CHAPTER 3

METHODOLOGY

Study Site

Phu Luang Wildlife Sanctuary (PLWS) is situated at 17°3′ to 17°24′ N and 101°16′ to 101°21′ E, in northeast Thailand. The center covers the areas of Wang Saphung, Phu Ruea, Dan Sai and Phu Luang districts of Loei province within an approximate area of 897 square kilometers (see Figure 1). The geographical features of the wildlife center consist of high mountains. While the highest peak rises up to 1,571 meters, the altitudes in the Sanctuary range from 400-1,500 meters. The weather on the upper slopes of the mountains is cool with an average temperature of about 4-24 °C. The forest types include coniferous forest, dry Dipterocarp forest, dry evergreen forest, lower montane rainforest, lower montane scrub, mixed deciduous forest, and tropical rainforest.

Climate of Phu Luang Wildlife Sanctuary (PLWS)

PLWS get full impact of the monsoon, causing heavy rainfalls during May-October. Annual precipitation averages 1,238 mm per year and temperature averages about 20-24 °C. In the coolest months of December and January, average temperatures are as low as 4-6 °C but frequently drop much

lower. The hottest months are from February to April. Relative humidity averages about 60-83 %.

Vegetation of Phu Luang Wildlife Sanctuary

Forest vegetation of PLWS can be classified into two major groups: the evergreen forest and the deciduous forest (Gardner, Sidisunthorn, & Anusarnsunthorn, 2000; Tem Smitinand, 2001; Thawatchai Santisuk, 2007). Types of forests are recognized as follows:

Tropical rainforest: This forest type is found between 400 and 800 m. The vegetation shows a high diversity of tree species, such as *Artocarpus lacucha, Hopea ferrea, Baccaurea ramiflora, Paranephelium xestophyllum, Garcinia speciosa, G. merguensis, Knema erratica, Pometia pinnata, Cassia bakeriana, C. agnes, Livistona speciosa, Dillenia aurea, Mesua ferrea, Syzygium megacarpum, Dracontomelon dao* and *Afzelia xylocarpa*. This type is best developed at the surroundings of the Head Quarter of PLWS and at Pha Kop.

Dry evergreen forest: This forest type occurs between 400 and 1,000 m. The vegetation is similar to the tropical rain forest with trees such as *Hopea ferrea, Baccaurea ramiflora, Paranephelium xestophyllum, Afzelia xylocarpa,* etc. The ground flora is composed of members of Araceae, Zingiberaceae and ferns. This type of forest can be found at the surroundings of Nam Tob Substation, Huai Baeng Forest Ranger Station and Pak Dang Forest Ranger Station.

Lower montane rainforest: This forest type occurs on high mountain ridges and slopes above 800 m. Dominant species of the upper storey belong to Fagaceae such as *Castanopsis tribuloides* and *Quercus oidocarpa* with the presence of Euphorbiaceae i.e. *Triadica cochin* and Lythraceae i.e. *Lagerstroemia calyculata*. The ground flora is composed of members of orchids, mosses and ferns. The best sites for this forest are the surroundings of Khok Huai Toei and Phuluang Wildlife Research Station.

