CHAPTER - 5

MATERIALS AND METHODS

5.1. Geography of the study areas

Chiang Mai, the third largest city of Thailand, is located about 700 km north from Bangkok in the central region of northern Thailand. It lies at 18° 50' N latitude and 99° 0' E longitude and at the altitude of 312 m from main sea level covering about 170,000 km^2 (Figure 6).
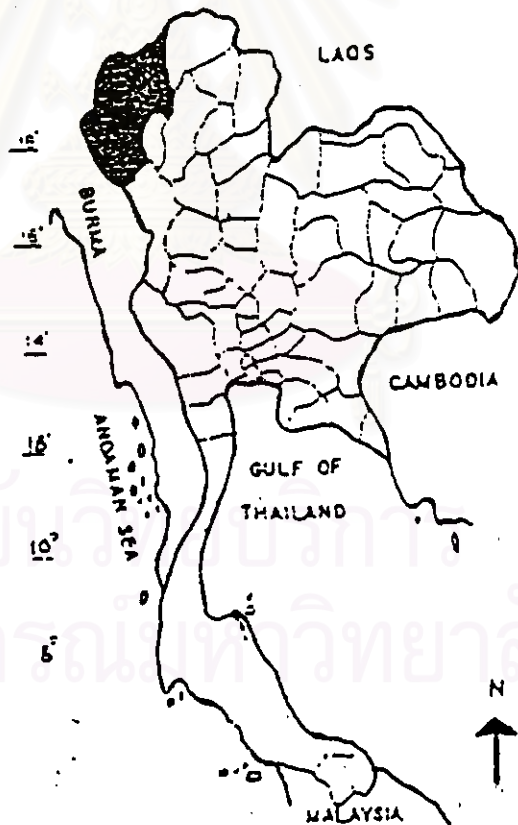
Figure 6. Map shows migratory behavior observation sites in three locations: Chiang Mai, Mae Tung Ting and Mae Hong Son indicated by dark color.

Topographically northern Thailand is divided into "uplands" and "low-lands". The upland regions include most popular mountains: Doi Inthanon, the highest mountain in Thailand and Doi Suthep. Doi Suthep is a national park, lies west of Chiang Mai rising to a height of 1610 m. The adjoining peak of Doi-Pui is about 1685 m. In these regions there are two types of forests viz. Deciduous and evergreen. The deciduous forests are found between 350-950 m elevation. Deciduous forests are mostly dominated by *Depterocarpus* species. Mixed deciduous forests are dominated by *Tectona grandis* L. f. (Verbenaceae). The primary evergreen forests are found from 950 m elevation to the summits 1350 m with tall canopy 30-50 m high (Figure 7). *Eupatorium odoratum* L. *Tridax.* Procuben L., *Memosa diplotricha* C. Wright ex. Saux. Var. and *M. pegra* L. (Leguminose, Memosoideae) are very commonly found at various elevations in disturbed areas from 350-950 m.
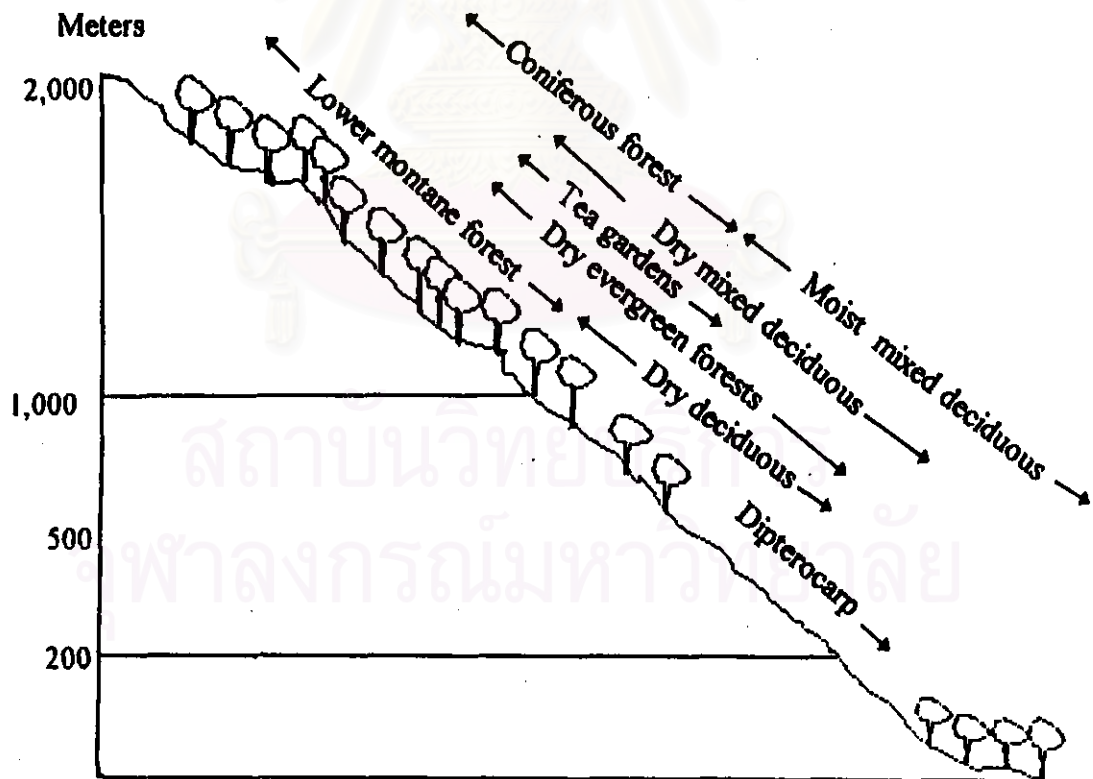
Figure 7. Schematic highland-lowland transact for the northern Thailand (Adapted from Kunstadter and Chapman, 1978).

