CHAPTER 3

MATERIALS AND METHODS

This research concerned several aspects of studies involving 2 species of indigenous plants, i.e. *Peliosanthes teta* Andr. and *Gymnema inodorum* Decne. and a species of local vegetable, *Basella alba* L. The studies aimed at surveying and collecting the plants from domesticated fields as well as natural habitats. Species identities were gathered from characterizations of the plant specimens collected from different locations. Genetical relationships among the ecotypes within species were analysed from such characterizations to determine varietal classification. The studies were thus allocated into 7 parts and the materials and methods of individual study can be described as follows:

1. Surveys and collections

Targeted areas of the surveys were in 8 provinces in the Upper-North of Thailand, i.e. Chiang Mai, Chiang Rai, Lampang, Lamphun, Mae Hong Son, Nan, Phayao and Phrae. Visits were made to villages, focusing on local farms, backyard gardens, roadside areas, public spaces and schools. Accessible natural habitats were also explored. This part of studies was carried out from May 2007 to April 2009.

Origins, evolutionary path and sustaining cultivation of domesticated varieties were gathered through interviews delivered to the owners of the planting fields, farms or gardens. Inquiries regarding the existence, distribution and exploitation of the plants in natural habitats were made to the officers in charge of the woods and public spaces. Sampling locations were indicated by global positioning system (GPS). Environmental status of domesticated areas as well as ecosystem of the natural habitats were recorded.

Information on consumption abilities, utilities and marketing were relatively collected from the field surveys. Market inspection, accountable for potential evaluation of the plants in terms of utilization, was conducted accordingly.

Accession samples were taken for cultivation in experimental plots inside Huai Hong Khrai Royal Development Study Centre, Doi Saket, Chiang Mai. Collected samples from various areas were grown under the same conditions. Growth and development of the plants were observed and recorded. Parts of the plants were consequently taken for characterization at their mature stage.

2. Growth pattern

Experimental plant accessions were grown from their propagating parts in the cultivated plots. Data in several aspects concerning their growth and development were collected throughout a growth cycle, for *Basella alba* L., or an annual growth cycle, for *Peliosanthes teta* Andr. and *Gymnema inodorum* Decne. Vegetative growth of the plants were followed in terms of leaf flushing and shoot sprouting. Reproductive growth was determined from the growth stages, i.e. emergence of inflorescences and durations of flowering and fruit setting. Botanical drawings of individual plant organs at certain stages of the growth were carried out. Illustrations of the growth cycle of each species were consequently composed from relevant drawings.

3. Morphological characterization

Morphology of the plant parts, i.e. root, stem, leaf, inflorescence, flower, fruit and seed of the 3 species was individually studied, following the methods described by Chayamarit (2002).

Records were made on the minimum, maximum and average values of the measurements taken from individual plant parts at mature stage, categorized as listed

below, of all accession samples of available experimenting plants. Five replications from each accession were encountered and evaluated standard deviations were accordingly presented.

3.1 Root

Records were made on the shape and the colour of the roots. Diameter and length of the roots were also measured.

3.2 Stem

Observations were made on the shape and the colour of the stems of *Basella alba* L. and *Gymnema inodorum* Decne. and stem diameter was measured at the height of 5 cm above ground. For *Peliosanthes teta* Andr., rhizome diameter was recorded.

3.3 Leaf

Leaf phyllotaxy were identified. Characters of leaf blade, leaf apex, leaf base, leaf margin as well as leaf colour were described. Measurement was made on the width, the length and thickness of the leaves. In *Peliosanthes teta* Andr. extra records were made on the leaf number and the amount of the veins appeared on the leaf.

3.4 Inflorescence

Inflorescence type and shape and colour of peduncle were examined. Measurements were made on the length and the width of inflorescence as well as the length and diameter of peduncle.

3.5 Flower

Floral symmetry, shape and colour of the flower, floral whorl arrangement and component of individual whorl were evaluated. Measurements were done on the width and the length of the flower, petals and sepals. Thickness of petals and sepals was also inspected.

3.6 Fruit and seed

Type, shape and colour of fruits and seeds were examined. Width, length and thickness of the two organs were measured.

Botanical drawings as well as digital photography of the plant parts at their mature stages were composed to illustrate morphological characters of the target plants. Numerical taxonomy was analysed from collected data of the leaf characters. Dendograms were consequently produced to exhibit genetic relationship of the accessions within species using the version 2.21i of the NTSYSpc programme developed by Rohlf (2005).

