Three

Aids to Identification of Bee Flora

Honeybees visit a number of plant species to collect food, i.e, nectar and pollen. So far, over 1,000 plant species are known to constitute the bee flora of the Hindu Kush-Himalayan region. In order to fulfill the objectives of beekeeping, proper planning, use, and skilled management of existing bee floral resources are essential. The identification and categorisation of bee flora are, therefore, necessary for proper bee management.

Identification of Bee Flora

The bee flora of an area can be identified through surveying, in the field, plants visited by honeybees, either by direct observation or through published reports. They can also be identified by carrying out pollen analysis of honey (melissopalynology). These methods are described in detail in the following text.

Direct Observation

The bee flora of an area can be identified by observing honeybees foraging on flowering plants. The nature of the plant, whether it is a source of nectar or pollen, can be determined by observing the bee's activity on the flower. If a bee thrusts its proboscis into the interior of the flower basin, the plant is taken to be a source of nectar. The availability of pollen, on the other hand, can be determined by observing bees collecting pollen and carrying it in loads on their hind legs. The status of the material, whether major or minor, is determined by the intensity of bee visits, and the occurrence of the plant species is determined by the density of the plant (number of plants/m^2 for herbacious plants and number of plants/ha for trees and shrubs). Such field surveys have been carried out by Dewan (1980; 1984), Kafle (1984), Shahid and Qayyum (1977), and Makhdoomi and Chohan

(1980) in order to identify the bee flora of Bangladesh, Nepal, and Pakistan respectively.

Published Reports

While making field surveys, it is not always possible to find honeybees foraging, and, hence, it is not always possible to determine whether a particular plant is a bee plant or not. In such cases, the plant is collected (the whole plant for herbs and a flowering branch for shrubs and trees), pressed, identified, and then compared to the published reports of its use by bees.

Melissopalynology

Another method of identifying bee flora in an area is by identifying the pollen in the honey and the bee pollen load sample of the particular area. Honeybees, while foraging on the flowers of different entomophilous plants to collect nectar, also collect some pollen along with it. This pollen is retained in the ripened honey. Thus, the honey which the bees subsequently store in honeycombs always contains a certain amount of pollen. The microscopic examination of pollen grains in honey is known as melissopalynology or pollen analysis. Identifying the pollen in honey helps to identify the honey sources and analysing the bee pollen load reveals the pollen sources of an area. Studies to identify bee plants through melissopalynology have been carried out by many researchers in the Hindu Kush-Himalayan region (Focke 1968; Deh-Feng and Wen-Cheng 1981; Yue-Zhen 1984; Saraf 1972; Atwal and Goyal 1974; Singh 1983; Singh 1989; and ICIMOD 1996).

The Need for Identifying Pollen

Studies on pollen analysis of honey are helpful in both the qualitative and quantitative analyses of honey (Louveaux et al. 1978). Quantitative analysis is useful for confirming the botanical origin of unifloral or multifloral honeys. The types of pollen in honey indicate the flowers from which the bees gathered the nectar. For example, honey samples are considered to be rich, poor, or extremely poor in pollen if the number of pollen grains per 10g sample of honey is above 100,000, 20,000-100,000, or below 20,000 respectively.

Analysing the pollen in honey also helps to identify the geographical origin of the honey, because local bee flora have characteristic plant associations that are reflected in the pollen grains present in local honeys

(Pfister 1895; Maurizio 1975; Nair 1985). Honey pollen analysis also helps to identify the season in which the honey was extracted and the plant sources of toxic honeys. In addition, it indicates the relative preferences of honeybees for individual plants that flower simultaneously.

How to Identify Pollen

In order to analyse the pollen in honey, an adequate knowledge of pollen morphology is essential. Pollen is a granular mass of male reproductive cells produced in the anthers of a flower. When seen with the naked eye, these appear as minute dust-like particles. However, each particle has its own special structure which indicates its origin. A brief account of pollen grain structure and the diagnostic features used in identifying pollen are given in the next section.

Structure of the Pollen Grain

A pollen grain is a living cell surrounded by two protective coats, the intine and the exine. The cell contains cytoplasm and a nucleus. On the surface are apertures or germinal pores or furrows. The detailed structure of a pollen grain is shown in Figure 3.1.

