CHAPTER 3 METHODOLOGY

3.1 Study area

Huai Kha Khaeng Wildlife Sanctuary (HKK) is a protected area located in Uthai Thani and Tak provinces in western Thailand at 15°00’ - 15°50’N and 99°00’ - 99°19’E. HKK is 2780 km^2 and is covered by a mixture of mixed deciduous forest, deciduous dipterocarp forest, dry evergreen forest and montane evergreen forest. Retaining connectivity with Thungyai Naresuan Wildlife Sanctuary, Mae Wong National Park, Umphang Wildlife Sanctuary and Pu Toie National Park, HKK is part of a network of 34 forested wildlife sanctuaries, national parks and protected areas, forming a large protected area called the Western Forest Complex (WEFCOM). In the eastern part of HKK, the study area starts from Huai Chang Tai stream, which is located on the Khao Khiao massif with a maximum elevation of 1347 msl. The study area is approximately 2 km from the Khao Nang Rum wildlife research station (KNR) (15°29’N, 99°18’E) (Figure 3.1). During the study period, total annual rainfall was 1625 mm, the highest was in September with 509 mm, whereas the lowest was in February with none. The annual mean air temperature was 26.3 °C, and maximum and minimum air temperatures were 39 and 18 °C, respectively (Khao Nang Rum wildlife research station, Department of Meteorology).

