CHAPTER 3

MATERIALS AND METHODS

3.1 COLLECTING SITES

Boletes were collected from eight national parks village forests in upper northern Thailand. The sites are in Chiang Mai (Doi Chiang Dao, Doi Inthanon, Doi Suthep-Pui and Sri Lanna), Chiang Rai (Khun Chae), Mae Hong Son (Huai Nam Dang), Nan (Doi Phu Kha) and Phayao (Doi Luang). A variety of collecting sites, representing different forest types that effect to diversity of boletes have been selected. Details of the 14 study sites in each location were presented in Table 3.1 and Figure 3.1.

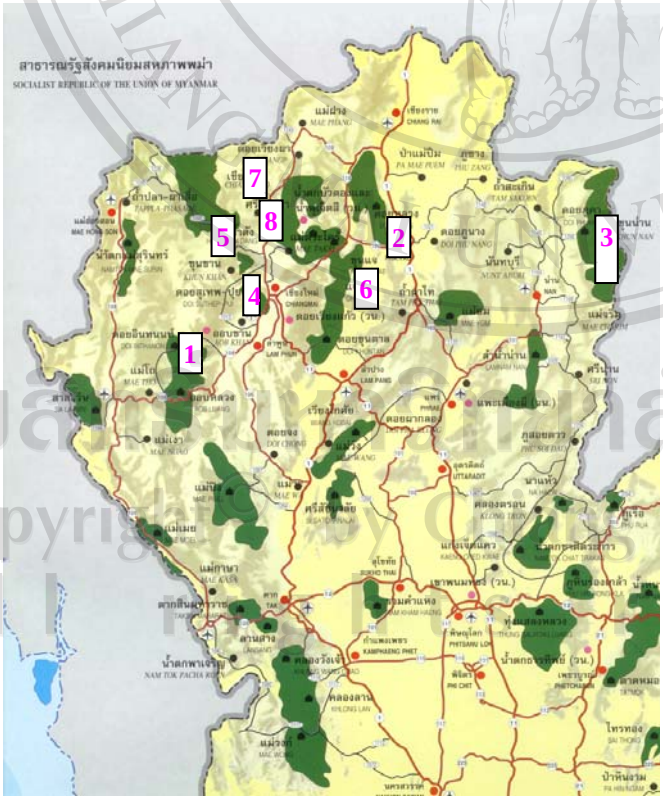
Table 3.1 Fourteen study sites of national parks in upper northern Thailand**.**

National Parks	Sites	Grids	Altitudes (m-amsl)	Dominant trees
Doi Inthanon	Mae Ya waterfall	N18 26.360 E98 35.875	523	*Dipterocarpus* spp.
	Wachiratan waterfall	N18 32.507 E98 35.766	829	*Quercus*, *Castanopsis*, *Lithocarpus echinops*
	25 km marker on Highway 1009	N18 32.507 E98 35.500	1076	*Pinus kesiya*, *Quercus*, *Castanopsis*, *Lithocarpus echinops*
	Ang Ka	N18 35.315 E98 29.255	2565	*Rhododendrons* spp. *Pandanus* sp. and *Cyathea*
Doi Luang	Jumpa Thong Waterfall	N19 12.899 E99 44.002	593	Bamboo *Dipterocarpus* spp. and *Pinus* sp.
Doi Phuka	nature trail	N19 12.254 E101 04.848	1330	*Pinus* sp. *Dipterocarpus* spp., scattered *Castanopsis*
Doi Suthep-Pui	Pha Lad	N18.48.62 E98 54.600	400	bamboo, *Dipterocarpus* spp.
	Sangasabhasri Lane to Huai Kok Ma village	N18 48.547 E98 54.592	1149	*Castanopsis* spp. and *Lithocarpus polystachyus*

Table 3.1 (Continued).

