3. MATERIALS & METHODS

3.1. MATERIALS

Materials for the study were honey samples collected from artificial hives of *Apis mellifera*, kept in apiary, pollens from corbicular loads as well as hive cells and pollens from the body-surface of the bees, those who did not carry any pollen loads on their pollen bags in corbicules of hind legs.

Materials were collected from different locations under North 24 Paraganas as here in below, as well as from adjoining areas –

North 24 Paraganas District	Nadia District
1. Berabereia 2. Chandpara, 3. Bagangram, 4. Gopalnagar, 5. Nischintipur, 6. Mondalpara, 7. Santoshpur	1. Bagula, 2. Majhdia

3.2. METHODS OF MATERIAL COLLECTION AND PRESERVATION

Honey samples had been collected month-wise by taking sample once in every week in a month. Honey samples had been collected with a Pasteur pipette from different hive cells at a time and from different boxes placed at a certain apiary.

Pollen samples were collected in three different ways, i) taking it from corbicular load, *i. e.* from the pollen basket on the tibia of the hind most legs, ii) from the pollen chambers in a hive and also iii) from the pollens clung on hairs throughout the body of honey bee.

Honey samples, taken in separate glass vials were preserved in low temperature inside refrigerator and the pollen pellets preserved in FAA (5: 5: 90 parts of Formalin, Acetic Acid and Alcohol).

3.3. CLEARING OF POLLEN SAMPLES FOR MICROSCOPY

Inner materials of pollens were cleared for better viewing of the features of pollen wall for the accurate characterization and identification of them. Clearing was done by the acetolysis method recommended by Erdtman (1960). Pollen samples, either in honey or in pellet were plunged into a mixture of Acetic anhydride and concentrated sulphuric acid, mixed in a proportion of 9:1. The samples were placed in separate centrifuged tubes. For the pollens in honey a certain amount of honey sample was diluted with hot distilled water and centrifuged at 2500 rpm for 10 minutes. The process was repeated twice or thrice after administering distilled water to the pellet. The pellet was then dried, as much as possible, and treated for acetolysis. The tubes loaded with pollen samples were kept in water bath at a temperature 70^{o}C for 3 – 5 minutes and centrifuged at a speed of 2500 rpm. for 10 minutes. Then the supernatant was discarded and the pellet was washed with double distilled water (DDH_2O) repeatedly twice or thrice by further centrifuging and discarding the supernatant.

3.4 PREPARATION OF GLYCERINE JELLY

50 gm of Gelatine, 150 ml of glycerine, 175 ml of distilled water and 7 gm of phenol crystals are the requisite for the preparation of the jelly. The first three constituents are mixed thoroughly and boil in a water bath for one or two hours. The phenol crystals should be added and mixed thoroughly. While still warm and molten, the glycerine jelly should be poured on a petridish making a thin, uniform layer of about 0.5 cm. thick. It is then cooled and preserved in refrigerator.

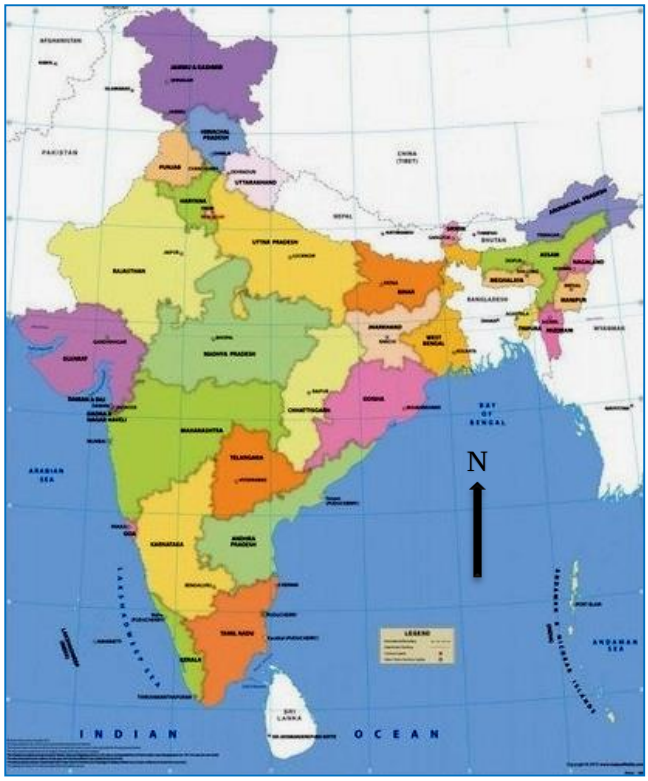
3.5. PALYNOLOGICAL PREPARATION OF HONEY SAMPLES