The migratory behavior of *A. dorsata* were observed at three locations: Chiang Mai, Mae Tung Ting and Mae Hong Son. Chiang Mai is the second largest city of Thailand whereas Mae Tung Ting (village) and Mae Hong Son is a province located on northwest from Chiang Mai city (Figure 6).

5.2. Meteorological data

Means of meteorological data (air temperature, relative humidity, rainfall and wind speed) of Chiang Mai (CM) and Mae Hong Son (MHS) for years 1996-1998 are presented in Table 5. These data were obtained from the weather station, northern regional meteorological station near Chiang Mai International Airport which was 3-15 km from my study sites in Chiang Mai (Appendix-I).

Table 5. Means of air temperature, relative humidity, rainfall and wind speed of Chiang Mai (CM) and Mae Hong Son (MHS) provinces recorded from 1996-1998.

Month	Air temperature (°C)		Relative humidity (%)		Rainfall (mm)		Wind speed (km/day)	
	CM	MHS	CM	MHS	CM	MHS	CM	MHS
January	20.1	19.5	64	71	0.0	00.0	1.6	0.4
February	22.0	21.0	66	61	47.5	00.0	2.6	0.9
March	26.2	26.4	58	57	19.1	12.5	3.3	1.2
April	28.2	28.2	63	53	209.6	7.4	3.6	0.7
May	28.4	30.5	71	62	117.1	117.0	3.9	0.8
June	27.7	28.0	77	75	107.5	125.3	3.9	0.9
July	27.4	27.0	78	82	142.0	213.6	4.2	0.5
August	27.4	26.7	89	84	266.0	314.6	3.6	0.5
September	26.7	26.7	80	82	257.2	192.0	3.1	0.3
October	26.1	26.8	76	80	156.8	80.0	2.9	0.2
November	24.9	24.3	75	80	61.0	10.7	2.1	0.2
December	22.0	23.0	69	77	0.0	0.1	1.6	0.3

There are three seasons: raining season (June-October), dry season (November-February) and hot-dry season (March-May). January is the coldest month with combination of a cold wind and thunder shower. Ambient temperature gradually increased from February and exceeded up to 40°C in May (hot season). Rainy season starts from June to October.

5.3. Study sites selection for genetic studies

5.3.1. Study site selections to test hypothesis-I

To test the hypothesis-I whether the colonies occupied the same nest site are related or not (see chapter 2). The health care center building (HCC) located in the central Chiang Mai City is used annually as a nest site by colonies of *A. dorsata*. Two locations on this building are particular favored for nesting; a third story window awing (Figure 8). Although there are 32 windows of identical size and design in the health care center building. The same window is consistently used for nesting.

A. *dorsata* swarm

Figure 8. *A. dorsata* swarm occupying a particular window frame of
the health care center building, Chiang Mai.