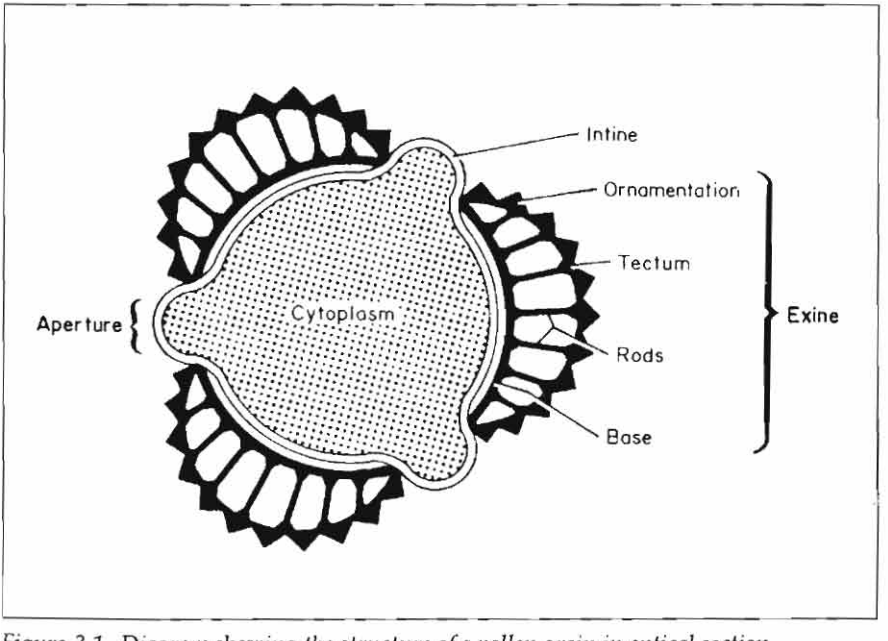

Figure 3.1 Diagram showing the structure of a pollen grain in optical section (Source: Sawyer 1981)

The inner layer or **intine** is thin, delicate, and very elastic. It is a semi-permeable membrane and does not stain. When the grain is seen in section, the intine can be recognised as a thin, clear line surrounding the cell contents. The outer layer or **exine** is thicker, more brittle, and often variously sculptured or provided with various modifications such as spines, outgrowths, or reticulation. The exine is made of an extremely durable material called **sporopollenin**.The exine is composed of four layers, as listed below.

i) ***Base***: This is a clear, uniform layer, the outer part of which can be stained to reveal a dark line in the optical section of the grain.
ii) ***Rods*** or ***Columns***: These are arranged radially from the base.
iii) ***Tectum***: This layer forms a roof over the rods. It may be an incomplete layer, leaving some of the rods free-standing.
iv) ***Ornamentation***: This is provided by a layer of spines, outgrowths, reticulation, and other processes on the tectum.

Usually, all the layers are not present. Those which are present show many modifications that are very useful in identifying a particular pollen grain.

Apertures are present on the surface of pollen grains. These are formed by the thinning or the absence of some of the layers of the exine. They are called **furrows** or **colpi**, if they are elongated and tapering towards the ends, or **pores** when they are round or oval (Figure 3.2). Pores and furrows often occur together, but at different levels of the exine. Apertures allow the grain to dry or to absorb water and thus to change into an

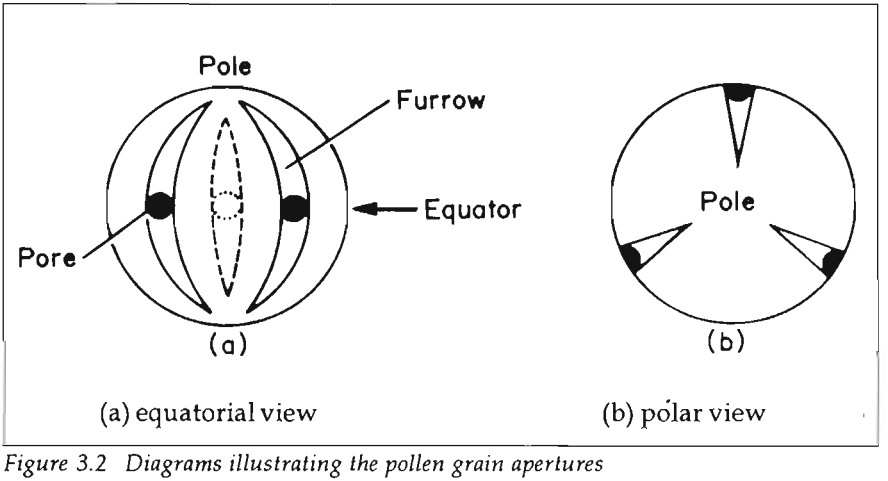

Figure 3.2 Diagrams illustrating the pollen grain apertures (Source: Sawyer 1981)

expanded state. They also provide an easy outlet for the pollen tube when the grain germinates. Therefore, they are also called germ pores or germinal furrows.

