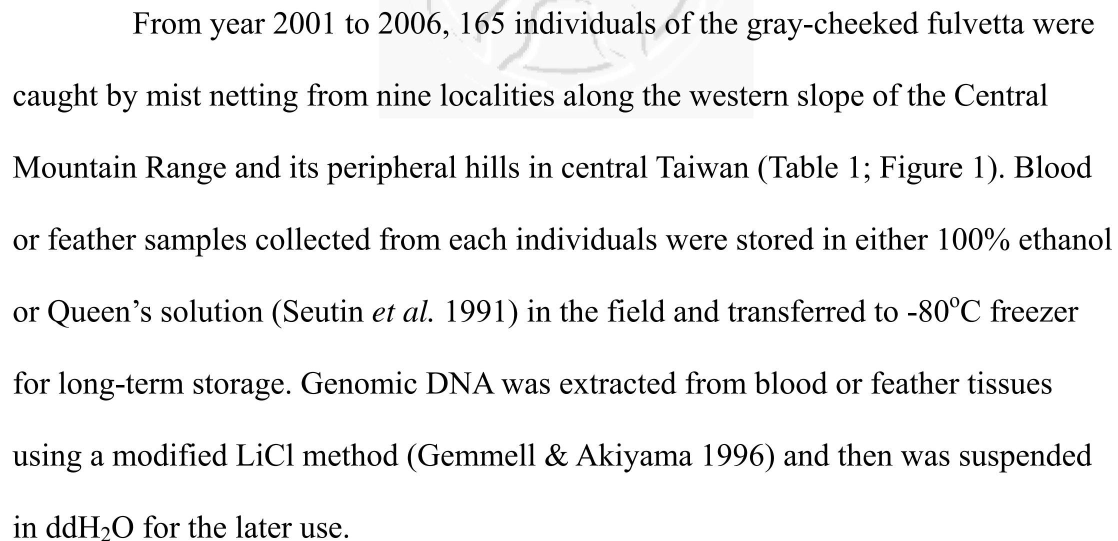
Materials and Methods



Study species, study areas and genomic DNA isolation

From year 2001 to 2006, 165 individuals of the gray-cheeked fulvetta were caught by mist netting from nine localities along the western slope of the Central Mountain Range and its peripheral hills in central Taiwan (Table 1; Figure 1). Blood or feather samples collected from each individuals were stored in either 100% ethanol or Queen's solution (Seutin *et al.* 1991) in the field and transferred to -80°C freezer for long-term storage. Genomic DNA was extracted from blood or feather tissues using a modified LiCl method (Gemmell & Akiyama 1996) and then was suspended in ddH_2O for the later use.

Screening for malaria infections

A nested PCR protocol (Waldenström *et al.* 2004) was conducted to amplify a 580 bp fragment of mitochondrial cyt-*b* gene from the malaria parasite *(Plasmodium* or *Haemoproteus spp.*) to detect infection in the samples. PCR reactions were repeated no fewer than three times for each sample, and PCR products were electrophoresed on a 1.2% agarose gel for visualization. Individuals typed positively at least twice were considered infected. The infectious state of an individual was classified as whether malarial DNA was present (1) or absent (0). PCR products of infectious individuals were then sequenced using MegaBASE^{TM} 1000 DNA analysis system (Amersham Bioscience, Piscataway, NJ). DNA sequences were aligned and assembled using software Sequencher (Gene Codes Corp., Ann Arbor, MI). Phylogenetic relationships among alleles, including five malarial sequences (i.e. genus of *Plasmodium* and *Haemoproteus*) downloaded from Blast network service

(National Center for Biotechnology information, Bethesda, MD), were reconstructed via neighbor-joining method in MEGA 3.0 (Kumar *et al.* 2004).

Screening of MHC class I variation

Polymerase chain reaction (PCR) primers (ex2-02F: CCA GAA TAT TGG GAT AGG AAC ACC and ex3-03R: CCT GGT GAT CTC AGC AGC GCC GTC) were designed based on conserved regions of exon 2 and exon 3 of MHC class I α chain of the great reed warbler (GenBank accession no. AJ005503 - AJ005510). This primer pair amplified a fragment of MHC class I gene comprising part of exon 2, the entire intron 2, and part of exon 3 (*i.e.* the peptide binding region, PBR). The PCR was set up in a volume of 12.5 μl containing 50-200 ng genomic DNA, 1 x PCR buffer (TOYOBO), 0.16 μM of each primers, 2.0 mM $MgCl_2$, and 0.25 U Blend Taq-Plus DNA polymerases (TOYOBO) with 0.05 μg of anti-Taq DNA polymerase antibody (TOYOBO). PCR conditions were set as 2 min at 94 ^{o}C, followed by 25 cycles of 20 s at 94 ^{o}C, 30 s at 55 ^{o}C, 1 min at 72 ^{o}C, then the next 35 cycles of 20 s at 94 ^{o}C, 30 s at 62 ^{o}C, 1 min at 72 ^{o}C, followed by final extension of 10 min at 72 ^{o}C in the iCycler thermal cycler (Bio-Rad, Hercules, CA). Samples containing PCR products were ethanol precipitated, mixed with a ROX-labeled internal size marker, MegaBASE^{TM} ET-900R, and then electrophoresed using the capillary electrophoresis-single strand conformation polymorphism (CE-SSCP) method (Arakawa *et al.* 1997) on a MegaBASE^{TM} 1000 DNA analysis system (Amersham Biosciences). The CE-SSCP procedure was carried out as follows: samples were denatured for 3 min at 94 ^{o}C, snap-cooled on ice, then injected (45 s, 3 kV) into the capillaries containing 3% MegaBASE^{TM} nondenaturing Long Read Matrix (LRM, Amersham Biosciences), and run in MegaBASE^{TM} 1 x Running Buffer at 27 ^{o}C and