4. Pollen morphology

The features and textures of mature pollens, i.e. polarity, symmetry, shape, size, sculpturing and aperture, were investigated through Scanning Electron Microscopy (SEM).

Mature pollens of the plant specimens were examined. Pollens were extracted from the anthers of different ages. They were then placed on a clean microscopic glass slide holding a drop of distilled water on it. After a glass cover slip was mounted, the slide was put under light microscopy for detection of mature pollens. Hereafter, the pollens collected from the anthers accommodating mature pollens were prepared for SEM investigation.

In this experiment, the pollens for SEM inspection were prepared relying on the procedure stated by Wongsawad *et al.* (1996) and Pehliven and Özler (2003). The pollens of individual species were taken from the plants of 3 accession samples representing separate provinces of collection. They were then kept in gelatinous capsules placed in the bottles containing silica gels. Later, they were sprinkled evenly over the surface of an adhesive carbon tape fixed on the stub. After those pollens were coated with gold in a sputtering chamber (JEOL JFC-1200 Fine Coater) the stub was then slid onto the specimen stage for examination under the SEM (JEOL Model : JSM 5410LV). Photomicrography of the pollens was undertaken for morphological analysis as suggested by Erdtman (1972), Fægri and Iversen (1989) and Simpson (2006).

5. Anatomical characterization

Anatomical structures of the plants were investigated from transverse and longitudinal sections of the plant parts, i.e. root, stem, leaf and flower. The sections were permanently prepared using paraffin embedding technique formulated by Johansen (1940). Chemical reagents and components of chemical solutions as well as the methods of preparation applied in this technique are shown in Appendix A.

The preparation procedure of permanent slides for histological studies of the plant parts are as follows:

5.1 Killing and Fixing

Tissues of the plant parts were sampled, killed and fixed in FAA solution which is a mixture of formalin/acetic acid/alcohol for at least 24 hours before being kept at room temperature. The fixed samples were then dehydrated with a series of alcohol mixtures as dehydrating agents.

5.2 Dehydration

Alcohols used for dehydration were ethanol/tertiary butyl alcohol. They were mixed and diluted with distilled water to make the concentrations of alcohol

ranging from 50% to 100%. The fixed samples were immersed sequentially in a series of dehydrants having the percentages of alcohol mixtures being 50%, 70%, 85%, 95% and 100%, each at the duration of 6-24 hours, as suggested by Sass (1966). When the whole process was complete dehydrated tissues were then ready for paraffin infiltration.

5.3 Paraffin infiltration

Paraffin infiltration started from passing dehydrated samples into pure tertiary butyl alcohol (TBA) for at least 24 hours before being infiltrated in a mixture of TBA and liquid paraffin (1:1) for 3 days at room temperature. While some paraffin chips were melted in a beaker kept in the hot air oven at 56^{o}C, the samples in TBA/liquid paraffin were placed in the same oven until TBA was completely evaporated. The samples were later transferred into small vials, half-filled with melting paraffin. They were left for a complete infiltration in the oven, at least for a week, before taken out for embedding.

5.4 Paraffin embedding

Melted paraffin (Paraplast) was prepared and kept in the hot air oven, waiting to be used for embedding. It was then poured into paper boats to cast a paraffin block where the samples were embedded. Orienting of the samples while embedding was quickly done with a needle heated over the flame. Air bubbles in the paraffin were, in the meantime, eradicated. The embedded paraffin blocks were left hardened before taken for sectioning.

5.5 Sectioning

Sectioning was done by rotary microtome. The paraffin block containing embedded samples were cut into cubes and mounted on wooden blocks saturated with paraffin. The thickness of the paraffin ribbons was adjusted to 15 micrometres. Selected ribbons of tissue sections were then pasted with albumin adhesive on the glass slides. The ribbons were left dried before moving on to staining.

5.6 Staining

Completely dried ribbons mounted on the slides were stained with Delafield's hematoxylin. They were first immersed in xylene until the paraffin totally dissolved away leaving only the mounted tissue sections. The slides were then hydrated in a series of ethanol and its mixtures starting from absolute ethanol/xylene (1:1) then onto 95% ethanol/xylene (1:1) before passing through diluted ethanol of 95%, 70%, 50% and 30%, respectively, each for at least 5 minutes. After 30% ethanol the slides were placed in staining jars half-filled with Delafield's hematoxylin solution for 5 minutes before going through dehydration process. This process started with distilled water onto a series of ethanol from 30% to 50%, 70% and 95%, respectively, each for at least 5 minutes. The slides were consequently immersed in a 1:1 mixture of xylene and 95% ethanol prior to absolute ethanol/xylene (1:1) then finished up in 100% xylene. After the whole process was done the slides were placed in the jars of xylene, ready for permanent mounting.