National Parks	Sites	Grids	Altitudes (m-amsl)	Dominant trees
	Doi Pui	N18 49.021 E98 53.404	1554	*Castanopsis* spp. and *Lithocarpus polystachyus* and *Pinus* sp.
Huai Nam Dang	nature trail	N19 19.095 E98 36.08	914	*Pinus kesiya*, *Dipterocarpus* spp., Bamboo & scattered *Castanopsis*
Khun Chae	nature trail	N19 4.2064 E99 23.3120	963	*Pinus* sp. *Dipterocarpus* spp., Bamboo & scattered *Castanopsis*
Pha Daeng (Chiang Dao)	Sri Sang Wan Waterfall	N19 37.729 E98 57.418	574	bamboo, *Dipterocarpus* spp.
	Longan orchard at Muang Ngai-Ban	N19 07.047 E98 43.615	914	*Dimocarpus longan* and grasses
Sri-Lanna	near Mae Ngad Dam, on Hwy 1323	N19 08.1638 E98 57.2503	1100	*Anisoptera costata*, *Toona ciliata*, *Hopea odorata*, *Tectona grandis*, *Xylia kerrii*, *Shorea obtusa*, *Shorea siamensis* and *Dipterocarpus* spp.

m-amsl = meter-above median sea level.



1=Doi Inthanon Naitional Park
2=Doi Luang National Park
3=Doi Phuka National Park
4=Doi Suthep Pui National Park
5=Huai Nam Dang National Park
6=Khun Chae National Park
7=Pha Dang (Chiang Dao) National Park
8=Sri-Lanna National Park

Figure 3.1 Study sites of boletes collection in this work.

The sites were ranged from dry dipterocarp forests in the lowlands to higher elevation pine-dry evergreen forests (Figure 3.2). There are 3 altitudinal ranges; lowland forests (<800 m amsl), mid-elevation forests (800-1200 m amsl) and highland forests (1200-2565 m amsl)

a. b. c.

Figure 3.2 Forest type in National Park: a. dry-dipterocarp. b. deciduous forest. c. mixed-pine forest.