Pollen Grain Features

Each pollen grain has its own peculiar structural features which helps us recognise its origin. The features that can be used in its identification are as follows.

Size: The size (diameter) of the pollen grain is expressed in microns or micrometres (μm), where 1μm=1/1000mm. The maximum diameter of a pollen grain includes the spines or other ornamentation. Sawyer (1981) used five size classes: i) very small (<20 μm); ii) small (20-30 μm); iii) medium (30-50 μm); iv) large (50-100 μm); and v) very large (>100 μm). However, not all pollen grains are round. Many are either elliptical or triangular, even elongated. Vorwohl (1990) suggested that instead of defining size by the diameter, it should be defined by the length and breadth. He suggested nine classes of length and breadth (Table 3.1).

Shape: Shape is the outline; the optical section seen under the microscope. A pollen grain can be round, oval, elongated, triangular, semi-circular or boat-shaped, and irregular or multi-sided (Figure. 3.3). The shape plays a very minor role in the identification of pollen grains, because it varies depending upon the position in which the grain lies and upon the viewing aspect. Thus, one kind of pollen grain may show several different shapes on the microscope slide. However, Sawyer (1981) considered shape an important feature in pollen identification.

Apertures: As described earlier, pollen grains have apertures on the surfaces. The shape (type) and the numbers of apertures are important characteristics of a pollen grain and can be used in identification. Furrows containing pores are counted as one aperture. In compound pollen grains, the numbers refer to each single grain sub-unit. Nine classes of **aperture number** (as given in Table 3.1) can be used in pollen identification. Regarding **aperture type**, Vorwohl (1990) used the following five classes in pollen identification (Table 3.1).

i) ***Pore***: Equatorial, a more or less isodiametric (round) aperture, e.g., all the plants in the family *Poaceae* and many plants in the families *Cucurbitaceae* and *Chenopodiaceae*.

Table 3.1 ***Diagnostic Pollen Grain Features Used for Identification***

1. Length and breadth	1	<10 µm
	2	10-15 µm
	3	15-20 µm
	4	20-25 µm
	5	25-30 µm
	6	30-35 µm
	7	35-40 µm
	8	40-50 µm
	9	>50 µm
2. Aperture number	1	1
	2	2
	3	3
	4	4
	5	5
	6	6
	7	7
	8	>7
	9	absent or not clear
3. Aperture type	1	Pore
	2	Colpa
	3	Colporate
	4	Syncolpate
	5	Heterocolpate
4. Exine Sculpture	1	Psilate, Faveolate, Fossulate
	2	Scabrate, Verrucate, Gennuate
	3	Echinate
	4	Clavate, Bacculate
	5	Rugulate, Striate
	6	Reticulate
	7	Fenestrate
5. Aggregation	1	1
	2	2
	3	3
	4	4
	5	5
	6	6
	7	7
	8	>7

Source: Vorwohl (1990).

ii) ***Colpa (Furrow)***: Equatorial, longitudinal aperture, usually tapering towards the ends, e.g., *Allium* spp and *Lamium* spp.

iii) ***Colporate***: Pores with colpa, e.g., *Symphytum* spp. Most probably there are not many monocolporate forms.