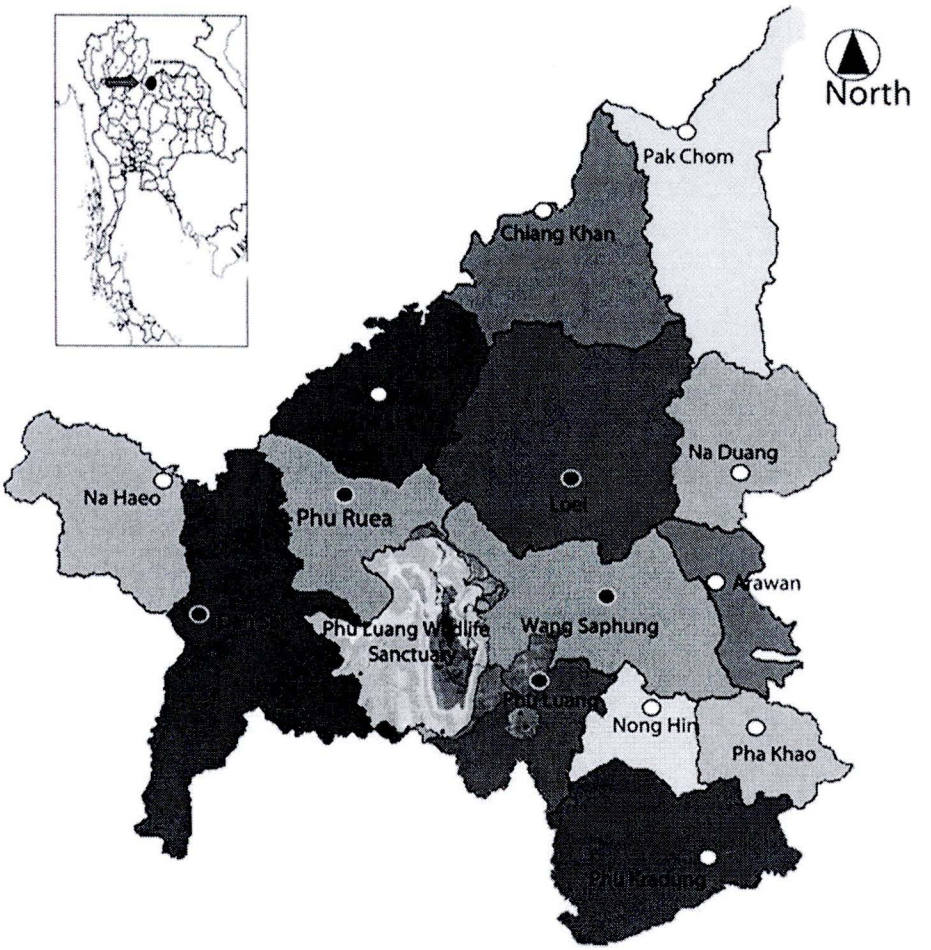

Figure 1 Map of Phu Luang Wildlife Sanctuary, Loei province, Thailand

Note. From *Loei Tourist Map*, Retrieved June 15, 2011, from http://www.loei.go.th/novabizz.com/map/16.htm

Coniferous forest: This type of forest occurs at altitudes above 1,000 meters. Two characteristic species are *Pinus kesiya* and *P. merkusii* which are scattered around the edge of the cliff. This forest type is best developed at the surroundings of Lon Tae Forest Ranger Station.

Lower montane scrub: This forest type occurs on high mountain ridges and slopes above 1,000 m. The dominant tree species are *Rhododendron lyi, R. simsii, Lyonia foliosa, Vaccinium sprengelii, Lithocarpus recurvatus* and *Agapetes saxicola.* This major forest type covers vast areas in PLWS. The best sites are at the surroundings of Khok Nok Kraba, Lan Suriyan, Khok Phrommachan, etc.

Dry Dipterocarp forest: This forest type is found between 400 and 600 m. The typical forest is dry, with frequent fires. The dominant tree species are *Dipterocarpus obtusifolius, Terminalia alata, Pterocarpus macrocarpus, Phyllanthus emblica, Cratoxylum* sp., *Schleichera oleosa, Vitex limoniifolia, Shorea obtusa* and *S. siamensis*. This forest occurs in a small area at Wang Saphung district and Dan Sai district. The explored sites are at the surroundings of Phu Khon Substation and Nam Ki Substation.

Mixed deciduous forest: This forest type occurs up to 1,000 m elevation, and its dominant species include some plants of economic and herbal value, such as *Pterocarpus macrocarpus, Afzelia xylocarpa, Xylia xylocarpa* var. *kerrii, Dipterocarpus turbinatus* and *Vitex pinnata.* This forest type occurs along the eastern border in Wang Saphung district and Phu Luang district and was explored at the surroundings of Huai Nam San, Nam Chan Forest Ranger Station and Phu Ho Forest Ranger Station.

Discolichens were collected on bark and rocks from all the seven different forest types including coniferous forest (CF), dry dipterocarp forest (DDF), dry evergreen forest (DEF), lower montane rainforest (LMRF), lower montane scrub (LMS), mixed deciduous forest (MDF), and tropical rainforest (TRF) (see Figure 2 and 3). A list of host plants is presented in Table 2. For Thai pronunciation and the English names of collecting sites the system of Smitinand (2001) was followed The collections were made on 23 sites in 4 districts: Phu Ruea District (PR), Dan Sai District (DS), Wang Saphung District (WS) and Phu Luang District (PL) (see Table 3 and Figure 4).