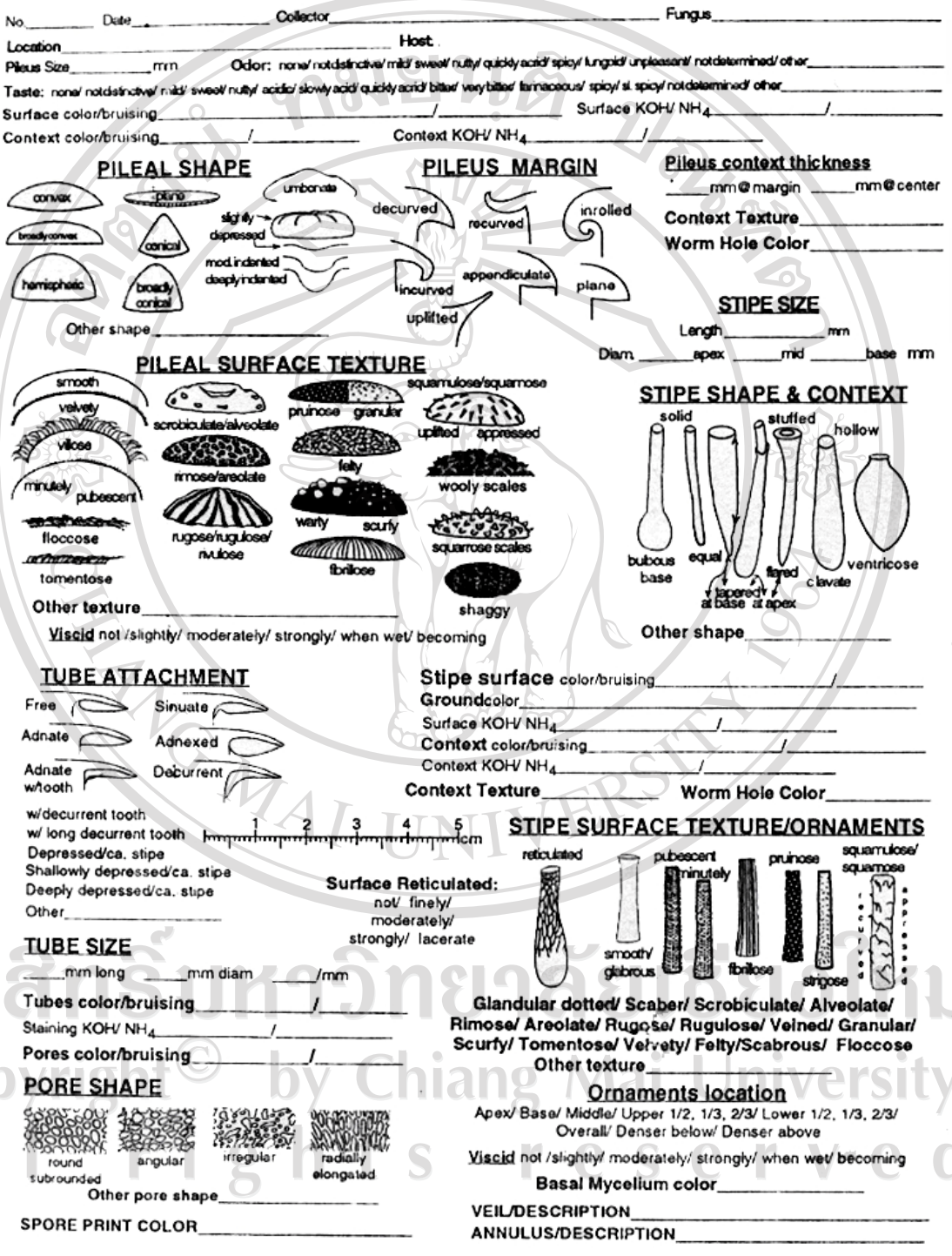
3.2 COLLECTING METHODS

Boletes were collected randomly from approximate 200×30 m^2 for each sampling sites during May-November in the year 2005-2006. Young mature and old individual fruiting bodies were collected with chisel, soil were removed with a fine brush and placed in brown paper bags in a basket, while data of associated host, forest types, temperature and humidity were recorded. The edible species sold in local and roadside markets nearby the study sites were noted and some interest species were also bought for examination. The tissues of all specimens were kept in 1.5 ml microcentrifuge tube with 300 µl 2X CTAB buffer at -20^{o}C. Specimens were dried at 45-50 ^{o}C overnight and kept in plastic boxes containing silica gel.

3.3 TAXONOMY METHODS

The collected mushroom were tentatively identified to genus and species by conventional morphological characters (Smithe, 1975) and chemical methods using the keys that provided by Corner (1972), Moser (1983), Ellis and Ellis (1990). The major morphological characters recorded in this study were including basidiocarp (pileus, pileus trama, tubes or hymenophore, annulus, stipe cuticle and stipe trama) and basidiospore as described in Chapter 2 (Thiers, 1975). Some species also needed to find clamp connection appearance. All morphological characters of each collected taxa were recorded in boletes annotation form (Figure 3.3) that provided by Mueller *et al*. (2004).

Several major genera appearing in the present study which are boletes group distinctly with tubulose hymenophore can be identified promptly to genus level by using a key to genera that provide by Halling (2008). The major morphological characters used to delimit all these genera have also been discussed in a field guide to the boletes by Thiers (1975) which now is available on website of California Mushroom. In the other hand some confusing species or rare species of boletes taxa were selected to study the phylogenetic relationship by using sequences analyses of 28S rDNA and ITS (see detail in section 3.5 phylogenetic study).



1 Bolete annotation sheet, in English. (B. Ortiz Santana)

Figure 3.3 An annotation form for recording macroscopic characters of boletes in this study (Mueller *et al*., 2004)

Draft Key to Some Bolete Genera (tubulose hymenophore group) (Halling, 2008).

1. Tubes and pores with clusters of cystidia in the hymenium that stain dark brown to blackish to occasionally vinaceous in a solution of KOH; associated only with Pinaceae……..……***Suillus***

1'. Darkly staining, clustered cystidia absent in hymenium; associated with diverse plant families..……**2**

2. Basidiocarp with a dry (rarely tacky), universal veil (be sure to check young specimens), collapsing to form a peronate annulus or annular zone and basidiospores smooth……..……**3**

3. Basidiocarp often with sulfur yellow pigmentation, staining blue; clamp connections absent……..……***Pulveroboletus***

3'. Basidiocarp lacking sulfur yellow pigments, not staining blue; clamp connections present……..…..……***Paragyrodon***

2'. Basidiocarp without a veil; if a veil is present, then spores ornamented……**4**

4. Stipe eccentric, lateral, or absent; tubes/pores radially elongated, decurrent ……..……**Gyrodon** (include *Boletinellus*)

4'. Stipe central; tubes/pores ±circular, adnexed, adnate, rarely decurrent..**5**

5. Pores white to pallid when young, becoming yellow at maturity; stipe hollow, with surface hyphae oriented circumferentially; spores ellipsoid, yellow in deposit..……***Gyroporus***