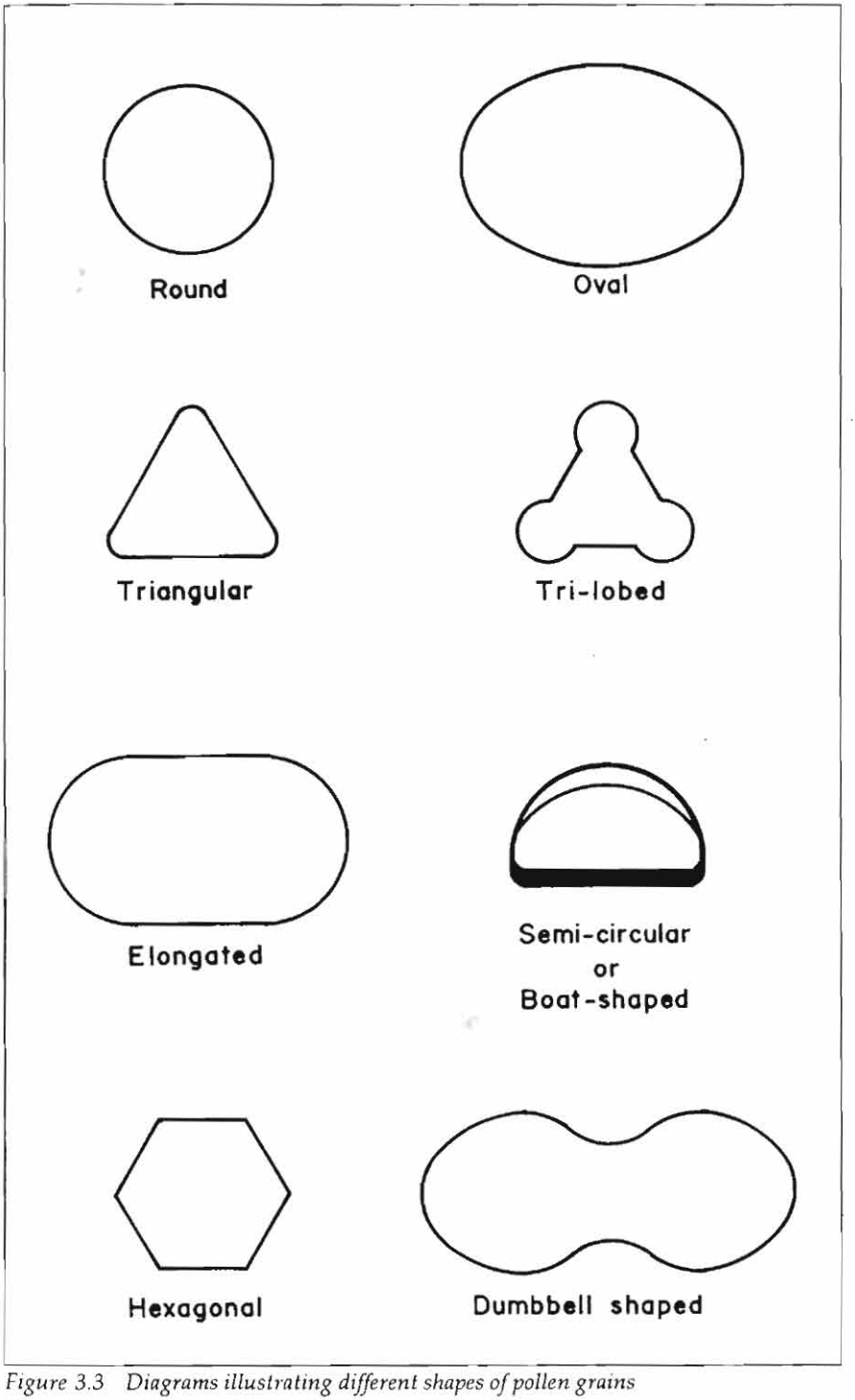

Figure 3.3 *Diagrams illustrating different shapes of pollen grains* (Source: Sawyer 1981)

iv) ***Syncolpate***: Apertures are formed as colpi or colporate where the ends of the colpi meet at the polefield, e.g., in some species of *Eucalyptus;* or colpi are like a band and meet with one another, e.g., *Mahonia* spp, *Berberis* spp, *Passiflora* spp, and *Crocus* spp.

v) ***Heterocolpate***: Both pores and colpi occur separately on the same pollen grain, e.g., *Lythrum* spp and *Phacelia* spp.

Sawyer (1981) recognised only four classes of aperture types to be important in pollen identification. These are: i) pores only; ii) furrows only; iii) united pores and furrows; and iv) irregularly occurring furrows.

Exine Sculpture (Exine Ornamentation) and Surface: The exine, the outermost coat of the pollen grain, is either smooth or variously ornamented with different outgrowths or projections, such as granules, various kinds of spines, and reticulum or window-like structures. The ornamentation of the exine has a major effect on the surface of pollen grain as viewed under the microscope.

The surface view of the pollen grain, as seen under the microscope, is regarded as the pollen surface. Since exine ornamentation is the outermost layer of the pollen, the surface view of this layer is considered to be the surface of the pollen grain. The surface of pollen grains, as seen under the microscope, can sometimes change with a change in focus. For example, the pollen grain of *Luffa cylindrica* may appear granular under one focus and reticulate under another.