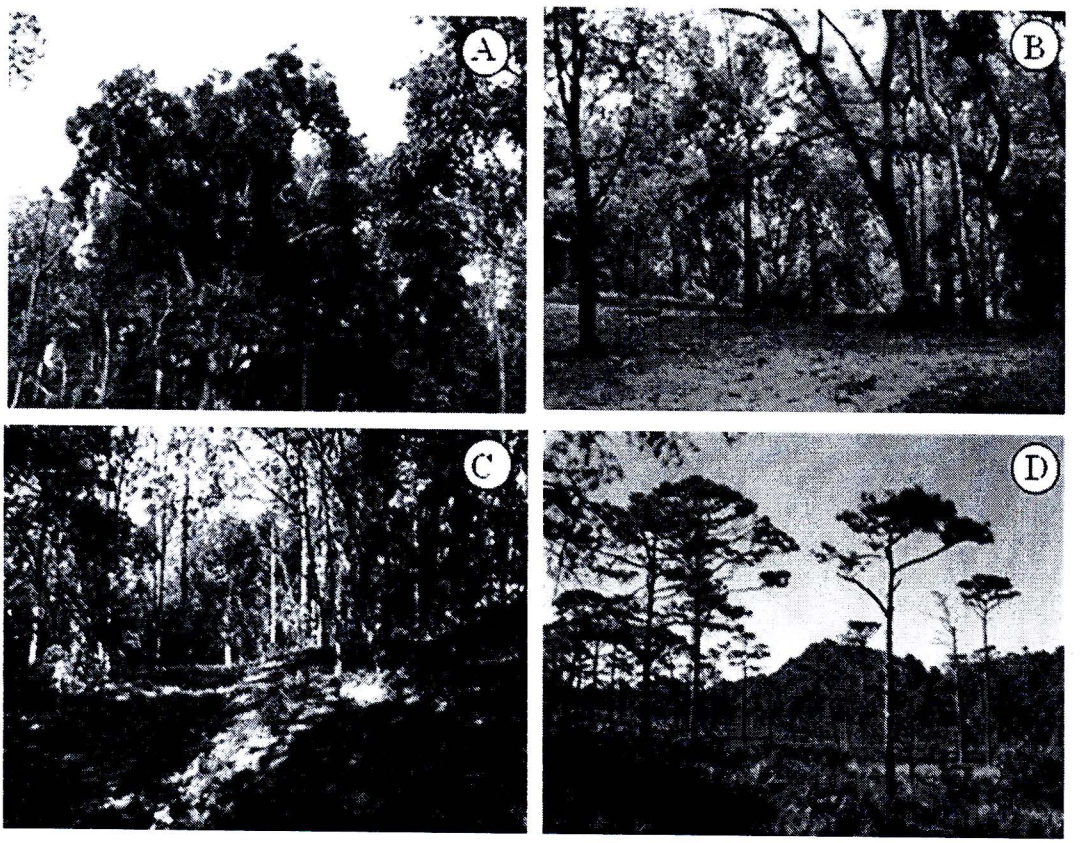

Figure 2 Seven forest types at Phu Luang Wildlife Sanctuary A. Tropical rainforest at 'Head Quarter of PLWS, B. Dry evergreen forest at 'Nam Tob Substation' C. Lower montane rainforest at 'Phuluang Wildlife Research Station' D. Coniferous forest at 'Lon Tae Substation'