5'. Basidiocarps without all the above combination of characters……**6**

6. Surface of stipe with numerous relatively small squamules, which are usually pallid when young but become dark colored (dark brown to black) at maturity……..…***Leccinum***

6'. Surface of stipe not as above……..……..……...……**7**

7. Pores white, yellow, or red; spores brown, olive, olive brown or bright yellow brown in deposit …..……...**8**

8. Spores elongate-fusoid, with longitudinal ribs or striations…..***Boletellus***

8'. Spores lacking longitudinal ribs/ striations……..……**9**

9 Hymenophore entirely pigmented pinkish red, orange red, brownish red; basal mycelium typically sulfur yellow..***Chalciporus***

9' Hymenophore not uniformly pigmented with pink to red or brown; basal mycelium variously colored……..……..……**10**

10. Spore deposit bright yellow brown; pileipellis hymeniform; pigmented portions of pileus and stipe turning blue in alkali solution……***Xanthoconium***

10'. Spore deposit with olive brown pigments; pileipellis usually a trichodermium; reaction to alkali various……..……**11**

11. Spores ovoid to short ellipsoid; clamp connections present; not always mycorrhizal……..……***Phlebopus***

11'. Spores fusoid-elongate; clamp connections absent; mycorrhizal with many plant families……**12**

12. Basidiospores alveolate-reticulate, reticulate or with pit-like perforations……***Heimioporus*** (=*Heimiella*)

12'. Basidiospores with smooth walls…...***Boletus*** (incl. *Xerocomus*, *Pseudoboletus*)

7'. Pores pink, vinaceous, gray brown to very dark brown or black; spores pinkish flesh color to vinaceous brown to reddish brown or black in deposit……..…..…**13**

13. Spores with some kind of obvious ornamentation (light microscope).**14**

14. Basidiocarp usually with black to dark gray scales on pileus and stipe; hymenophore black with age, often staining red first then black ***or*** slowly black directly……..……..**15**

15. Spores globose, reticulate or variously echinate……...….……***Strobilomyces***

15. Spores subglobose to ellipsoid, longitudinally costate, ridged, or winged……..…..…….***Afroboletus***

14'. Basidiocarp lacking dark colored scales, sometimes viscid, glutinous or tacky; hymenophore becoming flesh pink to grayish pink; spores elongate-fusoid, ornamented with pit-like perforations, fissures or large isolated warts…...…..***Austroboletus***

13'. Spores smooth……..……**16**

16. Hymenophore very dark brown; spores dark brown in deposit; context bluing when exposed……..…***Porphyrellus***

16'. Hymenophore flesh pink to grayish pink; spores flesh pink to grayish pink in deposit; context not bluing, but may oxidize to other colors……..…**17**

17. Portion of the pileus surface becoming extremely gelatinized with age; basidiocarps often not large and fleshy; hymenial cystidia not pseudocystidia…***Fistulinella***

17'. Pileipellis not gelatinous; basidiocarps usually robust; hymenial cystidia present as pseudocystidia……***Tylopilus***

3.4 STATISTIC METHODS

The overlap and complementarity of fungal communities from different national parks were calculated using the equation (Colwell and Coddington, 1994):

$$\text{Overlap (\%)} = \frac{\text{number of taxa shared between A and B} \times 100}{\text{total number of taxa observed in A and B}}$$

in which A denotes the number of boletes taxa found in one site and B denotes the number of boletes taxa in another sites.

3.5 CULTURE AND EDIBILITY INFORMATION OF SOME EDIBLE AND WILD BOLETES

The fresh sporocarps of wild boletes especially the edible species were cultured for vegetative mycelium. A pure culture of each collection was isolated from the tissues (small pieces that cut from section between the pileus and stipe) using half strength Potato Dextrose Agar (PDA) plate. The pure cultures were incubated at room temperature ($28^{o}C \pm 2^{o}C$) (Lumyong *et al*., 2007).

Cultivation of the *Phlebopus* species were examined by using the inoculation of sorghum seed covered with species of *Phlebopus* mycelia in Longan (*Dimocarpus longan*) seedling bags.

Wild mushroom hunters in the local area were interviewed for information (boleti's local names, associated plants, edible or poisonous, prices, etc) about boletes at each site of collection.