Pure honey sample (5 ml.) was diluted with 5ml. of absolute alcohol and for transferred to a 15 ml. conical pyrex centrifuge tube and centrifuged for 10 - 15 minutes at 2500 rpm. The supernatant was decanted, which contained honey sugars and other colloidal matters. The remaining sediment in the tube was mixed with concentrated glacial acetic acid and volume was made up to 10 ml. in the centrifuge tube. The mixture was again centrifuged and decanted. The process was continued thrice until the removal of even traces of sugar before acetolysis.

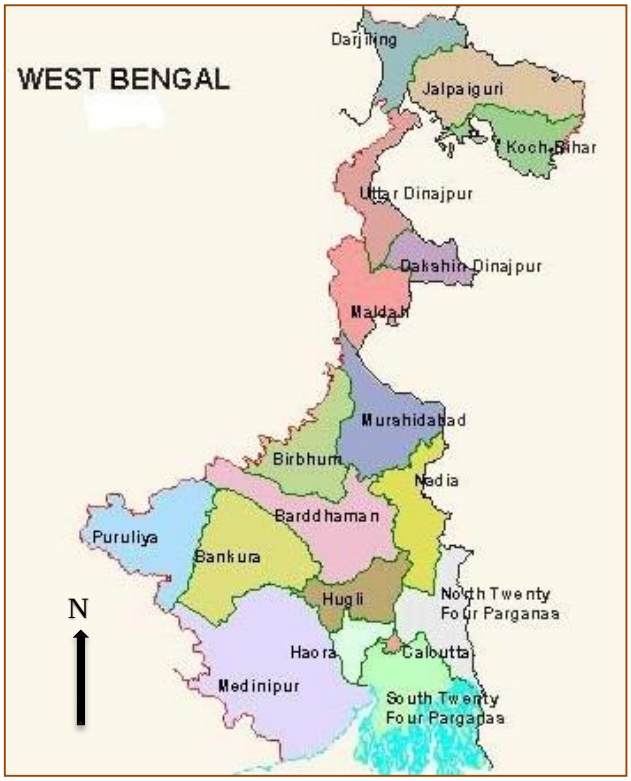
As recommended by Erdtman (1960) acetolysis solution (9 drops of acetic anhydride solution and 1 drop of concentrated Sulphuric acid) was added to the honey sediment and the tube placed into a wire basket were heated in a water bath to 80^{o}c for 5-10 minutes. Then the tubes were removed from water bath and the volume was made up to 10ml. with glacial acetic acid and was further centrifuged and decanted. The remaining precipitate was centrifuged with distilled water.

3.6. SLIDE PREPARATION

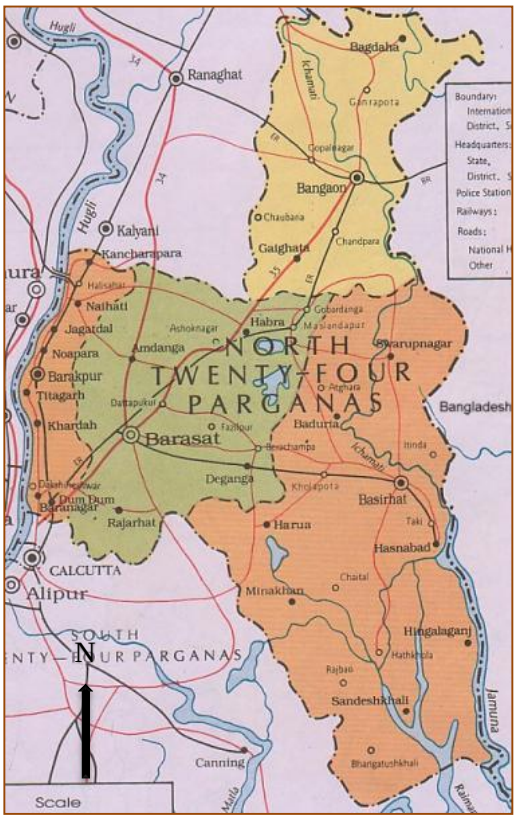
A little amount of glycerine jelly was taken on the tip of a platinum needle and touched with the pellet of acetolyzed pollens with care. The jelly with pollen materials attached with it was then transferred on a slide. After spreading the jelly evenly, distributing pollens in it, as much as possible, the material was covered with a glass cover slip on it. The cover glass was thereafter sealed on the slide with molten paraffin along the margin of it.



