend in pitch over the course of the song. The remaining two species can be distinguished on the basis of their frequency range (Stripe-throated Bulbuls have the lowest-pitched frequency-modulated songs in the group) and the organization of elements (Black-crested Bulbul songs tend to rise in pitch more dramatically than the other species). These four species are most similar to published descriptions of the Red-vented Bulbul (songs of 2 to 6 elements; frequency range of 0.9 to 4.5 kHz; Kumar, 2004) and the Common Bulbul (songs of 4 to 7 elements; frequency range of 1.2 – 3.5 kHz; Lloyd *et al*., 1999). Our acoustic descriptions can assist ornithologists, ecologists, and conservation biologists in future research endeavours. Based on these quantitative differences in the songs of the six species, acoustic recordings should provide a useful tool for monitoring bulbuls. Acoustic survey techniques may be particularly advantageous in dense habitats where visual monitoring is difficult, and where unobtrusive recordings may be collected at a distance from the animals (Waide and Naring, 1988; Haselmayer and Quinn, 2000; Grava *et al*., 2008).

Discriminant analysis of nine fine structural features revealed significant vocal differences between the six species. The acoustic features that contributed most strongly to our discriminant analysis may be the features that are most important for interspecific recognition. Features that strongly influenced discrimination included frequency measurements (both maximum and minimum frequency), temporal measurements (particularly song duration and inter-element interval), and compositional features (the number of types of element within a song). Acoustic distinctiveness is expected to increase when birds live in the same habitat (Kirschel *et al*., 2009; Seddon and Tobias, 2010), and may be particularly important in the close associations of a mixed-species foraging flock. Song is an important feature as a heterospecific pre-mating isolation mechanism (Irwin *et al*., 2001; Balakrishnan, 2010). All six bulbul species were regularly occurred together, creating a complex acoustic and social environment. Many prior studies have found acoustic, species-typical differences in closely-related, allopatric congeners (e.g. Kirschel *et al*., 2009; Valderama *et al*., 2007; Price and Lanyon, 2002).

Results of the species' pair-wise comparisons are consistent with the idea that song provides a mechanism for discriminating between species that are visually similar. It is well known that both visual and acoustic signals are important in avian mate choice (Bradbury and Vehrencamp, 2011), and both types of signals are expected to be used in distinguishing territorial opponents and prospective mates. All six study species were subdivided into three pairs of visually-similar bulbuls, based on similar patterns of plumage (assessing plumage similarity through visual inspection; careful spectrophotometric comparisons is an important avenue for future research). The first pairing included Black-headed and Black-crested Bulbuls, both of which have bright yellow body parts and glossy black heads (Figure 5). These species were regularly found in the same areas of mature forests where vegetation is dense and comprised of multiple storeys. Discriminant analysis readily distinguished these two species on the basis of both length and frequency measurements; Black-headed Bulbuls sing longer, trilled songs whereas Black-crested Bulbuls sing shorter songs made up of varied frequency-modulated elements that rise in pitch. The second pairing included Streak-eared and Stripe-throated Bulbuls, both of which have plain, brownish-grey plumage (Figure 5). These two species co-occur, often in dense vegetation of deciduous forests, where their cryptic plumage colour makes them difficult to observe. Discriminant analysis readily distinguished these two species on the basis of both length and frequency measurements; Streak-eared Bulbuls have longer, trilled songs whereas Stripe-throated Bulbuls have shorter, lower-pitched songs made up of varied frequency-modulated elements. The third pairing included Red-whiskered and Sooty-headed Bulbuls, both of which share similar plumage patterns consisting of black facial ornaments and bright red undertail coverts (Figure 5). These two species also co-occur, frequently singing from treetops in urban areas and secondary forests. Discriminant analysis showed less separation between this pair of species owing to the similarly complex, frequency modulated songs given by both. Nevertheless, discriminant analysis significantly separated the two species, primarily on the basis of song length; Red-whiskered Bulbuls have longer songs with more elements uttered in rapid succession than Sooty-headed Bulbuls.