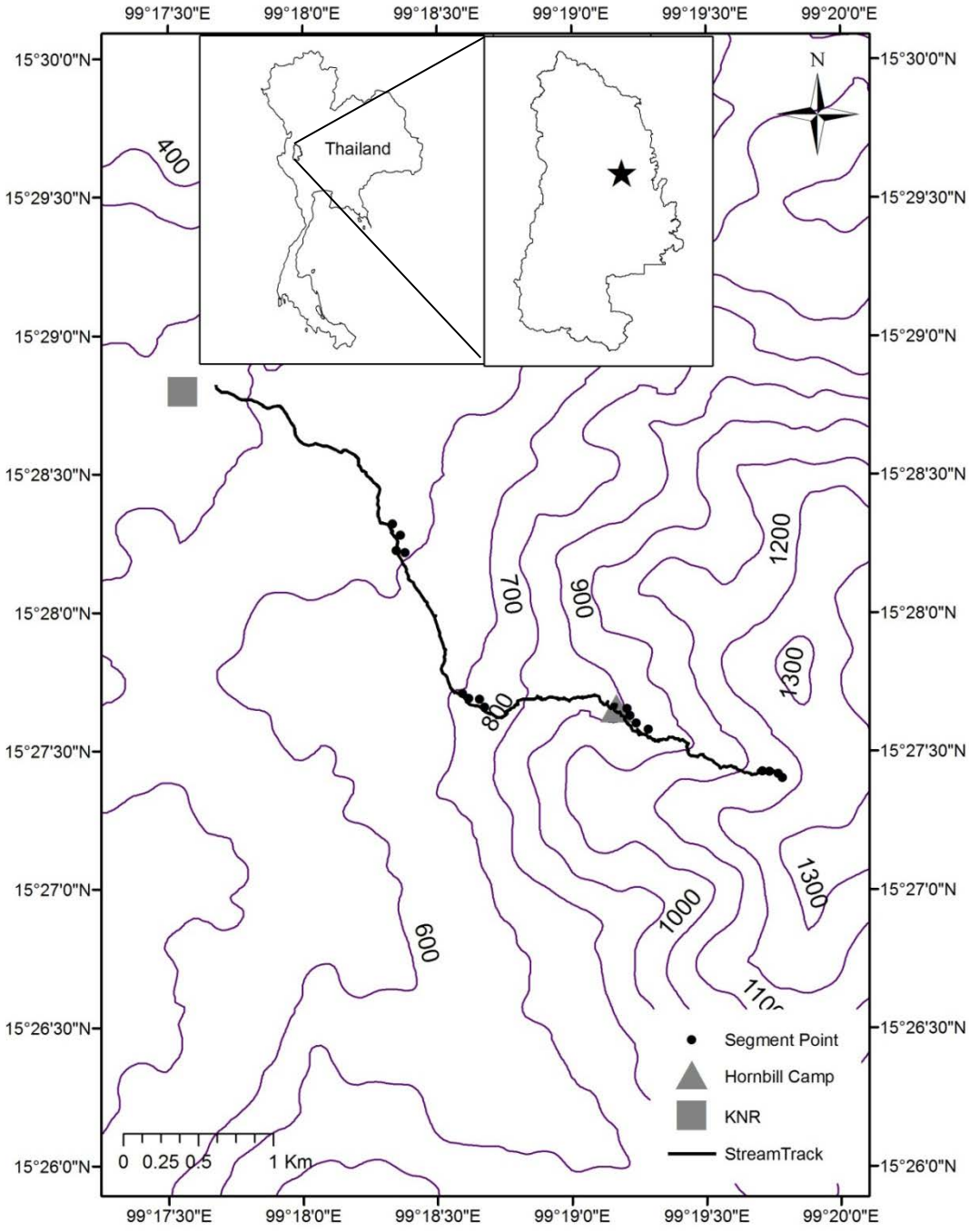

Figure 3.1 Map showing the topography (grey contours) and the stream (black line) of the study area in the Huai Kha Khaeng Wildlife Sanctuary (grey square = Khao Nang Rum wildlife research station, grey triangle = Hornbill Camp (Camp), black circles = 50 m segment locations along the Huai Chang Tai stream at 550, 700, 900 and 1100 msl).

3.2 Sampling methods

3.2.1 Anuran survey

The study area was divided into 4 sections (roughly 200 m elevation intervals) from 550 - 1150 msl, which I labeled as 550 (550 - 600 msl), 700 (700 - 750 msl), 900 (900 - 950 msl) and 1100 (1100 - 1150 msl), covering evergreen and hill evergreen forest starting at evergreen forest near the transition zone with mixed deciduous forest at approximately 500 msl. Each section is located approximately 1 km apart (Figure 3.1). The altitude of each elevation section was obtained from direct field measurements using an altimeter (Oregon - Scientific model EB833). In each elevation section, the 200 m transects were divided into four 50 m segments, 10 m apart set up along the Huai Chang Tai (Figure 3.2). In total, there were sixteen segments. Visual encounter surveys (Heyer *et al*., 1994) were conducted after sundown along a given transect between 19:00 to 21:00 each night. Three observers searched for 20 - 30 min per segment; species, number of observed frogs, frog snout-vent length (SVL), weight (I weighed the first 10 adult frogs encountered per particular species to the nearest 0.01 g during July to October 2013) and position found were recorded. The frogs were photographed and identified to species using a field photographic guide (Chan-ard, 2003) and taxonomic literature (Frost, 2013). The surveys were conducted twice per month along each transect from November 2012 to October 2013.

3.2.2 Environmental factors

5 x 10 m plots were established along each segment (n = 16) to measure the density of understory vegetation (stems/50 m^2). Density was estimated by counting the apex of all individual plant stems intercepting four height strata: 0 - 20 cm (ground level), 21 - 100 cm (1 m), 101 - 200 cm (2 m) and above 200 cm (> 2 m) (modified from Keller *et al*., 2009). At each segment I collected stream characteristics (width (m) and depth (m) which were used to calculate stream volume (m^3)), water pH and canopy cover (%). These microhabitat features were recorded at every 10 m along a segment, in total six checkpoints (0, 10, 20, 30, 40, & 50 m) per segment (Figure 3.2). Rate of stream flow (m/s) was recorded at each transect segment. The mean obtained from each 200 m transect was used for analysis. Slope (degrees) was measured by using a Suunto (PM-5/400PC) clinometer. The vertical height of the single steepest waterfall along

each transect was also recorded. Air temperature maximum, minimum and average (°C) during the survey period was measured at the KNR as well as at the Camp (Figure 3.1), which was located on the Khao Khieo massif approximately 900 msl adjacent to the Huai Chang Tai. During each nighttime survey, at the beginning and at the end of each transect segment, water temperature (°C), soil (pH, relative humidity and surface temperature (°C)) and air (relative humidity (%) and temperature (°C)) were recorded. Data on monthly total rainfall (mm) and mean air temperature for 550 msl were collected from KNR, and at 700 - 1150 msl these were also collected from the Camp from November 2012 to October 2013. Monthly total rainfall and mean air temperature from KNR were then plotted using a climate diagram in order to define the wet and dry seasons during this study (Walter *et al*., 1975).