5.3.2. Study sites selection to test hypothesis-II

Similarly to test the hypothesis-II whether the aggregated colonies on single support are genetically related or not related (see chapter 3), three aggregations were studied. Aggregated colonies were collected from three nest sites. The first a water tower of Maejo University that was located about 35 km north from Chiang Mai city (Figure 9). Second, was the window of third floor of the health care center building and third a branch of a tree in Mae Tung Ting village.

Figure 9. Three aggregated colonies nesting together on a water tower of
Maejo University, Chiang Mai.

5.4. Genetic analysis of samples

5.4.1. Collection of brood samples to test hypothesis-I from the same nest site

Brood samples were obtained from the five individual colonies that seasonally re-occupied the window of the health care center from 1993-1998 (Table 6). Unfortunately, the 1994 sample was not collected.

Table 6. Sampling sites and collection date (years) of brood from the particular window of the health care center building (HCC).

Nest Location (Window site)	Collected date	Bees stages	Total number of colonies
Window of HCC	15 / Oct. / 1993	Adults	1
Window of HCC	12 / Oct. / 1995	Adults	1
Window of HCC	27 / Feb. / 1996	Pupae	1
Window of HCC	22 / Feb. / 1997	Pupae	1
Window of HCC	22 / Mar. / 1998	Pupae	1

5.4.2. Collection of brood samples to test hypothesis-II

Three aggregated colonies on a water tower of Maejo University, two aggregated colonies on the health care center building and two colonies nested on a single branch of a tree from Mae Hong Son were collected (Table 7).

The nest distance between two out of three colonies on water tower of Maejo University was 0.2 m and one colony was about 2.5 m apart. Similarly, the distance between two colonies nested on the health care center building was 3 m and two colonies nested on a single branch of tree was 0.75 m.

Table 7. Brood samples from aggregated colonies collected date and sites.

Sampling sites	Collection year	Nests sites location	Bees stages	Number of colonies
Mae Tung Ting	1996	Tree	Pupae	2
Maejo University	1997	Building	Pupae	3
Health care center building	1997	Water tank	Pupae	2

5.4.3. Brood sampling methods

When a colony re-occupied the window of the health care center building, sealed brood size of 3×5 cm containing about two hundred pupae was collected by cutting the comb. Brood was then wrapped in aluminum foil and stored in liquid nitrogen before transferring to the Bee Biology Laboratory. All the samples were stored at -75° C at the Malaria Research Laboratory at Chulalongkorn University, Bangkok.

5.4.4. Brood samples analyses

In many analyses reported here it was necessary to test hypothesis concerning the relatedness of queens heading various colonies. As queens could not be sampled directly, it was necessary to infer queen genotype using a sample of worker brood. Ten to 20 bees per colony were used to extract DNA.

5.4.5. DNA extraction

Eppendorf centrifuge tubes were numbered, pierced and placed on ice. Each pupa was then removed from the frozen brood comb with clean forceps. The head of each pupa was detached from its body and cut into two (Figure 10). During the head detaching processes, the scalpel and forceps were regularly washed in 100% ethanol to minimize the possibility contamination between bees. One half of the head was stored in a deep freeze for future use and other half was placed in an Eppendorf tube and

finely ground with a sate stick for 5-10 seconds in order to release head tissues. After crushing the head, 1 ml of boiling 5% chelex® 100 resin in 0.1 M TE (BioRad Sydney) was added (Appendix-IIIa). Then the Eppendorf centrifuge tubes containing chelex® resin and DNA extraction were allowed to float in boiling water for 15 minutes (Walse et al., 1991). Samples were stored at 4°C in refrigerator.

Figure 10. One half part of the head of a pupa including mouthpart.

(Ant = antenna, E= eye, Lrn = Labrium, and Md= mandible).

5.4.6. Primers selection

Three microsatellite loci: A88, A14 and B124 designed by Estoup et al., 1994 were used to determine the genotype of each worker (Table 8).

Table 8. Primers A88, A14 and B124.

