CHAPTER 3

MATERIALS AND METHODS

3.1. Collection

3.1.1. Collection sites

The specimen collections have been done since October 2004 to October 2005 in five sites covering four Provinces in Northern Thailand (Figure 16).

1. Chiang Mai Province
    - Doi Suthep-Pui National Park, latitude 18.48.00 longitude 98.56.00
    - Queen Sirikit Botanic Garden, latitude 18.92.00 longitude 98.94.00
    - Mae Jo, latitude 18.55.00 longitude 99.00.00
    - Fang, latitude 19.57.00 longitude 99.10.00
2. Uttradit Province
    - Sak Yai National Park, latitude 17.40.00 longitude 100.41.00
3. Phetchabun Province
    - Nam Nao National Park, latitude 16.40.00 longitude 101.33.00
4. Chiang Rai Province
    - Wiang Pa Pao, latitude 19.02.00 longitude 99.20.00

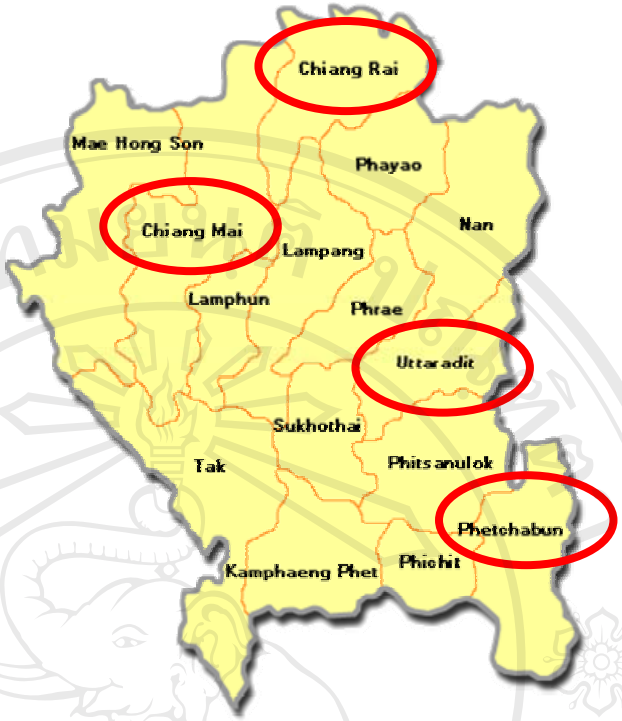

Figure 16 The map of Northern Thailand (Smitianand, 1977).

3.1.2. Collection Methods

Specimen collection was carried out by observing of cercosporoid symptoms on the leaves surface. The symptoms is characterized by frog eye spots, stripes, shot hole effect. Leaf spots may be absent or be present in every degree of distinctness from a faint discolouration on the upper leaf surface to definitely defined and often characteristically marked lesions. When no leaf spots are visible, an effuse Fruit bodies of the fungus ordinarily shows on the lower leaf surface. Fruit bodies of the cercosporoid genera on the leaves surface is observed by using hand lens with 10x magnification. The specimens that showed the cercosporoid symptoms were stored in the plastic bags. Collecting bags are sealed and labeled some information including name of host plants, collecting site, collector and collection date.

Direct observation was carried out after arriving from the field by using dissecting microscope for Fruit bodies body examination and stereo microscope with 1000x magnification to observe morphological structures of cercosporoid genera. The rest of samples were kept in the moist chamber for further observation. On returning to the laboratory, the material is incubated for a week and either studied immediately or air dried and stored for study a later. Air drying enabled single spore isolations to be made, which would not be possible with oven dried specimens.

3.2 Observation and identification

3.2.1 Observation of specimens

The fungal structures was checked by means of freehand section or by using a microtome. A small piece of the infected tissue from each specimen was mounted in water or lactophenol for examing conidiomata, conidiophore, conidia, conidial scars and hilum. Microscopic observations were made from fresh material at the day of collection. For a dried specimen was needed for additional observation. Resuming the shrunken structure of these fungi. A small piece of the leaf tissue was soaked in a drop of lactophenal. All of these were carried out on slide and heating it for a few seconds above a small flame and cooling down for further handling, next the fungus was scraped away from the leaf tissue with a blade and mounted in a drop of lactic acid for light microscopy observation. Measurements of conidia, conidiophore and other fungal structures were 30 replicates for each fungus and drawing of fungal structures of taxonomic value were prepared under 400 magnification. The morphology of conidia, conidiophore, stromata etc, were described and illustrated

with a drawing and photographic pictures by using Olympus DP 11 digitally camera. Identification species are mainly based on Braun's concept (Braun, 1995)

3.2.2. Preservation of specimens

Dried samples have immediately been observed. Each specimen was pressed between clean paper sheets and dried for preservation and change paper sheets every day until specimens is completely. Once fully examined, the piece of material with all of its markers, is placed in a resealable envelope with the following details:

Herbarium number

Host name

Collection site

Collector

Date

The material is stored in labeled a box in a dry environment and naphthalene balls are used to keep insect infestation at a minimum. Material which is not used, is destroyed.