Diagrams of different exine ornamentations and surface patterns are shown in Figures 3.4 and 3.5 respectively. According to Sawyer (1981), exine ornamentation and surface are two different features, both of which are important in identifying pollen. He identified nine classes of exine ornamentation (thin, medium with no rods, medium with spaced rods, medium or thick with coarse external rods, a layer of closed thin rods having long thin spines, large broad-based spines, small or very small spines or warts, and other projections) and five classes of surface (such as smooth or indefinite, granular, striated, net or pitted, and having isolated dots due to spines or other projections). However, Vorwohl (1990) considered exine ornamentation only as the most important feature in pollen identification and reported seven classes of exine sculpture (Table 3.1). These are described below.

i) ***Psilate, Faveolate and Fossulate***: When exines are smooth, they are known as psilate, e.g., *Betula* spp and *Pyrus* spp. Exine sculpture

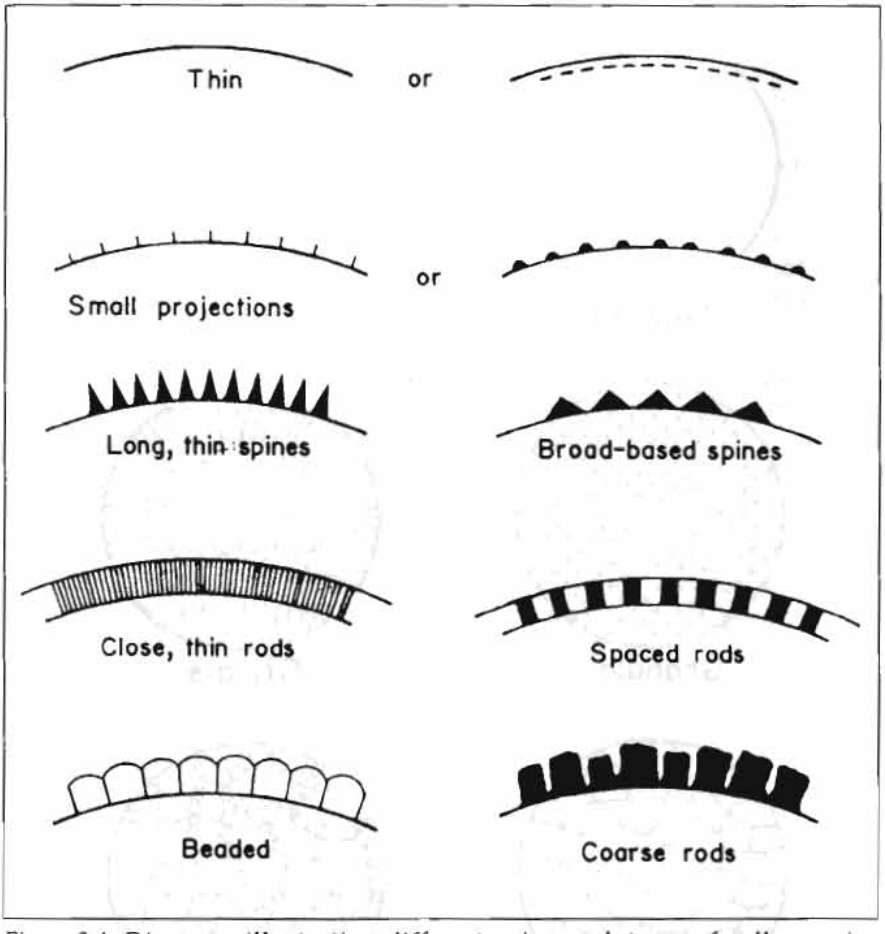

Figure 3.4 Diagrams illustrating different exine sculptures of pollen grains (Source: Sawyer 1981)

with little pits is reported as faveolate and is known as fossulate with relatively larger pits. In viewing the surface, the pollen grains with psilate, faveolate, and fossulate exine sculpture appear smooth or sometimes indefinite.

ii) ***Scabrate, Verrucate and Gennuate***: Exines with little warts and diameters of not more than one µm are scabrate, e.g., some species of *Quercus*. In verrucates, the diameter of the warts is more than one µm, e.g., members of the *Ranunculaceae* family, some *Asteraceae*, and *Nigella* spp. Gennuate is an exine sculpture with round warts which are narrower at the base, for example, *Ilex* spp. In optical sections, this kind of exine appears to have small projections. Pollen grains with this kind of exine sculpture appear granular in viewing the surface.