Microsatellite locus	Core sequences	Primers
A88	$(CT)_{10}TC(CCTT)_2$	5'CGAATTAACCGATTTGTCG3'
	$(CTTT)_3 ..(GGA)_7$	5'GATCGCAATTATTGAAGGAG3'
A14	$(CT)_3 ...(GGT)_9$	5'GTGTCGCAATCGACGTAACC3'
		5'GTCGATTACCGATCGTGAC3'
B124	$(CT)_8TCCTCTTC...(CT)_{14}$	5'GCAACAGGCGGGTTAGAG3'
	$CCTC(GC)_3... (GGCT)_8$	5'CAGGATAGGGTAGGTAAGAG3'

5.4.7. End-labeling of primers with γ ^{33}P

These loci: A88, A14 and B124 were separately end-labeled radioactively using polynucleotide kinase and with γ-phosphate ^{33}P-ATP (Bresatec Adelaide) in the following way (Table 9).

Table 9. End-labeling the primers A88, A14 and B124

Reagents	Volume (μl)
dH2O	15.75
Primers (A14, A88 or B124)	7.0
10×PNK (buffer)	3.5
γ^{33}P-ATP (Ci mmol^{-1})	7.0
T$_4$PNK	1.75
Total	35.0 μl

The reaction mixtures were incubated at 37°C for 30 minutes. After that the tubes were again incubated at 90°C for 2 minutes to stop the reaction.

5.4.8. Polymerase chain reaction (Amplification of DNA)

PCR reactions were made in the following way (Table 10).

Table 10. Polymerase chain reaction mixture.

Reagents	Volume (µl)
dH$_2$0	3.9
Tth plus (10 × Reaction buffer)	32.5
MgCl$_2$ (1.5 mM)	19.5
dNTPs (1.0 mM)	26.5
Cold Primer I A88 (not radioactive-labeled)	6.5
Cold Primer II B124 (not radioactive-labeled)	6.5
Hot Primer II A88 (radioactive-labeled)	32.5
Hot Primer I B124 (radioactive-labeled)	32.5
Taq+plus polymerase (Biotech Inter, Perth)	2.6
Total	162.5µl

After preparing the PCR reaction, 5 µl of the chelex DNA extraction and one drop of mineral oil were added to 5 µl of PCR reaction mixture. The mixture was then spun for a few seconds and then thermocycled under the following conditions. Denaturing at 94°C for 4 minutes for 1 cycle; 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds for 30 cycles; 72°C for 10 seconds for 1 cycle (Oldroyd et al., 1996).

5.4.9. Gel preparation

6% acrylamide gels were prepared according to Table 11. Unpolymerised acrylamide was warmed and then 10% ammonium persulfate and N, N, N', N', tetra methyl ethylenediamine (TEMED Appendix-IIIb) was added before pouring the gel

on the front plate. Meanwhile the front plate was cleaned with saline (Appendix-IIIc). After that the gel was poured and covered with the back plate. Then the shark-tooth combs were inserted between the front and the back plates and then clamped with clips and left for 12:00-16:00 hours.

Table 11. 6% polyacrylamide gel.

Reagents	Volume (μl)
Urea	37.89 g
Polyacrylamide (40%)	13.5 ml
5×TBE (Denaturing acrylamide mix)	18.0 ml
dH$_2$O	30.0 ml
Ammonium persulaphate (10%)	390.0 μl
TEMED	100.0 μl
Total	589.39 μl

5.4.10. Gel loading and running

The gel running tank (electrophoresis apparatus) was first cleaned with 90% ethanol and then the gel plate was firmly fixed in the gel running tank. After that, 1× TBE buffer solution (composition of 100ml 5×TBE + 400 ml dH$_2$O per chamber) was poured in the upper and lower buffer chambers. Then the tank was left for a few minutes to check whether there was any leakage. If leakage occurred, then the crack was sealed with blue tac. If there was no leakage, the gel was pre-warmed for 20-30 minutes. Before loading the samples, each lane (chamber of shark-tooth comb) was flushed with a buffer solution using a syringe to remove urea and then immediately bromophenol blue marker (stop blue dye) was loaded in each lane to warm up the gel.

Before loading the samples, they were denatured by heating at 94°C for 3 minutes. Meanwhile, each lane was again flushed with a buffer solution to remove

urea residues. After flushing out the residues from each lane, 2 μl of each sample was gently loaded into each lane using a different tip in order to avoid contamination of DNA between the samples. After completion of the sample loading, the upper and lower chambers of the gel running tank were covered and power switched on. The gel was run at a constant power of 40 watts (current=33 am, voltage=1,350-1,400v) for 4:00-4:30 hours.