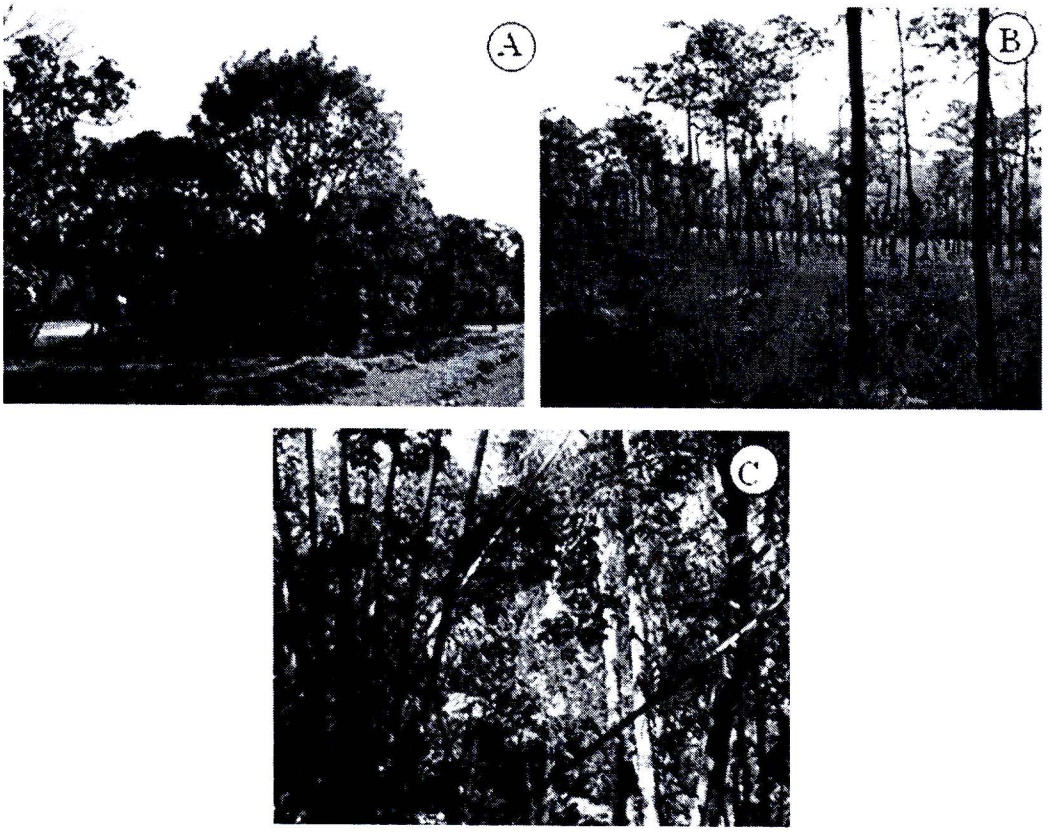

Figure 3 Seven forest types at Phu Luang Wildlife Sanctuary A. Lower montane scrub at 'Khok Nok Kraba Substation' B. Dry Dipterocarp forest at 'Phu Khon Substation' C. Mixed deciduous forest at 'Huai Nam San'

Table 2

Host Lists of Discolichens at Phu Luang Wildlife Sanctuary

Scientific names	Families
Acacia farnesiana (L.) Willd.	LEGUMINOSAE-MIMOSOIDEAE
Afzelia xylocarpa (Kurz) Craib	LEGUMINOSAE-CAESALPINIOIDEAE
Agapetes saxicola Craib	ERICACEAE
Aglaonema nitidum (Jack) Kunth	ARACEAE
Artocarpus lacucha Roxb.	MORACEAE
Baeckea frutescens L.	MYRTACEAE
Cassia bakeriana Craib	LEGUMINOSAE-CAESALPINIOIDEAE
Castanea tribuloides (Sm.) A. DC.	FAGACEAE
Dipterocarpus obtusifolius Teijsm. Ex Miq.	DIPTEROCARPACEAE
Dipterocarpus retusus Blume	DIPTEROCARPACEAE
Fagraea ceilanica Thunb.	GENTIANACEAE
Glochidion rubrum Blume.	EUPHORBIACEAE
Lithocarpus calathiformis Rehder & Wilson	FAGACEAE
Lithocarpus trachycarpus (Hickel & A. Camus) A. Camus	FAGACEAE
Lithocarpus truncatus (King) Rehder & Wilson	FAGACEAE
Lyonia foliosa (Fletcher) Sleumer	ERICACEAE
Mesua ferrea L.	GUTTIFERAE
Persea kurzii Kosterm.	LAURACEAE
Pinus kesiya Royle ex Gordon	PINACEAE
Pinus merkusii Jungh. & de Vriese	PINACEAE
Pterocarpus macrocarpus Kurz	LEGUMINOSAE-PAPILIONOIDEAE
Quercus lineata Blume	FAGACEAE
Rhaphiolepis indica (L.) Lindl. Ex Ker	ROSACEAE
Rhododendron lyi H. Lév.	ERICACEAE
Rhododendron simsii Planch.	ERICACEAE
Shorea obtusa Wall. Ex Blume	DIPTEROCARPACEAE
Shorea siamensis Miq.	DIPTEROCARPACEAE
Sladenia celastrifolia Kurz	THEACEAE
Styrax benzoides Craib	STYRACACEAE
Syzygium thorelii (Gagnep.) Merr. & L.M. Perry	RUBIACEAE
Ternstroemia gymnanthera (Wight & Arn.) Bedd.	MYRTACEAE
Wendlandia tinctoria (Roxb.) DC.	THEACEAE

