Chapter III

MATERIALS AND METHODS

The present dissertation has two distinct arena for studies. First is the collection of the study materials. It is completely outdoor works that include spotting the plants, collecting pollen-bearing part and outdoor processing of the materials so that it can reach the laboratory in good condition. The second major part of the work is in the laboratory where host of works including identification of voucher specimens, processing of polliniferous materials for different types of studies, their description and understanding their scientific knowledge contribution is done.

The entire work is done entirely on freshly collected materials only and no specimen was collected from any herbarium.

3.1 FIELD SURVEY

Extensive field surveys were carried outduring the years 2013 to 2017 in different areas of Terai-Dooars and foot-hill regions. Basically, the surveys were conducted throughout the years covering all the three different flowering seasons of the year namely pre-monsoon, post-monsoon and winter for four consecutive years as the flowering season of different species are varied. Once missed, it is then essential for one year to collect the polliniferous materials in its next flowering season. All plants are not growing on road-sides or on easy to pursue areas. This has taken a long time to complete the field collections. Polliniferous materials, mainly the fully matured flower-buds or freshly blooming flowers were collected from different types of woody plants, including trees and woody climbers. So, materials were collected only in their flowering phenophase and the flowers are the basic essential plant-part for the present dissertation.

3.1.1. Collection of specimens

For the present work specimens were collected in two forms:

(i) Voucher specimens, in the form of flowering twigs, were collected for identification of plants and to process those into the mounted herbarium-sheets. Specimens were collected in triplicate and were kept inside the polythene bags; and

(ii) For pollen morphological study well developed matured flower buds were collected and kept in small zip-locked polythene bags. For overall collection and processing of specimens Jain and Rao (1977), Bridson & Forman (1998) and Das (2018) were basically consulted.

When twigs are kept in polythene bags (substitute of vasculum) it was sprinkled with little amount of water, then inflated the bag by blowing with mouth and finally tied the mouth of the bag with rubber bands (Das 2018). On the other hand, for

pollen extraction, anthers were taken out from the well matured flower-buds with the help of forceps and preserved in glacial acetic acid (Erdtman 1952). Same field numbers were maintained for these two sets of materials.

3.1.2 Record of field data

During collection, all the specimens were recorded in a *Field Note Book* (FNB) and the specimens were tagged with the serial number as it was recorded in the FNB. Some important field-data like date and place of collection, and essential field characters (Das 2018) etc. were recorded in FNB.

3.1.3 Processing and drying of specimens

Collected specimens were cleaned and trimmed to suitable sizes, labelled with field-number andthen dried under heavy wooden Plant Press within blotters (old newsprints and/or blotting papers). Before pressing, the specimens were treated with diluted formaldehyde (2:3, formalin : water) to prevent the fragmentation of specimen and avoid the chances of microbial attack including fungi and bacteria and also of herbarium-beetles (Bridson & Forman 1998; Das 2018). During the time of extreme humidity, especially during the monsoon months, a Hot Air Oven was sometimes used at 40º – 45º C for quick drying.

3.1.4 Poisoning of specimens

After drying the specimens were poisoned with 4 – 6 % ethanolic solution of Mercuric Chloride ($HgCl_2$) and dried again within the blotters.

3.1.5 Mounting on Herbarium-sheets

After poisoning the specimens were first checked for the condition of the labeled field number, then mounted onstandard herbarium sheets or mount-boardsusing polyvinyl-acetate (PVA) glue (commonly available brand 'Fevicol') and kept under pressure for a day. The standard size of Herbarium-mount/sheet is 43 x 29 cm. Each herbarium-sheet was then provided with a 'herbarium-label', preferably to its lower-right corner and was provided with basic data for the specimen including name of taxon, place and date of collection, family, habit, habitat, flowering and fruiting, altitude, name of the collector and determinator (Jain & Rao 1977; Bridson & Forman 1998; Das 2018). Mounted herbarium-sheets were then temporarily stored in a steel cabinet.

3.1.6 Identification

Mounted specimens were identified through the consultation of different types of literature, mostly floras (including Hooker 1872 – 1888; Prain 1903; Hara 1966, 1971; Ohashi 1975; Hara & Williams 1979; Hara *et al.* 1982; Grierson & Long 1983, 1984, 1987, 1991, 1999, 2001) available and by matching with the previously

identified specimen at the NBU and CAL Herbaria. Sometimes, for the doubtful specimens, experts from different areas were consulted.