Map of India



Map of West Bengal



Map of North 24 Paraganas

3.7. PALYNOLOGICAL PREPARATION OF CORBICULAR LOAD

After visiting the polliniferous plants, the honeybees return to their hive. Such honeybees were captured and the pollen load was collected from their hind legs and preserved in a 70% alcohol for further analysis of pollen.

Pollen samples from corbicular load were centrifuged for 10 - 15 minutes at 2500 rpm. After decanting the supernatant, the sediments were dissolved with glacial acetic acid and again centrifuged for 10 - 15 minutes. After discarding the supernatant, the remaining sediments were treated with acetolysis solution (9 drops of acetic anhydride solution and 1 drop of concentrated sulphuric acid). The centrifuge tubes placed into a wire basket were heated in a water bath to 80^{o}c for 5 -10 minutes. Then the tubes were removed from water bath and again the volume was made up to 10 ml. with glacial acetic acid and was further centrifuged and decanted the supernatant. The remaining precipitate was again centrifuged with distilled water.

Slide preparation

Slide was prepared with glycerin jelly as described above (3.6).

3.8. PREPARATION OF POLLENS FROM SURFACE OF BEE BODY

The pollens of the nectariferous plant get attached to the romentum of the bees which visit the plants for nectar. Those honeybees were captured while they return to their hives and were preserved in 70% alcohol. Thus the pollens attached to the surface of the bee body got placed in the alcohol and was utilized for further analysis of pollen.

Pollen samples from bee body surface were centrifuged for 10 - 15 minutes at 2500 rpm. After decanting the supernatant, the sediment was dipped in glacial acetic acid and again centrifuged for 10 - 15 minutes. After discarding the supernatant, the remaining sediment was treated with acetolytic solution (9 drops of acetic anhydride solution and 1 drop of concentrated sulphuric acid). The centrifuge tubes, placed into a wire basket, were heated in a water bath to 80^{o}C for 5 -10 minutes. Then the tubes were removed from water bath and the volume was made up to

10ml. with glacial acetic acid and further centrifuged and decanted. The remaining precipitate was centrifuged with distilled water.

3.9. PREPARATION OF REFERENCE SLIDES

Slides for palynological study were carried out with the pollen samples obtained from the flowering plants of known identity.

Slide preparation

Slide was prepared with glycerin jelly as described above (3.6).

3.10. OBSERVATION UNDER MICROSCOPE AND STUDY OF POLLENS

Pollens from the prepared slides were scrutinized under right magnification for visualizing pores, colpa etc. Microscopic observations as well as photography were carried out with Leica DML 1000 bright field trinocular microscope. Pollens from different collections were, thus, identified. Relative presence of all pollen species in a sample of seasonal collection was determined by counting them under different fields of observation from the same slide. A thorough study of the preparations with samples were carried out for different seasons of a year, and thus, the studies, continued for three consecutive years, yielded foraging calendar of *Apis mellifera* in the area under study.