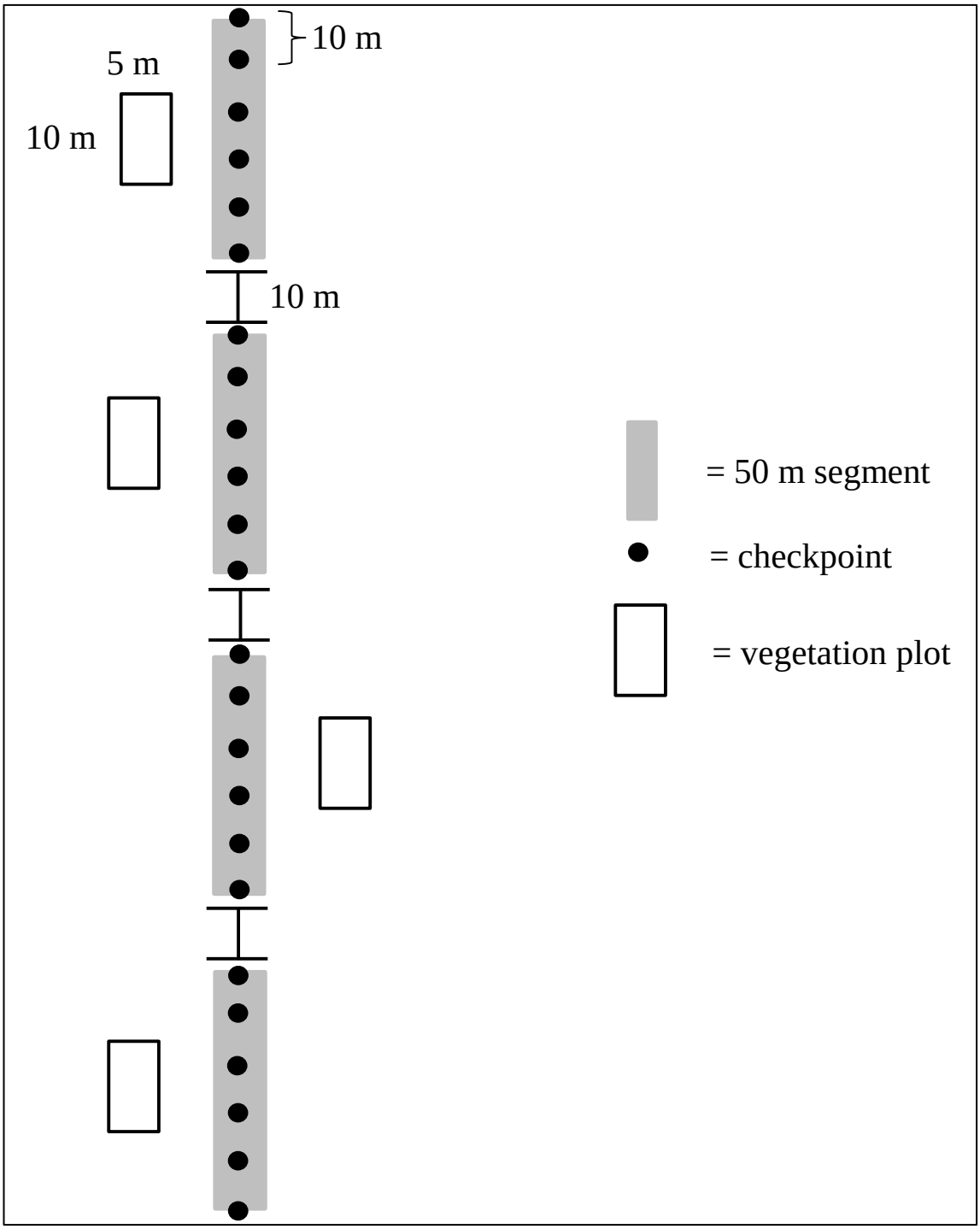

Figure 3.2 Diagram of stream segments with vegetation plots and checkpoints at each elevation.