All the recorded plant families were classified following Angiosperm Phylogeny Group (APG-III, Chase & Reveal 2009). Dichotomous identification keys, correct names, protologue, basionyms, synonyms, local/ common names, reference to voucher specimens, local distribution etc. were provided during enumeration.

For Family delimitation mostly Cronquist's (1988) and APG-III (Chase & Reveal 2009) has been followed. For the up-to-date nomenclature www.plantsoftheworldonline.org; www.theplantlist.org and IPNI were extensively consulted.

However, for Enumeration part all the families, genera under each family and species under each genus were arranged alphabetically for easy handling, especially for the non-taxonomic readers.

3.1.7 Storage

After the completion of the current work, the first set of voucher specimens will be deposited in NBU-Herbarium and duplicates will be deposited to CAL.

3.2 TECHNIQUE OF POLLEN PREPARATION

Anthers were plucked from mature flower buds just before blooming to avoid contamination by pollens of other plants. In case where mature flower buds were not found, partially opened flowers were taken. All the flower samples were collected from different stretches of vegetation in the Terai-Dooars region of West Bengal. Anthers were preserved in glacial acetic acid in small glass-vials till further processing (Erdtman 1943).

3.2.1 Processing of Polliniferous materials

All the polliniferous materials i.e. anthers were subjected to acetolysis method of Erdtman (1960) and as modified by Chanda (1966) and Nair (1970). At first the pollen bearing parts of the flowers were kept in 5 ml glass-vials with glacial acetic acid. The vials were stored within boxes before the final preparation of grains.

3.2.2 Acetolysis

The pollinifeorus materials were crushed with distilled water using a stainless steel sieve (80 mesh) and collected in a test tube and centrifuged at 4000 rpm for five minutes. Supernatant decanted and pellet collected. Glacial acetic acid was added to the pellet and centrifuged at 4000 rpm for five minutes. The supernatant again decanted and the pellet collected.