Evidence in acoustic significant for genetic drift between sympatric speceis

A comparison of acoustic distances of all six species demonstrates that species-specific vocalizations provide information for pre-mating isolation of all the six *Pycnonotus* species. Matrix analysis using the Mantel test showed substantial positive correlation between acoustic and genetics distances, although results were statistically non-significant. The results predict that species-specific vocalizations have evolved relatively with genetic differentiations which have also been shown in several species of passerines (e.g. *Regulus* group; Päckert *et al*., 2003, Oropendolas group; Price and Lanyon, 2002). These results support the idea that acoustic divergence reflects the role of genetic drift leading reproductive isolation between closely related species (West-Eberhard, 1983; Slabbekoorn and Smith, 2002; Irwin *et al*., 2008).

Mitochondrial DNA is a useful resource for studies of genetic relationship among species (Avise, 1986). It has been widely used for phylogenetic constructions to explain genetic evolution and genetic relationship in several groups of avian species e.g. Blackbirds (Lanyon and Omland, 1999), Doves (Johnson *et al*., 2001), *Phylloscopus* warblers (Bensch *et al*., 2006), and Fulvettas (Pasquet *et al*., 2006). Molecular techniques provided a powerful tool for species analysis, particularly within this highly diverse group of the family Pycnonotidae. Phylogenetic hypothesis of the bulbuls have suggested three distinct lineages of the family Pycnonotidae comprising: 1) monotypic African genus *Calyptocichla*, 2) a large African clade, and 3) a large Asian clade (Pasquest *et al*., 2001; Moyle and Mark, 2006; Johansson *et al*., 2007). They also proposed that most *Pycnonotus* species could be grouped into the large clade of Asiatic bulbuls (except *P. barbatus*, *P. nigricans*, and *P. capensis*, see Pasquest *et al*., 2001; Moyle and Mark, 2006 for detailed analyses). However, due to the numerous and morphologically heterogeneous members of the Asiatic genus *Pycnonotus*, many species were not sampled and detailed analyses within this clade lacked. This study of six *Pycnonotus* species has added the first 16 mtDNA sequences of three Streak-eared Bulbuls and three Sooty-headed Bulbuls. This genetic information would be useful for further robust phylogenetic analysis of this group.

By comparing acoustic and genetic distances, the results show a non-statistically significant positive correlation between acoustic and genetic features, suggesting that variations in song structure among the six species of *Pycnonotus* reflects phylogenetic relationship. Inter-specific comparisons between acoustic and genetic features show that species-specific vocalization is significant for members of the genus *Pycnonotus* through reinforcement of pre-mating isolations mechanisms (Price, 1998; Irwin *et al*., 2001). This acoustic-genetic relationship underlies the song divergences in these sympatric *Pycnonotus* species and contains evolutionary information on these birds' song-related phylogeny (Price and Lanyon, 2002; González *et al*., 2011). Molecular analyses of the 16s mtDNA revealed that all six *Pycnonotus* species are closely-related and share a common evolutionary history; genetic distance using MCL of all six species ranged from 0.023 to 0.063. Molecular results of this monophyletic group of sympatric *Pycnonotus* species support previous phylogenetic studies (Pasquest *et al*., 2001; Moyle and Mark, 2006). Surprisingly, analysis of song structures shows that *Pycnonotus* songs rapidly change over short periods of evolution. The song structures of all six species can be subdivided into two discrete song types: 1) trilled songs (Black-headed Bulbul and Streak-eared Bulbul), and 2) frequency-modulated tones (Black-crested Bulbul, Stripe-throated Bulbul, Red-whiskered Bulbul, and Sooty-headed Bulbul). It is possible that genetic relationship plays a minor role in song development in passerines, conversely song differences in *Pycnonotus* may be shaped by multiple selective pressures e.g. song learning, social competition, sexual selection, and habitat adaptation (Wiley and Richards, 1978; West-Eberhard, 1983; Grant and Grant, 1996; Catchpole and Slater, 2008; Kirschel *et al*., 2009; Tobias and Seddon, 2009).