3.11 TERMINOLOGIES FOR DESCRIBING POLLEN MORPHOLOGY

Morphological features of pollen in respect of size, shape, aperture, polarity, symmetry, exine structure and sculpture have been taken into consideration in the present text following the standard terminologies proposed by Erdtman (1952), Faegri and Iversen (1975), Kremp (1965), Moore, Webb and Collinson (1991):

3.11.1 Polarity

Spores and pollen grains develop distinct polarity as they are produced in pollen mother cell in tetrad. The portion of the grain facing the centre of tetrad is its proximal pole and the opposite end is considered as the distal pole. An imaginary axis joining proximal and distal poles is the polar axis and that goes through equator is the equatorial axis. The equatorial axis of a pollen grain, viewed from the pole, is described as its amb (ambitus).

3.11.2 Symmetry

Pollen grains may be symmetrical and it may either be radially or bilaterally symmetrical. Isopolar pollen grains with radial symmetry have more than two vertical planes of symmetry and one horizontal plane of symmetry. While, radially symmetrical hetero-polar grains have more than two vertical planes of symmetry and devoid of horizontal planes of symmetry. Isopolar grains with bilateral symmetry possess one or two vertical plane(s) of symmetry and one horizontal plane of symmetry. In contrast, hetero-polar pollen grains having bilateral symmetry exhibit one or two vertical plane(s) of symmetry and no horizontal plane of symmetry. Bilaterally symmetrical pollen grains are provided with a second equatorial axis.

3.11.3 Size

Size of pollen grains of different species varies quite considerably. It ranges between 5 µm to 200 µm. In case of radially symmetrical grains with circular or triangular or polygonal amb the equatorial diameter (E) and polar diameter (P) are taken into consideration, while in bilaterally symmetrical grains a second equatorial diameter (E1) is also measured. In saccate grains the measurement of height, breadth and depth of colpus, sacci and the entire grain are taken.

3.11.4 Shape

Shape of a pollen grain widely varies for different species. In radially symmetrical pollen grains the amb may be circular, triangular or polygonal. In case of triangular or polygonal amb the

side may be convex, concave or straight. The amb may be square, too. Radially symmetrical pollen grain appears as rotational ellipsoid with polar axis as the axis of rotation. In rare cases pollen grains may be filamentous. The shape of pollens varies with the ratio of polar (P) and equatorial (E) diameters. In consideration of the value of P/E × 100 the different classes recognized are as follows:

Shape classes	**(PA/ED) × 100**
Per-oblate	<50
Oblate	50-75
Sub-oblate	75-88
Oblate-spheroidal	88-99
Spheroidal	100
Prolet-spheroidal	101-114
Sub-prolet	114-133
Prolet	133-200
Per-prolet	>200

3.11.5 Types of aperture

Apertures are the areas on pollen wall where the exine is either thin or completely devoid of. Pollen grain may be provided with one or more aperture(s). Aperture may either be simple or compound. In simple aperture the outer and inner margins of aperture are congruent with each other. Different types of it are as follows:

Lete: When the aperture is slit like and is situated at the proximal end that is termed as lete. In case of the presence of single such slit is called monolete, while in case of the presence of three such slits on a pollen it is called as trilete.

Porus: It is a simple circular aperture on the equatorial plane having ratio of length and breadth ≤ 2. Pollens provided with pores are called as porate.

Leptoma/Ulcus: It is the thin area over exine functioning as an aperture, however differing from typical apertures. Such aperture with regular outline is termed as leptoma and with irregular outline is referred to as ulcus.

Sulcus: The aperture with furrow like appearance and oriented at right angle to polar axis is called as sulcus. The sulcus as a simple furrow or a triradiate furrow occurring at distal pole is termed as trichotomosulcate and when appears as gutter-like equatorial or subequatorial furrow all around the spore it is called zonosulcate.

Colpus: It is the furrow-like apertures, usually meridional in placement i.e. parallel to the polar axis.

Pore: More or less circular aperture is called as pore.

In many instances the outer and inner margins of aperture are not congruent i.e. not having the same outline in shape and/or size and is considered as a compound one, which may be of the following types:

Pororate: In this case the aperture possesses an outer irregular poral part and an inner circular oral part.

Colporate: The outer part of aperture or ectoaperture is furrow-like i.e. colpate and the inner oral part or endoaperture is more or less circular. This type is again divided into the three following types:

1. **Endoaperture circular:** In this case the oral aperture is more or less circular in outline.