5.4.11. Autoradiography

After finishing the gel running process, the gel mould was removed from the running tank electrophoresis apparatus and separated using scissors and a spatula. Then the front gel plate was immersed in a tray containing gel fixer (10% v/v acetic acid) for 20 minutes in order to leach the urea. It was rinsed with cold tap water for 10-15 minutes and dried at 80°C for 20-30 minutes and then left to cool. After drying, the front gel plate was covered with a plastic mesh and a sheet of Kodak X-Omant film was tapped over the dried gel. The film was left in a dark room for 24:00 hours at room temperature (28°C) in a lead line x-ray cassette. Sometimes if necessary it was exposed more longer than 24:00 hours depending on room temperature. Then the film was developed using an automatic developer machine.

5.5. Study sites selection for migratory behavior observations

To observe the migratory behavior of *A. dorsata* known nest sites were surveyed in three locations: Chiang Mai, Mae Tung Ting and Mae Hong Son (Figure 11). At Mae Hong Son and Mae Tung Ting traditional honey hunters were asked about the locations of nests and old nest sites.

5.5.1. Observations of migratory pattern of *A. dorsata*

Colony migration was observed from June 1995 to August 1998 at Chiang Mai city (n = 13 nests), Mae Tung Ting (n = 42 nests) and Mae Hong Son (n = 2 nests).

All established nest sites were visited monthly and the presence or absence of colonies were recorded. The number of colonies arriving or departing from established sites were also recorded. Departing swarms were classified according to the following criteria:

    i. Migration was caused by colony fission if the deserted combs had queen cells (preparing for swarming),

    ii. Migration was caused by biotic factors, i.e. predator-parasite pressures, if the colony had lost their whole comb or some part of comb, or

    iii. Migration was caused by abiotic factors, i.e. unfavorable environmental factors such as temperature, rainfall, wind etc.

5.5.2. Determining predator pressures

Based on questionnaire surveyed the three locations: Chiang Mai, Mae Tung Ting and Mae Hong Son were classified in two sites:-

    5.5.2.1. Undisturbed sites (colonies not harvested)
    5.5.2.2. Disturbed sites (colonies harvested)

5.5.2.1. Undisturbed sites

Chiang Mai city was classified as an undisturbed sites (not harvesting areas). Building owners never allow *A. dorsata* nests to be destroyed due to religious beliefs (Figure 11). After honey harvesting, the harvested colonies were observed till they absconded and recorded.

5.5.2.2. Disturbed sites

Mae Tung Ting and Mae Hong Son were classified as disturbed sites (colonies harvested). *A. dorsata* colonies were seasonally harvested during the main honey flow season (March-April) (Figure 11). After honey harvesting, the harvested colonies were daily observed till they absconded and recorded.
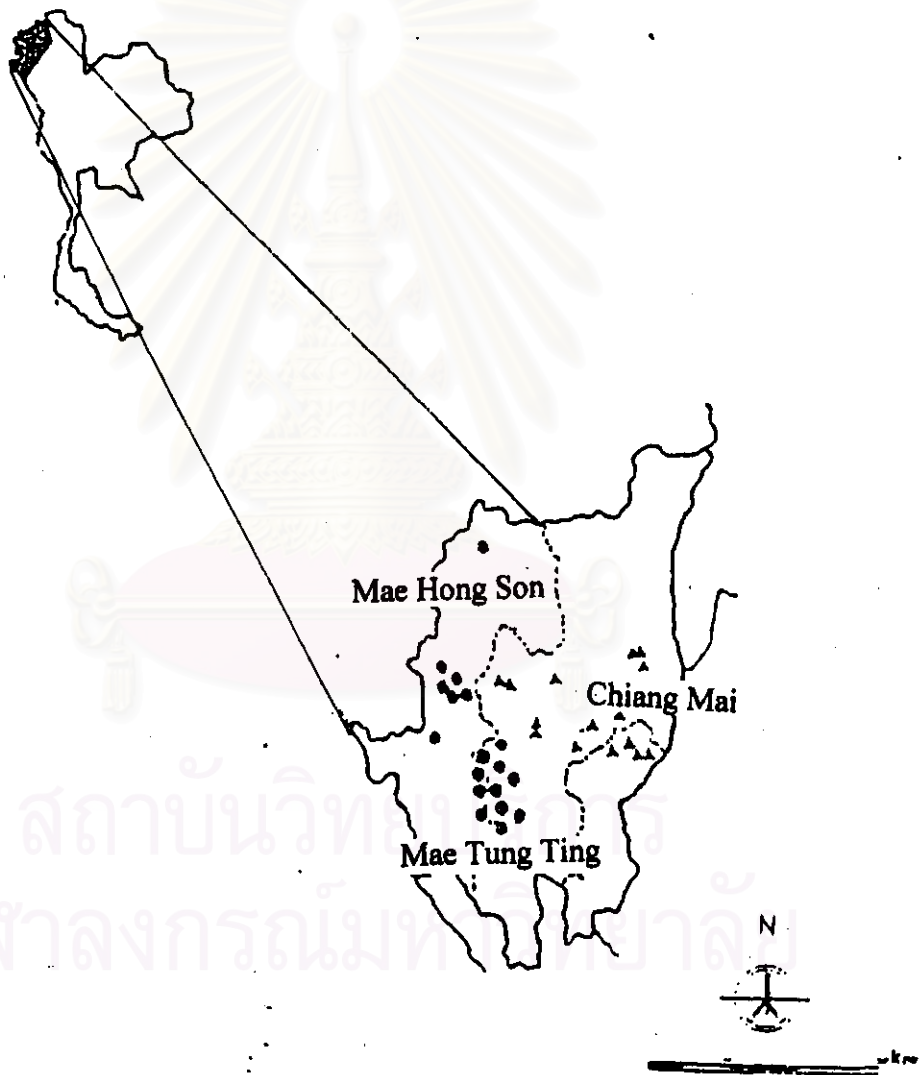