5.7 Mounting

Stained sections on the slides from xylene jars were quickly mounted with glass cover slips using Canada balsam as an adhesive. They were then dried before microscopic investigation.

5.8 Microscopic investigation

Permanent slides of the tissue samples were investigated under the Olympus BX50 Compound Light Microscope. Photomicrographing from Olympus DP21 Microscope Digital Camera was conducted for histological studies.

6. Karyotypic characterization

Somatic chromosomes of each plant species were investigated. The plants used as representatives of individual species were randomly selected, 24 in number, from collected plant accessions. Root tip squash technique for chromosome observation was used in preparing meristematic tissue of selected plants, as described by Feulgen and Rossenbeak (1924), Dyer (1979) and Singh (1993), later modified by Vitayasak (1996).

Chemical reagents and preparing methods of solutions used in this investigation appear in Appendix B. The procedures of karyotype studies are as follows:

6.1 Chromosome investigation

The process of chromosome investigation composed of 7 parts as follows:

6.1.1 Root-tip sampling

The growing roots of the plants, appearing translucent white, with the tips opaque cream to white, were collected by the length of 0.5-1.0 cm. At this part, trials of sampling time were made to obtain the most suitable time of sample taking. Root-tips were scheduled to be taken at 1-hour intervals from 7.00 to 12.00 a.m. The samples were then washed clean from soil by shaking gently in distilled water before being removed to vials containing clean distilled water, waiting to be pre-treated.

6.1.2 Pre-treatment of root samples

Trials of pre-treatment duration in para-dichlorobenzene solution (PDB) were engaged in this part of experiment to obtain the most suitable duration resulting in the maximum number of cells at metaphase stage. The root-tip samples were taken out of distilled water, wiped dried then immersed in PDB for 1, 2, 3, 4, 5, 6, 7 or 8 hour(s), at 10^{o}C prior to fixation.

6.1.3 Fixation

Pre-treated root-tips of various treatments as stated in 6.1.2 were washed in distilled water for 5 minutes then forwarded to the vials containing Carnoy's solution for five minutes to preserve the tissues and prevent distortion of the chromosomes.

6.1.4 Maceration

Preserved root-tips were macerated in hydrolytic solution of 1N HCl in warm bath at 60^{o}C for five minutes. The root-tips were then washed in distilled water before taken out for staining.

6.1.5 Staining

Macerated root-tips were stained in carbol fuchsin solution at 10^{o}C. Here, trials on staining durations were made and the treatments were allocated into those of 30 minutes, 1, 2, 3, 4, 6, 8 and 10 hour(s) of staining. Stained root-tips of different treatments were squashed thereafter.

6.1.6 Squashing

Stained root-tips were squashed following the Feulgen's squash method modified by Shiotani (1994). The root tips were individually placed on a clean glass slide with a drop of stain on it. The root caps were removed before the tips were thoroughly squashed. The excess tissues and dye were discarded before mounting. The squashed tissues were gently pressed with a needle, mounted with glass cover slips and sealed with wax or nail polisher, ready for investigation.

6.1.7 Microscopic inspection

The slides were placed under light microscopy for chromosome checking. Chromosome counts were made from the cells showing intact and well scattered chromosomes. Chromosome numbers were recorded from the cells with the exact counts from at least 10 cells per specimens. Microscopic photographs of the chromosomes were taken for karyotypic studies.

6.2 Karyotypic studies

For karyotypic studies, chromosome number, karyogram, idiogram and karyotype formula of each experimental species were figured out as described by Vitayasak (1996). Clear and sharp photomicrograps of the cells containing mitotic-metaphase chromosomes were selected. From a diploid set, individual chromosome was then measured for its length of short arm and long arm. Relative length (RL) and centromeric index (CI) of the chromosome were then formulated from the length of the whole chromosome (LT) which included those of the short arm and long arm (Ls + Ll) and the total length of the chromosomes (∑LT).

$$\text{Relative length (RL)} = \frac{Ls + Ll}{\sum LT}$$

$$\text{Centromeric index (CI)} = \frac{Ll}{LT}$$

Chromosome pairing within a diploid set was carried out when the RL and CI of the chromosomes were tabulated. The paired chromosomes were then arranged vertically with their centromeres aligned in descending order of the chromosome length. The haploid set was therefore produced with its diagrammatic karyogram.