3.2.3 *Bd* sample collection

Between September - October 2013, I collected skin swabs from the ventral, sides, feet and leg surfaces of 50 encountered adult anurans with sterile cotton swabs (JCB Mentip, Japan). Anurans were handled using a new pair of disposable gloves for each individual to avoid contamination of the samples and collectors. Each individual was then released at the site. Each sample was fixed in 70% ethanol, placed in a microtube and stored at room temperature. The samples were then processed at the Wildlife Forensic Science Unit laboratory, Department of National Parks, Wildlife and Plant Conservation (DNP) for *Bd* DNA extraction and PCR analysis.

3.2.3.1 Extraction of *Bd* DNA from swab samples

Potential *Bd* DNA was extracted from the swab samples following the protocol from the ArchivePure DNA Kit for genomic DNA purification from buccal cells (5 PRIME, Germany) with some modification described by the Conservation Research and Education Division of Thailand's Zoological Park Organization (ZPO) (Kamolnorranart *et al*., 2010). A measurement of DNA concentration was carried out using NANODROP 1000 Spectrophotometer (Thermo Scientific, USA) and the quality of genomic DNA was analyzed by agarose gel electrophoresis (see Appendix C). Each swab was placed in a microtube containing cell lysis solution and proteinase K solution (20 mg/ml) (Qiagen Company) and incubated at 55 °C for 60 min. The cotton swab was then removed from the solution and 1.5 µl RNase A solution was added. The solution was then incubated at 37 °C for 15 min. Afterwards, the sample was cooled on ice for one min. Proteins were removed by adding protein precipitation solution, then placed on ice for 5 min and precipitated by centrifugation. DNA in the supernatant was precipitated in isopropanol (2-propanol) and glycogen solution (20 mg/ml) and stored at -20 °C overnight. The DNA pellet was collected and washed with 70% ethanol then air dried. After the DNA pellet was dry, it was dissolved in DNA hydration solution (50 ng/µl) and incubated at 65 °C for 5 min. DNA was stored at 4 °C and was later used as the source of the DNA template for the PCR assay as described below.

3.2.3.2 Conventional PCR assay

All samples were screened for chytrid fungus detection with conventional polymerase chain reaction (PCR) of the ITS-1/5.8S DNA region of *Bd* using two specific primers for *Bd* (Annis *et al*., 2004; Garner *et al*., 2005; Goka *et al*., 2009). The sequences of the primers were Bd1a, 5'-CAGTGTGCCATATGTCACG-3' and Bd2a, 5'-CATGGTTCATATCTGTCCAG-3' (Annis *et al*., 2004; Goka *et al*., 2009).

PCR was conducted with 5 µl of each DNA template in a total reaction volume of 25 µl. The PCR reaction mix contained 12.5 µl of the TopTaq Master Mix Kit (200 µM of each dNTP, 1.5 mM $MgCl_2$, and 1.25 units of TopTaq DNA polymerase purchased from Qiagen, Germany), 1.5 µl of each primer and 2.5 µl CoralLoad Concentrate. PCR conditions included an initial denaturation for 3 min at 94 °C, followed by a 30 s denaturation at 94 °C, 30 s primer annealing at 50 °C, and 30 s extension at 72 °C for 30 cycles, and a final extension for 10 min at 72 °C. PCR products were separated on 1.5% agarose gels and bands of the DNA fragments were viewed under UV light.

3.3 Data analysis

3.3.1 Anuran richness and relative abundance

The species diversity and similarity at each elevation were determined by Shannon and Wiener's species diversity index and Morisita's Index of Similarity, respectively (Krebs, 1998). Both indices were estimated using Program SPADE (Chao and Shen, 2010). The expected richness of each elevation was estimated using the Chao 2 estimator (Krebs, 1998; Fu *et al*., 2006; Hutchens and DePerno, 2009; Walther and Moore, 2005). The Chao 2 also provides the least biased estimates of species richness when there are only a small number of samples (Colwell and Coddington, 1994). Species accumulation curves were generated based on the Chao 2. Abundance at each elevation was calculated from the total number of observed frogs at each transect. Estimates of species richness and 95% confidence intervals through rarefaction analysis were made using the Chao 2 to determine whether estimated species richness changed along the elevation gradient using the total number of observed frogs found across all elevations. From rarefaction curves, a significant difference in estimated species richness is indicated by an absence of overlap in the confidence intervals of rarefaction

curves along elevation at the maximum sampling effort (number of observed frogs) (Colwell *et al*., 2004). For the Chao 2, the species accumulation curve and the rarefaction curve were estimated using Estimate S Version 9.1 (Colwell, 2009). Statistical differences in species, abundance of observed frogs and environmental factors among elevations were determined by one-way analysis of variance (ANOVA) followed by Student's *t*-tests. Kruskal-Wallis tests followed by Mann-Whitney *U*-tests were used instead of ANOVA when variables were not normally distributed.