3.2.3. Single Spore Isolation

Single spore isolation of each new fungus encountered are attempted refer to Choi *et.al* (1999). A glass container or glass slide is sterilized with ethanol and wiped with a towel on which ethanol (70%) has been sprayed. A sterilized pipette is then used to transfer about 6 drops of sterilized water into the glass container or onto the glass slide. The suspension is then prepared as above. Sixteen squares are marked on the bottom of the water agar plate. The prepared homogenous spore suspension is then

transferred with a sterilized pipette, onto the surface of the water agar plate, with a drop placed above each of the drawn squares. Alternatively about six drops of the suspension can be pipetted onto the center of the agar plate and this can be carefully shaken to spread the suspension. If this method is followed, it is a good practice to mark the outer help to locate germinating spores later. A small drop of the suspension should be used at this stage to make a permanent slide and to check that the correct fungus has been selected. The unsealed plate is incubated at 25 ^{o}C for 12-24 hours. It is not sealed as this allows some of the surface water to dry out. If the plate is sealed with parafilm, water will accumulate on the surface of the agar and will increase the chance to contamination. The spores are checked within 12 hours and then every 24 hours to establish germination. Once of the spores have germinated, a sterilized glass needle is used to pick up a small piece of agar containg a spore. In order to establish that the spore is the one desired, and maintain quality control, a slide is prepared and examined under the compound microscope. If the spores do not germinate after 12 hours, seal the plate with parafilm and examine periodically. Ten germinated spores are transferred and distributed evenly onto two PDA plates and incubated at 25 ^{o}C until their colony in diameter, are about 1 to 2 cm. A small piece of mycelium with agar can then be cut and transferred to another PDA plate and the culture is checked after a few days, if there is no contamination, a pure culture has been obtained. Cultures can then be stored on the desired media. Spore suspension can be obtained by removing a few segments from the gills and agitating them in sterilized water. Isolation can also be carried out from sterile tissue within the cap. A few pieces of sterile tissue can be aseptically torn from the split Fruit bodies structure and placed on water agar. Conidia of *Cercospora* and allied genera can be picked up directly from

the substrate using fine forceps or a needle. It is better to pick up spores only (not conidiophores), as this reduces the chance of contamination. The spores are placed in sterilized water and agitated in order to provide a spore suspension.

3.2.4. Identification Procedures

Identification species are mainly based on the recent concepts (Deighton, 1967a, 1967b, 1971, 1973a, 1973b, 1974, 1976, 1979, 1983, 1987; Pons and Sutton, 1988; Braun, 1988, 1989, 1990, 1994, 1997 and Crous and Braun, 2003). In most cases the specimen could be identified to at least genus level with the above references. Further identification required examination of the relevant literature. Sources are often suggested in the above references and the Dictionary of Fungi (Kirk *et al.* 2001) is found particularly up-to-date and useful, as is the Bibliography of Systematic Mycology on CD ROM (Produced by I.M.I., C.A.B. International Wallingford, U. K.), the 'searchable' Index of Fungi on the internet is also invaluable.

3.2.5. Description of New Taxa

If, after consulting all of the relevant literature and seeking the advice of other mycological taxonomists, the fungi could not be identified, it was considered novel. Thorough descriptions are made on standarised record sheets and usually 30 conidia, conidiophores and conidiogenous cells and stromata are measured. Otherwise where sufficient material is not available, ranges are given.

3.2.6. Presentation of Results

Cercospora and allied genera are presented in alphabetical order. For each genus, a small summary account is given and the following information is presented for each species. “Host substrate” and “Known distribution”, with information from the literature listed first and the relevant references cited, after which data from this study is listed. “Material examined” with countries in alphabetical order, and collecting sites listed in numerical order of the herbarium number. Finally, remarks justifying the identification are made. For new species or genera, a full description is presented, with the etymology of the specific epithet given and the holotype indicated in the “Material examined”. Several unusual or rare species, or those which are poorly documented in the literature are illustrated andandor a description given.