3.6 PHYLOGENETIC STUDY

3.6.1 DNA EXTRACTION

A small tissue from the flesh section between the pileus and stipe of fruit body was cut with a sterilized knife and place into a 1.5 ml centrifuge tube containing 300 µl 2X CTAB buffer and keep in $-20^{o}C$ for DNA extraction. The specimens were ground with 200 mg of sterilized quartz sand then 2× CTAB extraction buffer were added for adjusted to 600 µl. Contents were then incubated at $60^{o}C$ in a water bath for 30 min with gentle swirling. The solution was then extracted two or three times with an equal volume of chloroform : isoamyl (24:1) at 13000 rpm for 30 min until no

interface was visible. The supernatant phase containing the DNA was precipitated by addition of 2.5 volumes of absolute ethanol and kept at –20^{o}C overnight. The DNA pellet was washed (70% ethanol) 2 times, dried (under vacuum), and resuspended in TE buffer (1 mM EDTA, 10mM Tris-HCl, pH 8) and mixed together with RNase A (1 mg/ml^{-1}). In addition some tissues specimens were extracted for DNA by using DNA extraction Kits (NucleoSpin® Plants II, Macherey Nagel, Catalog no. 740770.50) following manufacturer's protocol.

3.6.2 PCR AMPLIFICATION AND SEQUENCING OF 28S rDNA AND ITS

Approximately 900 nucleotides at the 5´ end of the nuclear large ribosomal subunit gene (28S rDNA) were amplified by OPERON primer pairs LROR (5'- ACCCGCTGAACTTAAGC -3') and LR5 (5'- TCCTGAGGGAAACTTCG -3') (Vilgalys and Hester, 1990). A portion of the ITS region was performed using a pair of universal primer ITS5 (5'- GGAAGTAAAAGTCGTAACAAGG -3') and ITS4 (5'- TCCTCCGCTTATTGATATGC -3') (OPERON) (White *et al.*, 1990). Genomic DNA 3 µl was used in a standard 50 µl PCR mixture (25mM $MgCl_2$, 10 Mg-free buffer, 2.5 µM dNTPs, 1.5 µM primers, and 1.5 unit of *Taq* DNA Polymerase-BioLabs M0267-S) under the following thermal conditions: 94^{o}C for 30s, 35 cycles of 94^{o}C for 30 s, 60^{o}C for 30 s, and 72^{o}C for 1.5 min. Amplicons were checked on 1% agarose gels stained with ethidium bromide under UV light. Negative control reactions omitting DNA were included in all sets of amplifications to monitor for potential contamination by exogenous DNA. PCR products were purified using NucleoSpin® Extract II PCR clean-up Kit (Macherey Nagel, Catalog no. 740609.50) following manufacturer's protocol. The amplified 28S rDNA and ITSs fragments were directly sequenced.

Sequencing reactions were performed and sequences determined automatically by Macrogen Company in Korea using PCR primers mentioned above.

3.6.3 PHYLOGENETIC ANALYSES

A total of 44 collections of boletes taxa were analysed along with reference fungal sequences, from different fungal families, which was downloaded from GenBank. Nucleotide sequences of the 28S rDNA and ITS were initially aligned using BioEdit (Hall, 1999), Clustal X 1.83 with default parameter settings (Chenna *et al*., 2003). The alignments were then manually optimized using Se-al v2.0a11 (Rambaut, 1996). Maximum parsimony (MP) and Neighbor joining (NJ) analyses were conducted using MEGA4 (Tamura *et al*., 2007). Tree searches were carried out using the heuristic method with a random stepwise addition and tree bisection and reconstruction branch-swapping algorithm.

MrModeltest v2.2 (Nylander, 2004) was used to determine the best-fit DNA substitution and gamma rate heterogeneity for each dataset. The best-fit model was used in Bayesian anayses with gaps treated as missing. Bayesian analysis was carried out, using the program MrBayes v3.13 (Huelsenbeck and Ronquist, 2001), for two replicates of 1 million generations, with 6 chains sampling every 100 generations. Branch support was estimated by performing 1000 MP bootstrap replicates (Felsenstein, 1985) and Bayesian posterior probabilities was calculated from the consensus of 18,000 trees after excluding the first 2000 trees as burnin by using PAUP*4.0b10 (Swofford, 2002).