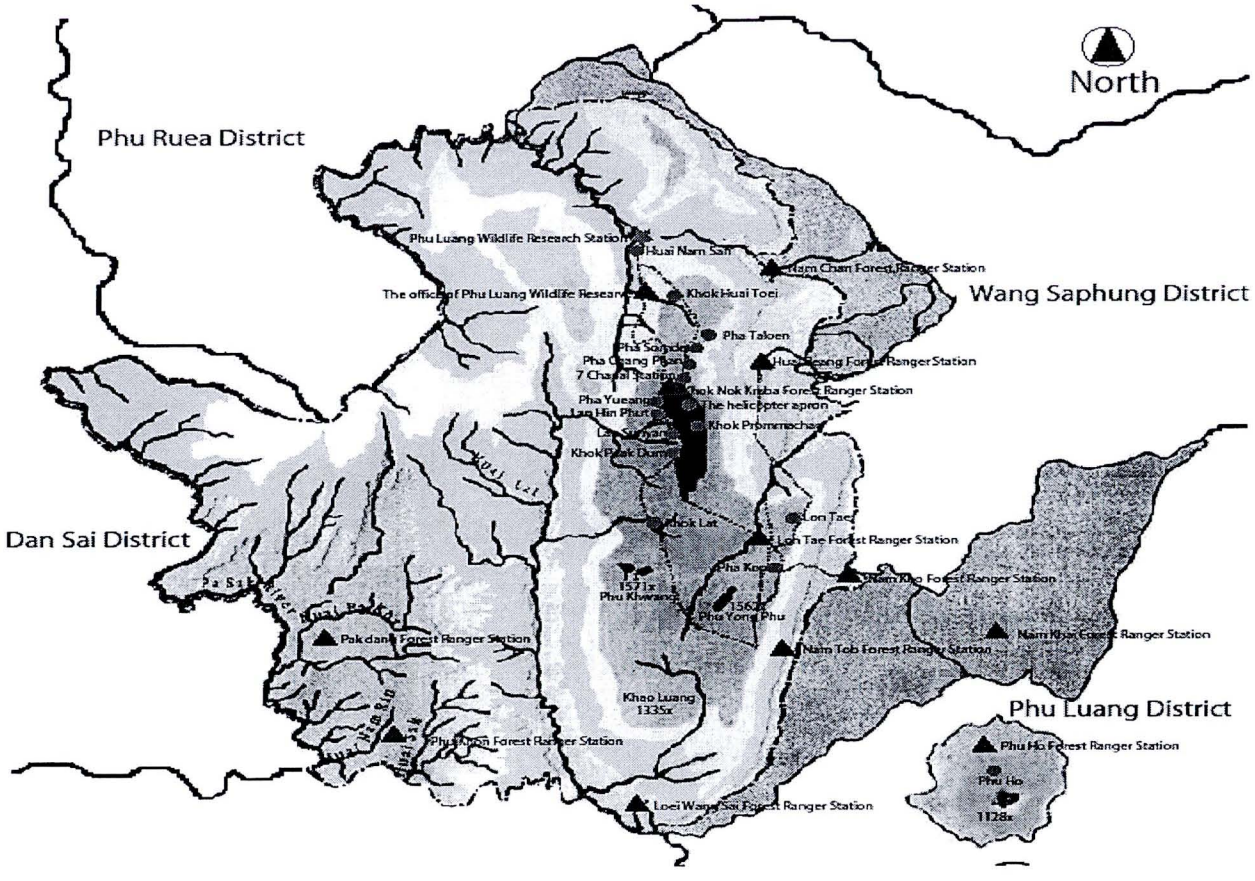

Figure 4 Map of collecting sites of discolichens at Phu Luang Wildlife Sanctuary, Loei province, Thailand

Note. From Tourism Authority of Thailand, 2000.