The size of the chromosomes, categorised as large (L), medium (M) and small (S) was classified based on the longest and the shortest chromosomes of the same set. The medium sized chromosomes were those having the length less than half of the average length of the longest and shortest chromosomes while the small sized were those having the length of their chromosomes less than half length of the longest chromosome.

The CI was applied to signify the type of the chromosomes based on the position of the centromere, being as follows:

CI = 0.500-0.599 : metacentric chromosome
CI = 0.600-0.699 : submetacentric chromosome
CI = 0.700-0.899 : acrocentric chromosome
CI = 0.900-1.000 : telocentric chromosome

From RL, CI, chromosome size and centromere position, the karyotype formula were then constructed from the diploid set of the chromosomes.

7. Isozyme pattern investigation

Isozyme patterns of individual plant species were investigated via electrophoresis technique proposed by Schmidt (1980), Shields *et al*. (1986) and Chokthaweepanich (2002) involving 8 enzyme systems, i.e. acid phosphatase (ACP), esterase (EST), glucose dehydrogenase (GDH), glutamate oxaloacetate transminase (GOT), leucine aminopeptidase (LAP), malate dehydrogenase (MDH), peroxidase (POX) and shikimate dehydrogenase (SKD). The plant proteins used for determination were obtained from the leaves of the mature plants grown in cultivation plots.

This study comprised 6 parts. Chemical reagents including preparation methods of solutions and other agents used for the whole experiment were indicated in Appendix C. The procedures conducted here are as follows:

7.1 Protein sample preparation

Samples of protein were extracted from mature leaves of each experimenting species. The leaves occurred on the third lowermost up to the sixth nodes of the vines or the stems were those harvested for protein extraction. They were kept cool from the field to the extraction room. The leaves were washed with distilled water and

dried before weighing. One gram of the leaves was finely cut and ground with liquid nitrogen in a prechilled mortar. Three millilitres of extraction buffer and 0.1 gram of polyvinylpolypyrrolidone (PVPP) were poured in. A homogenate was filled into an eppendrof tube and centrifuged at 14,000 rounds per minute for 30 minutes at 4^{o}C. Supernatant layer was then transferred into another eppendrof tube and stored at -20^{o}C.

7.2 Polyacrylamide vertical slab gel preparation

The gel molds were cleaned with 95% ethanol before being constructed. Thereafter, 7.5% acrylamide gel solution was filled into the gel molds and a well form (comb) was inserted into the gel solution, immediately. Air bubbles occurring in the solution or on the well form should be meanwhile eliminated. Polymerization was allowed to complete within about 10 minutes before the well form was carefully removed. The wells, now appearing on the top part of the gel were ready for sample loading. Hereafter, the electrophoresis cell was composed and electrode buffer was poured into the chamber.

7.3 Sample loading

Each enzyme sample was mixed with the marker dye solution (9:1). Twenty microlitres of the mixture were loaded into a well using micro-syringe. Cares were well taken while loading the samples to avoid cross-contamination of the samples.

7.4 Electrophoresis

Electrophoresis was performed in a Mini-Protean® 3 Cell Electrophoresis unit (Bio-Rad). The chamber was connected with the power supply and kept at 4^{o}C all through. The gels were run at approximately 120 V and 80 mA until the samples had migrated to 1.5 cm above the lower rim of the gel. Thereafter, the power supply was turned off, and the gel was taken out for staining.

7.5 Staining

Staining recipes were prepared for individual enzyme while the gels were running. When the power supply was switched off, the gels were carefully removed from the molds and then placed into staining boxes. Staining solution was then poured onto each gel. The boxes were gently shaken and kept in the dark. When the bands appeared, the stained gels were then rinsed out and kept in distilled water until inspection.

7.6 Data analysis

Isozyme banding patterns from the gel profiles were then transformed into zymograms according to the relative to front (Rf) of the movement between the enzymes and the marker dye solution.

$$Rf = \frac{\text{Distance of enzyme movement}}{\text{Distance of marker dye solution movement}}$$

Profiles of isozyme patterns and zymograms of each enzyme system produced from individual species were recorded. The bands across all enzyme systems and entries were coded as present (1) or absent (0). Similarity for qualitative data (SimQual) was calculated from Jaccard's similarity coefficient (Sokal and Sneath, 1963). Cluster analysis was produced through sequential, agglomerative, hierarchical and nested (SAHN) clustering method using unweighted pair-group method with arithmetic averages (UPGMA) implemented in the numerical taxonomy and multivariate analysis system computer programme package, NTSYSpc version 2.21i developed by Rohlf (2005) to place specimens of individual species into groups and present them as dendrograms (Sneath and Sokal, 1973).