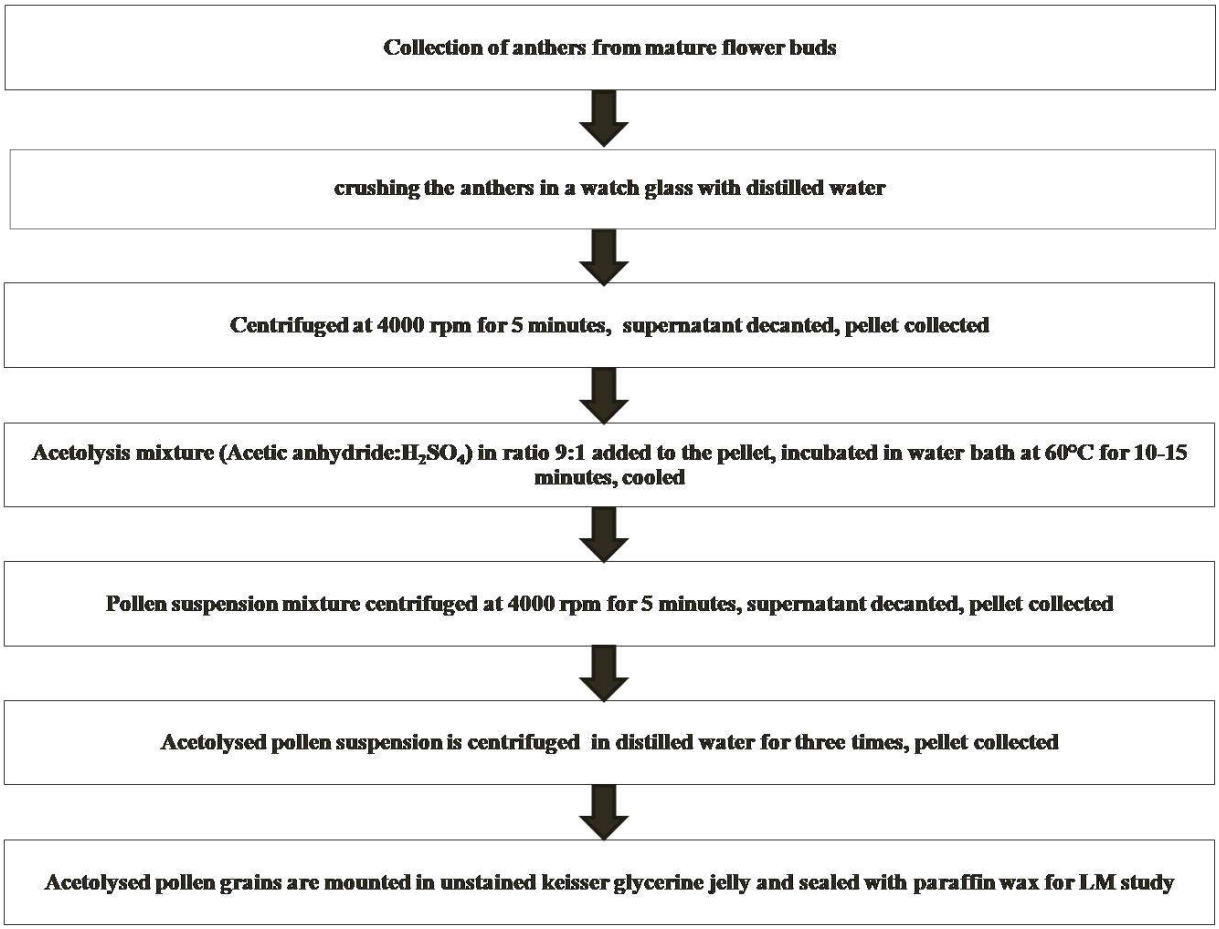

Figure 3.1. Flow-chart showing acetolysis process of pollen grains.

3.2.3 Preparation of permanent slides

After the acetolysis process, a minute piece of unstained Keisser Glycerin Jelly (Keisser 1935) were taken on the tip of a sterilized inoculation-needle and touched at the residue of the acetolysed sample and then placed on the centre of a grease free glass slide. Then a glass cover-slip was placed on the jelly. Little amount of high melting point wax is kept touching the cover-slip and then heated slowly over a narrow flame. Caution was taken for not over heating as this may cause boiling of the jelly and spreading of the sample out. During the process, wax will melt and will enter the space between slide and the cover-slip. Then the prepared slides need to cool down and a label is attached to one end of the slide. For pollen-slides labels contain data on both sides, i.e. on face or upper side as well as on the glued side.

3.2.4 Light and Scanning Electron Microscopic studies

The pollen slides were then examined and photographed with a trinocular Magnus light microscope under Oil Immersion Objective and 10/15X eye-piece. For the Scanning Electron Microscope study, a drop of the acetolysed pollen grains was

smeared on a grease free cover slip and air dried (sometimes dried under a UV lamp) for critical point drying (CPD). Then it was coated with platinum (10 nm) in an S150 Sputter Coater (Bazarragchaa & Yuon 2012) and scanned under the Zeiss Evo 18 SEM microscope at the Centre for Nanoscience and Nanotechnology, University of Calcutta and at IISER, Kolkata.

3.2.4 Morphological Analysis

Detailed morphological studies were conducted under light microscope using oil-immersion objective and different layers were viewed through LO analysis. The measurements were taken from the scanning electron micrographs and also from light microscopes using in-build facilities. The measurements of the polar axis, equatorial diameter, polar diameter, length and breadth of colpi, length and breadth of ora, exine thickness were recorded on 20 matured pollen grains per specimen with LM (Zeiss Axioskop 40 x 1000 magnification) and photographed. The mean and standard deviation (SD) were calculated using Excel software (Microsoft Office 2007) and the data have been presented as [Minimum (Mean) Maximum ±SD] format. UPGMA analysis were performed using the pollen morphological data for the families kept under groups in the artificial pollen key. This was done for the better understanding the phenetic relationships within these groups. For this PAST3 software was used. The pollen grains were described following the terminologies used by Potonié (1934), Erdtman (1943, 1945, 1947, 1952), Fægri & Iversen (1950), Iversen & Troels-Smith (1950), Van Campo (1958), Erdtman & Straka (1961), Fægri & Iversen (1964), Reitsma (1966), Praglowski & Punt (1973) and Nilsson & Muller (1978).

3.3 PREPARATION OF KEY

A detailed artificial dichotomous key, based on wide range of pollen micro-morphological features, both of LM and SEM has been prepared to facilitate easy identification of the taxa under the present study. As far as possible, clustering was avoided and attempts were made to reach directly to each species.