2. **Endoaperture lolongate:** In this case the aperture is elongated and oriented parallel to the long axis of the ectoaperture.
3. **Endoaperture lalongate:** In this type of aperture the endoaperture is elongated and oriented at right angles to the long axis of the ectoaperture. In some cases, the adjacent colpi of the endoaperture fuse with each other constructing the synorate condition. The endoaperture attains gutter-like appearance round the pollen grain.

Syncolpate/Syncolporate: In this type of aperture the ends of colpi fuse at either of the two or both of the poles.

Parasyncolpate/Parasyncolporate: In this case instead of direct fusion of apertures, they get branched and the branches fuse forming a triangular or polygonal apocolpal area i.e. colpus-free area at the pole.

Spiraperturate: In this type the aperture spirally covers the entire area of the pollen.

3.11.6 Margin of aperture:

In consideration of margin the apertures are of two types, (i) tenuimarginate, when the margin is thin and (ii) crassimarginate, when the margin is thick.

3.11.7 Position of aperture:

Position of apertures varies from species to species:

1. Proximal: cattaperturate/catatreme.
2. Proximo distal: anacataaperturate/anacatatreme.
3. Distal: anaaperturate/anatreme.
4. Equatorial: zonoaperturate/zonotreme.
5. Globally distributed: panto/periaperturate/pantotreme.

Pollens with angular amb having aperture on equator is designated as Zonoaperturate. When apertures are present on the corners of amb the pollen is Angulaperturate. Pollens having straight or convex sides and apertures situated at mid points are referred as Planaperturete, whereas when the sides are concave and the apertures are situated on the mid points of the sides the grain is called as Sinoaperturate. Zonoaperturate pollen grains having lobate amb and apertures placed on the notches are termed as Fossaperturate.

3.11.8 Subdivision of pollen surface with respect to apertures:

Based on the nature of the aperture, pore or aperture, the area of pollen grain in between two apertures is named as mesoporium/mesocolpium. The areas on the surface of zonoaperturate pollen grain devoid of any aperture at the polar region is designated as apoporium/apocolpium.

3.11.9 Exine stratification:

Exine of a typical angiosperm pollen grain is provided with two distinct layers, the inner nexine and the outer sexine. The sexine is comprised of an inner columellate layer subtended by a roof like structure tegillum. The nature of tegillum may be of varied nature for different species as complete (pertectate), incomplete (subtectate) or totally missing (intectate).

3.11.10 Surface of exine: Surface of the exine of different pollens may be provided with various ornamentations with specific types of projections or depressions. Surface features may be of the following types:

I. Sculpturing elements absent

1. Surface smooth – Psilate
2. Surface with depression or pits
   - 2.1. Pits isodiametric
     - 2.1.1. Diameter <1µm—Punctate

2.1.2. Diameter >1µm--Foveolate

2.2 Pits horizontally elongated along the surface

2.2.1. Pits irregularly arrayed – Fossulate

2.2.2. Pits parallel in arrangement – Canaliculate

2.2.3. Pits forming reticulations – Negatively reticulate

II. Sculpturing elements present

1. Sculpturing elements vertical, isodiametric in cross-section (Apiculate)

1.1. Size <1µm – Granulate.

1.2. Size >1µm

1.2.1. Broad base

1.2.1.1.Blunt apex

1.2.1.1.1. Height is less than or equal to width – Verrucate.

1.2.1.1.2. Height is greater than width – Baculate.

1.2.1.2. Pointed apex

1.2.1.2.1. Height is less than or equal to width – Conate.

1.2.1.2.2. Height is greater than width – Spinate.

1.2.2. Constricted base

1.2.2.1.Height is less than or equal to width – Gammate.

1.2.2.2.Height is greater than width – Clavate.

1.2.2.3.With slender shaft and a bulbous head – Pilate.

2. Projections horizontally elongated along the surface, with radial length at least twice the width (Murate)

2.1. Muri irregularly disposed – Rugulate.

2.2. Muri parallel to each other – Striate.

2.3. Muri forming reticulations (the area surrounded by muri is known as lumina) – Reticulate.