10 kV for 200 min. When the CE-SSCP procedure was completed, the program FRAGMENT PROFILER v1.0 was used to score the MHC class I alleles. The MHC typing procedure was conducted more than twice for each sample to check for consistency between runs. We defined each clear peak ranging from about 450-760 bp in length in chromatogram as an allele, although, the orthology of alleles was unknown. It should be noted that the set of primers used only amplified a subset of total MHC alleles (data not shown), however, following Westerdahl *et al.*(2004), we consider that the set of amplified alleles should act as a good index of the MHC diversity for each individual.

Screening for microsatellite variation

Five microsatellite loci, ALMO34, ALMO138 (Yao *et al.* unpublished), LSGATA7, LSGATA21 (Yeung *et al.* 2004) and GC-GATA10 (Huang *et al.* 2004), were used in this study (Table 2). Each 10 μl PCR reaction mixture containing 50-100 ng genomic DNA, 1 x PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.01% (w/v) gelatin and 0.1% triton X-100), 0.5 mM dNTP, 0.2 μm of each primer, 1.5 mM $MgCl_2$ and 0.4 U Taq DNA polymerase (Amersham Biosciences) were subjected to the following thermoprofile: 2 min at 94 , followed by 40 cycles of 20 s at 94 ^{o}C, 62 ^{o}C-0.3 ^{o}C per cycle for 20 s, 30 s at 72 ^{o}C, and final extension for 1 min at 72 ^{o}C using the iCycler thermal cycler (Bio-Rad). PCR products were electrophoresed on a MegaBASE^{TM} 1000 DNA analysis system (Amersham Biosciences). Allele size was scored using the software GENETIC PROFILER 2.0 (Amersham Biosciences). To verify evolutionary independence of the five loci, linkage disequilibrium test between pairs of loci was carried out using GENEPOP 3.4 (Raymond & Rousset 1995). To compare level of diversity of microsatellites with that of MHC, multilocus

microsatellite heterozygosity was calculated as the number of heterozygous loci (score '1' for each heterozygous locus and '0' for each homozygous locus) divided by the total number of loci following Westerdahl *et. al.* (2005).

Data analysis

Relationship between genome-wide variability and MHC diversity

Spearman's correlation was performed to calculate correlation between MHC diversity and the genome-wide variability (i.e. microsatellite heterozygosity) to determine whether there was any association between the two marker systems at the individual level. In addition, average percent difference (APD) was used as an index of within-population genetic variation (Yuhki & O'Brien 1990), which was defined as the average percentage of alleles that differed between individuals, were calculated for MHC and microsatellites according to Yuhki (1990); Pearson's correlations between MHC and microsatellite APD values from populations of 9 localities were then calculated.

Relationships between malarial infection, genetic variations and altitudes

To detect altitudinal adaptation at MHC class I loci, three statistical tests were implemented. First, I calculated the proportion of infected individual within altitudinal populations as prevalence of malarial infection, and implemented a likelihood ratio test (LRT, G-test)(Sokal & Rohlf 1995) to determine whether the probability of being infected with malaria differed between altitudes. Second, forward stepwise logistic regression (*P*-to-enter= 0.1, *P*-to-remove= 0.05) was performed to identify MHC alleles significantly associated with malarial infection in endemic

localities. In addition, odds ratio was calculated by EXCEL to compare the likelihood of getting malarial infection in individuals carrying a specific MHC allele or not. In my study, an odds ratio greater than one implies that individual carrying the specific MHC allele is more likely to be infected; an odds ratio less than one implies that individual carrying the specific MHC allele is more resistant to malarial infection.Thirdly, correlations were sought between the relative frequency of specific MHC alleles under malarial selection and corresponding intensity of malaria selection in the environment, so I examined the association between frequencies of the specific MHC alleles and altitudes by implementing a LRT. All statistical tests were performed in JMP 5.0 (SAS institute) software.

Genetic differentiation between populations among altitudes

I analyzed genetic differentiation of MHC and microsatellites between populations from different altitudes. I treated each MHC and microsatellite allele as either present (1) or absent (0) in a population and performed an Analysis of Molecular Variance (AMOVA) in Arlequin ver. 3.0 (Excoffier *et al.* 2005). The AMOVA estimates genetic structure indices using information on genotypes and their frequencies through non-parametric permutation procedures (Excoffier *et al.* 1992). F_{ST} statistical tests from AMOVA allowed us to obtain estimates of the overall genetic differentiation among altitudinal the gray-cheeked fulvetta populations based on MHC and microsatellite alleles.