Figure 11. Map shows colonies distribution and colonies harvested and not harvested sites. (• = harvested) and ▲ = not harvested).

5.5.3. Determining parasitic mites pressure

Adult protective curtain formation bees were collected directly from the nests in the late evening around 17:30 hours after they stopped flying. They were killed and preserved in 90% ethanol. Then the dead bees were washed with 90% alcohol two to three times and the alcohol was examined for mite under a microscope. After that the dead bees were individually examined under a dissecting microscope. Sealed brood was randomly cut from fresh comb and checked for the presence of mites by opening the caps of sealed brood. Similarly, when colonies migrated leaving behind the empty combs were immediately checked for mites using a hand magnifying glass. If mites were present, they were counted and recorded.

5.5.4. Measurement of broodnest temperatures

Broodnest temperature 3 colonies of *A. dorsata* was measured in January for 24:00 hours at the interval of 2 hours. A hand thermometer was tied on the tip of bamboo stick with tape and inserted between the curtain formation bees (nurse bees) and brood of *A. dorsata* colonies for 5 minutes located next to the particular window of health care center building. Another thermometer was hung next to the colonies of *A. dorsata* to determine ambient temperature.

5.5.5. Manipulation of old combs

Two deserted combs 1.5 m high and 1.8 m wide were collected from Chiang Mai University and cut into four pieces 0.30-0.46 m and fixed with wire and glue around the health care center building except the regularly used one. One piece of comb was pasted around 2.7 m south about 5 m high next to a particular window, 1 piece was on second floor just below a particular window around 3 m high, 1 piece was on east side around 4.8 m far from particular window around 5 m high and 1 piece was around 10.9 m on the north side of a particular window of health care center building (Figure 12). All these windows were randomly selected using head tail coin