Evidence in divergences of acoustic and body size as habitat adaptation

Comparisons between acoustic and morphometric distances suggest that body size may be a distinctive feature in the genus *Pycnonotus*. Multi-species comparisons add to understand song differentiation between closely-related birds, which may have evolved correspondingly with morphological adaptation e.g. Darwin's finches (Podos, 2001), Neotropical birds (Martin *et al*., 2011), and Hummingbirds (González *et al*., 2011). Sex determination using universal primers P2/P8 offer advantages for comparative morphological study, particularly in sexual monomorphic species where sex identification is visually difficult (Griffiths and Korn, 1997; Griffiths *et al*., 1998; Kahn *et al*., 1998; Leppert *et al*., 2006; Wu *et al*., 2007; Chang *et al*., 2010). Sexual dimorphism based on body size of all six *Pycnonotus* species agrees with Amiot *et al*. (2007), who suggested that size differences of this group is difficult to determine in the field. Through the findings of this study it is recommendable to determine sex of all six species by using DNA dimorphisms.

The relationship between acoustic and morphometric distances indicates that song structure of all six sympatric *Pycnonotus* species become more divergent with increasing morphometric distance. A classic example of body size influencing acoustic structure has been studied in Darwin's finches which provided strong evidence that beak morphology can shape a bird's signal evolution i.e. trill rate and song frequency Podos (2001). Body weight is also important for driving acoustic signals because of physical and energetic constraints Podos (2001). This is further supported in studies of distress calls and songs of Neotropical birds (Martin *et al*., 2011), who found a significant negative correlation between body weight and acoustic signal frequency. In this study of six sympatric *Pycnonotus*, the wing and tarsus length as variables representing flight and moving apparatus were important morphometric features in relation to acoustic variations for both males and females rather than the vocal apparatus (i.e. beak and body weight). In contrast to Podos (2001) and Martin *et al*. (2011), the beak and body weight of the six *Pycnonotus* were not important factors, probably because all six species have relatively close foraging niches; they are small frugivores, feeding within flocks, and foraging in similar fruiting trees. Field observations on habitat preferences suggests that all six species have slightly different preferred micro-habitats i.e. Black-headed Bulbuls and Black-crested Bulbuls are regularly associated with mature forests; Streak-eared Bulbuls and Stripe-throated Bulbuls prefer to share bushy vegetations, and Red-whiskered Bulbuls and Sooty-headed Bulbuls forage in urban areas. Therefore, habitat-induced acoustic and morphological adaptation may be a possible predictor for acoustic-morphological correlation illustrating that acoustic variation has evolved in correspondence with foraging morphology. This finding demonstrates that acoustic deviations in the six species of the *Pycnonotus* studied here have evolved through morpho-ecological adaptive processes, and indirectly from ecological competition and habitat partitioning. Therefore, it can be concluded that ecological adaptation also is a factor influencing song divergences of the six species studied, but further experimental work on the function of their inter-specific competition and ecological adaptation remains.

Further investigations

The study results provide new knowledge about the song structure of all six *Pycnonotus* species, which potentially could play a significant role for species recognition in flock living birds. In addition, song is potentially important for the reproductive isolation in order to increase the genetic drift between closely-related six species. However, the magnitude of genetic and morphological interactions with acoustic features is unclear, as the results are based on a small set of congener species. Given minor interaction between the acoustic-genetic relationship and the acoustic-morphological relationship, this correlation is statistically non-significant. Further research for more accurate distinctions are required by adding more species in future studies and may eventually lead to statistically significant results for the genus *Pycnonotus*.