Since the survey of segments varied in length of time searched, the number of observed frogs of each species was calculated based on the relative abundance per unit time (number observed/hour searched). All sub-adults were excluded from this study. For field effort, the number of hours in the field influenced significantly the number of frogs observed ($r = 0.561$, $r^2 = 0.31$, $n = 96$, $P < 0.001$). I then used an offset (log(time in searching)) to account for this (Behangana *et al*., 2009).

3.3.2 Species composition

Differences in species composition among elevation sections were determined by non-metric multidimensional scaling based on the Bray-Curtis index (NMDS) (Bray and Curtis, 1957; Keller *et al*., 2009; Kruskal, 1964; Magurran, 2004). I estimated the partition of community variation explained with each axis by correlation of Bray-Curtis distance measurements of the respective axis with the community matrix. Finally, I correlated environmental vectors onto these ordination axes (Venables and Ripley, 2002). Twenty random starting configurations were used for constructing the ordination diagram, with the final configuration that minimized the stress of the ordination configuration being retained for plotting. Significance in variation in community composition was assessed using the analysis of similarities (ANOSIM) permutation test (Clarke, 1993). ANOSIM was used to indicate dissimilarity in species composition between two sites, and the ranges were from 0 (highly similar between groups) to 1 (highly different between groups) (Clarke and Gorley, 2001). The following criteria (Clarke and Gorley, 2001) were used to interpret the data:

$R \geq 0.75$ indicates clear separation in species composition

$R = 0.74$ to 0.5 indicates separation with some overlap

$R = 0.49$ to 0.25 indicates separation with strong overlap

R < 0.25 indicates no difference in species composition

The tests were performed using the vegan-package in R statistical and programming environment (version 3.0.2, R Development Core Team, 2009).

3.3.3 Relationship between species richness and abundance and environmental factors

The relationship between the anuran species richness and species abundance along the elevation gradient in conjunction with environmental factors (i.e., elevation, stream characteristics and density of understory vegetation etc.) and interactions between the factors were determined by Generalized Linear Mixed Models (GLMMs). Since I repeated the surveys by sampling the same transects twice a month for one year, GLMMs provide a more flexible approach for analyzing non-normal data when there are random effects (Bolker *et al*., 2008). Anuran abundances were likely to be correlated because I sampled the same transects multiple times; I therefore incorporated species identity as a random effect and allowed the intercept coefficient to vary across each transect to account for spatial autocorrelation. For species richness, I used the month as a random effect. I found over dispersion in the variance, thus data for abundance was approximated using a negative binomial distribution whereas for species richness I used a Poisson distribution. Independent variables were standardized by subtracting the overall mean and dividing by two standard deviations in order to improve model convergence and aid interpretation of coefficient estimates (Diez and Pulliam, 2007). I used all combinations of explanatory variables to build a set of candidate models. Variables that were highly correlated ($r > 0.7$) were each removed, in turn, from the analysis, and model comparisons were made, with the variables from the models with lower Akaike information criterion value retained (Burnham and Anderson, 2002). For species richness, I used ΔAIC_c for comparison among the models (Burnham and Anderson, 2002). For abundance, due to the relatively high uncertainty ($\Delta AIC_c<2$) among the set of top models, I then used Akaike model weights for comparison among the models (Burnham and Anderson, 2004). Model averaging was then used to estimate the coefficients for covariables in the models. I used 95% confidence intervals to identify variables with significant influence on species richness and abundance. The tests were performed using the 'lme4' package in R statistical and programming

environment (version 3.1.0, R Development Core Team, 2009). The descriptions for the environmental variables are shown in Appendix A Table A.1.