method. All the pieces of old combs were pasted around the health care center building when the colonies were absent. Before fixing the pieces of the old combs, the deserted comb was removed from a particular window site by cutting from its support.
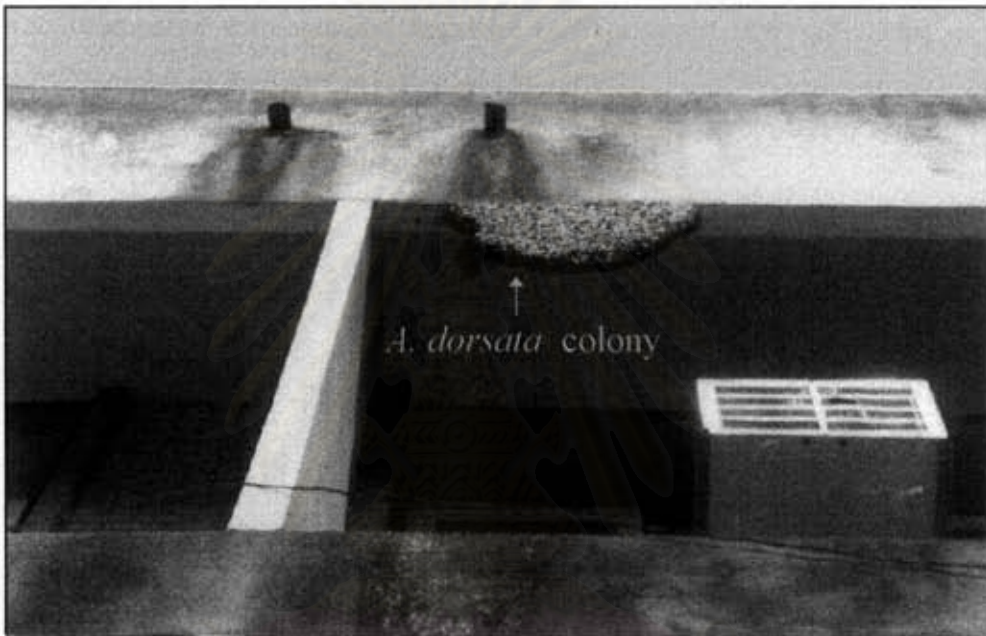

Figure 12. *A dorsata* swarms nested on the window frame of the health care center building, Chiang Mai.

5.6. Statistical analyses

5.6.1. Genetic data analysis

To determine whether the returning queen was the same individual as had occupied the nest in the previous season or her daughter, it has been hypothesized that the successive queens were identical by comparison of the inferred queen genotypes. If a particular queen returned to the same nest site, workers would reveal similar microsatellite genotypes in subsequent seasons. If a daughter queen returned as her

mother, inferred queen genotypes should show one common allele at each locus studied.

5.6.1.1. Scoring of microsatellite bands

Each allele was numbered using control DNA sequences guidelines (Appendix-IV) and compared with standard sequence of M13 mp18 DNA of 7.3 kb which run next the samples in the same gel (Figure 13).

Figure 13. Banding pattern produced by microsatellite PCR in different
line of *A. dorsata* using A88. (Lane 1-4 DNA marker and
size 90-160 bp).

If the sample had one band (allele) then the individual was considered homozygous, but when the sample had two alleles then the individual was considered heterozygous. If more than two bands were observed in a lane, then these types of bands were discarded considering the sample preparation error or sampling loading error.

5.6.2. Migratory behavior data analyses

5.6.2.1. Spearman correlation tests

Spearman rank correlation tests were used to determine the colony migration association with mites.

5.6.2.2. Chi-square tests (goodness-of-fit-tests)

Migratory data which had one variable were subjected to Chi-square test for Goodness of fit to compare the number of colonies migrated frequencies between disturbed and undisturbed sites.

$$\chi 2 = \sum \frac{(O-E)^2}{E}$$

Where ,

O = observed frequencies

E = expected frequencies

5.6.2.3. 2×2 Chi-square contingency tests

The number of colonies persistence in colonies harvested and not harvested sites were subjected to 2×2 contingency table to determine the significance impact of predators on colonies migration.

$$\chi 2 = \sum \frac{(O-E)^2}{E}$$

Where,

O_{ij} = observed value ith row and jth column

E_{ij} = expected value (E_{ij} calculate from NP =. N = total sample numbers;

P = probability). Expected value (E_{ij}) was calculated from observed value.

E_{ij} : $C_j \times r_i/N$; $r_i \times C_j/N$

r_i = total of rows

C_j= total of column

N= total number of individuals in rows and columns

df = (k - 1)

5.6.2.4. Analysis of variance tests (ANOVA)

ANOVA tests were performed to determine significant of climatic parameters with colony migration by using SPSS window 97 software program (Norusis. 1996). First, the number of colonies observed each month was compared with climatic parameters such as temperature, relative humidity, rain and wind. Eventually, all climatic factors were used to determine the interaction between environmental factors and colony migration.

5.6.2.5. Multiple regression tests

Simple multiple regression tests were performed to determine the significant correlation of environmental factors and colony migration using SPSS window 97 software program (Norusis, 1996).