Table 3

The Collecting Localities at Phu Luang Wildlife Sanctuary

Forest type	Localities	Altitude (m)	Grid	Districts
1. Lower montane scrub	1. Pha Taloen	1,480	17° 16' 53" N, 101° 31' 21.6" E	Phu Ruea
	2. Pha Somdet	1,494	17° 17' 21.3" N, 101° 31'37.6" E	Phu Ruea
	3. Khok Phrommachan	1,487	17° 16' 53" N, 101° 31' 21.6" E	Phu Ruea
	4. Khok Nok Kraba Forest Ranger Station	1,250-1,485	17° 16' 53" N, 101° 31' 21.6" E	Phu Ruea
	5. Lan Suriyan	1,473	17° 16' 43.8" N, 101° 31'07.2" E	Phu Ruea
	6. Huai Lad	1,501	17° 16' 14.4" N, 101° 31'15.5" E	Phu Ruea
	7. Seven Chanal Station	1,468	17° 16' 46.1" N, 101° 31'08.3" E	Phu Ruea
	8. Pha Chang Phan	1,507	17° 16' 23.5" N, 101° 31'08.3" E	Phu Ruea
	9. Helicopter Apron	1,487	17° 16'23.5" N, 101° 31' 08.3" E	Phu Ruea
	10. Khok Paek Dam	1,520	17° 15'07.7" N, 101° 31' 45.5" E	Phu Ruea
2. Coniferous forest	11. Lon Tae Forest Ranger Station	1,343	17° 11'12.7" N, 101° 33' 58.3" E	Wang Saphung
3. Tropical rainforest	12. Head Quarter of Phuluang Wildlife Sanctuary	700	17° 20' 18.5" N, 101° 30' 32.3"E	Phu Ruea
	13. Pha Kop	1,245	17° 11' 32.2" N, 101° 33' 42.6"E	Wang Saphung
4. Mixed deciduous forest	14. Huai Nam San	787	17° 20' 35.9" N, 101° 30' 16.9"E	Phu Ruea
	15. Nam Chan Forest Ranger Station	397	17° 20' 49.0" N, 101° 33' 29.0"E	Wang Saphung
	16. Phu Ho Forest Ranger Station	569	17° 06' 37.9" N, 101° 38' 28.2"E	Phu Luang

Table 3 (continued)

Forest type	Localities	Altitude (m)	Grid	Districts
5. Lower montane rainforest	17. Khok Huai Toei	1,240	17° 19' 45.8" N, 101° 31' 26.1"E	Phu Ruea
	18. Phuluang Wildlife Research Station	923	17° 20' 34.8" N, 101° 30' 26.0"E	Phu Ruea
6. Dry Dipterocarp forest	19. Phu Khon Substation	700	17° 14' 32.7" N, 101° 18' 28.2"E	Dan Sai
	20. Nam Ki Substation	355	17° 09' 50.4" N, 101° 36' 38.5"E	Phu Ruea
7. Dry evergreen forest	21. Nam Tob Substation	360	17° 15' 36.2" N, 101° 34' 52.6"E	Wang Saphung
	22. Huai Baeng Forest Ranger Station	390	17° 16' 48.4" N, 101° 34' 00.1"E	Wang Saphung
	23. Pak Dang Forest Ranger Station	750	17° 10' 42" N, 101° 21' 48" E	Dan Sai

Materials and Data Collections

This survey started in August 2005 and ended in June 2010 in all the 7 forest types present at PLWS. Discolichens from trees, dead wood, rocks and other substrates were collected by means of a pocket knife or hammer and chisel. The fresh samples were wrapped in soft tissue paper and put in a temporary envelope, where the primary collection site information was noted. The specimens were keeping in cloth bag for ventilation, that to prevent from damage during transportation. These 700 specimens are deposited in the herbarium at Ramkhamhaeng University (RAMK).

Preparation of Specimens

The specimens were dried under open air for a several weeks or in an oven at 40 °C for a week to inhibit growth of fungi. The dried specimens were kept in pockets stored in a box. The sizes of pockets and boxes varied according to the size of the specimens. Index cards 7.5 x 12.5 cm were inserted below the samples within the pockets. The label paper was stuck on with information including the specimen number, TLC number, scientific name, family, collecting locality, substrate, forest type, collector, determinator and the date was printed.

Morphological and Anatomical Observations

Macroscopic Examination

The morphological characters; color, size, shapes and surface texture of the thallus and reproductive structures were observed under low magnification hand lens of 10x or 20x and stereomicroscope (Olympus SZ30). The data were recorded and compiled on data sheets waiting for cataloguing to genus–species identification.

Microscopic Examination

The anatomy of the thallus including reproductive structures, apothecium and pycnidium, were performed by free-hand sections with a razor blade and investigated with a light microscope (Olympus, CH) at 40-1,000x magnifications. The stratified layers of the thallus and of the reproductive structures were measured with an ocular micrometer and illustrated as a photograph or drawing, using a drawing tube. Iodine (I) was used for color staining of the ascus walls (Lugol's solution, Iodine 0.25%). Calcium oxalate crystals were also observed under the light microscope.

Illustrations (Drawings and Photographs)

The measurements and drawings of the macroscopic and microscopic characters were performed under the light microscope by an ocular micrometer and drawing tube (Olympus, CH) at 40-1,000x magnifications. A stereomicroscope (Olympus, SZ30) equipped with eyepieces of 4x to 100x

were used for study the details of thallus and apothecia. Drawings of sections were also made by means of a camera lucida (Olympus BH2-DA). Characteristic aspects were recorded in data sheets. Photos of the thallus and reproductive structures were taken with a Nikon Coolpix 7600 adapted to a dissecting microscope at the Department of Biology, Ramkhamhaeng University, Bangkok.

Microchemical Tests

Spot or color tests of lichen substances were carried out on the thalli and apothecia. From a small area of the thallus, the cortex was removed with a razor blade to uncover the medulla. Subsequently, the reagents, 10% aqueous solution of potassium hydroxide (K), or sodium hypochlorite (C), or a saturated solution of *para*-phenelenediamine (P.or Pd.) or K reagent immediately followed by C reagent (KC), was applied with a sharpened match-stick, thin glass rod, or a haemalytic capillary tube. The color reactions for lichen substances were examined and recorded.

Chemical Examination

Some of the chemical products of lichens can be determined by spot or color tests, however sometimes lichen substances are undetectable, owing to a very small amount of chemical constituents and therefore this procedure is not sufficient to identify the substances precisely. Thus, thin layer chromatography (TLC) was applied, using the standard method of White &

James (1985, pp. 1-41) and Elix and Ernst-Russell (1993, pp. 1-163) to confirm a lichen substance.

Small pieces of thallus and apothecia were placed into small test tubes and three to four drops of acetone were added to extract lichen substances from the thallus. Some of the solvent was evaporated in a warm water-bath to get concentrated solutions. These solutions are then spotted on the TLC-plate using a capillary tube for each sample. Merck silica gel 60 F-254 pre-coated glass plates, 20 x 20 cm TLC-plate for each solvent system were used. A 2B pencil was used to mark a base line 2 cm from the bottom edge. 19 points were made on each notch at 0.9 cm intervals, which begin at 1.9 cm from the edge of the plate.

The treated TLC-plates were placed into the solvent tanks and left the solvent moved to the top of plate. The plates were then taken out of the tank and dried at room temperature. Pigments were observed and marked under daylight. Spot detection and color note were marked under ultra violet light at 254 nm and at 365 nm. The lichen substances were developed after applying 10% sulphuric acid solution with a brush and heat the plate in an oven or on a hot plate at 110 °C for about 10 to 15 minutes. After heating, the spots were marked under ultraviolet light at 365 nm for checking characteristics of the spots. Identification of lichen substances was determined from R_f classes, color spot in daylight and under ultraviolet fluorescence both before and after heating or relative R*f* values using WINTABOLITES computer program. In some cases comparison with spot tests were needed to confirm the identification.

Table 4

The Control Lichen Used for Routine Identification of Lichen Substances was Standard-Rf

Standard-*Rf*	A	B'	C
Protocetraric acid	3	19	5
Salazinic acid	10	7	4
Physodalic acid	14	35	23
Squamatic acid	13	23	28
Hypoprotocetraric acid	22	40	19
Virensic acid	36	56	38
Notatic acid	34	44	38
4-*O*-Methylhypoprotocetraric acid	39	51	45
Norstictic acid	40	32	30
Zeorin	52	43	43
15-α-Acetoxyhopan-22-ol	60	48	48
Usnic acid	70	65	71
Atranorin	75	73	79

Three standard solvent systems, A, B' and C were used for routine examinations. The basic solvent systems were prepared according to Kalb as follows

A = Toluene (180 ml): 1, 4-Dioxan (45 ml): Acetic acid (5 ml) (T. D. A.)

B' = n-Hexane (140 ml): tert-Butyl-Methylether (72 ml): Formic acid (18 ml) (T. D. B'.)

C = Toluene (195 ml): Acetic acid (35 ml) (T. D. C.)