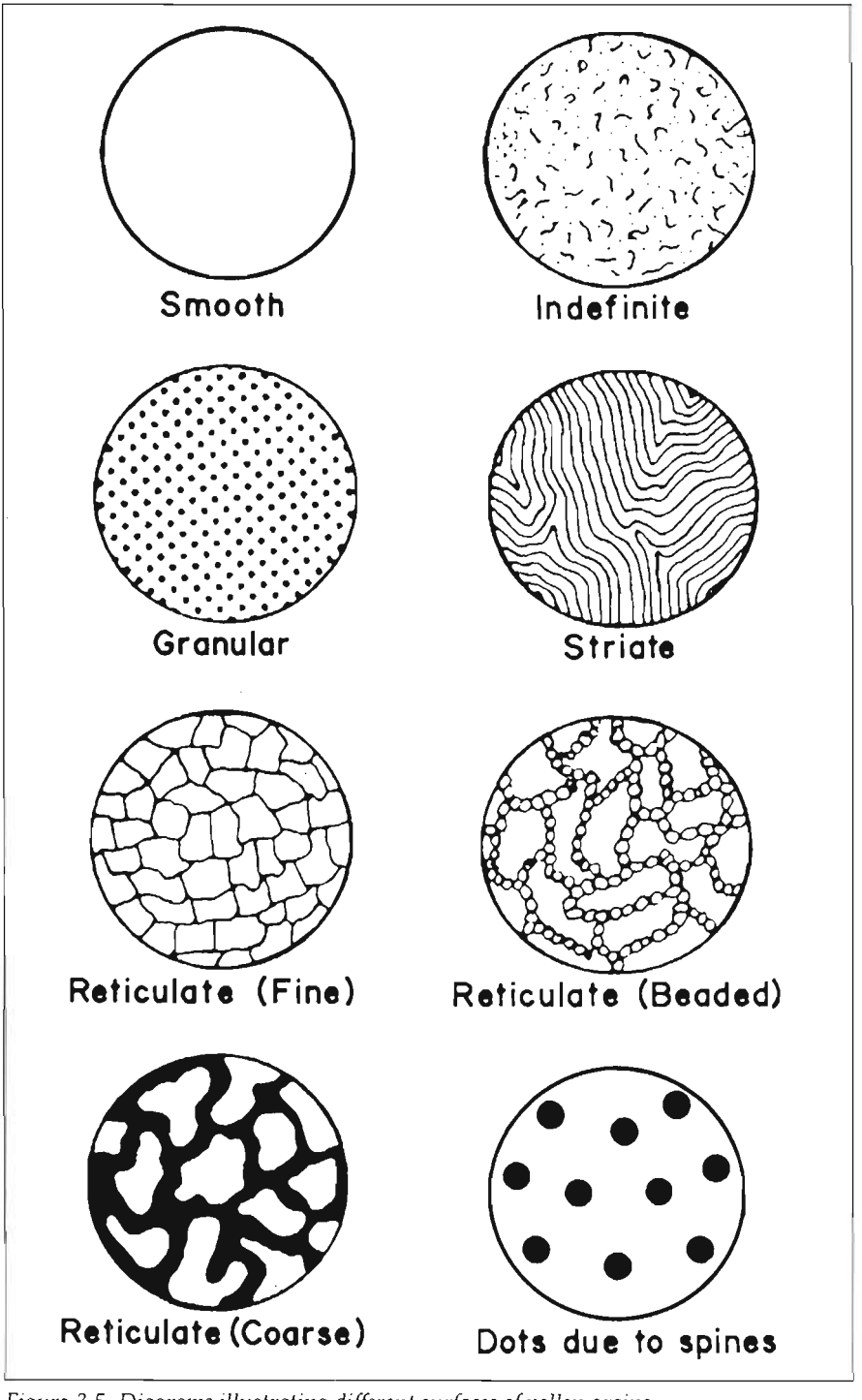

Figure 3.5 Diagrams illustrating different surfaces of pollen grains
(Source: Sawyer 1981)

iii) ***Echinate***: Exines provided with different kinds of spines, e.g., *Malvaceae, Cucurbitaceae, Campanulaceae,* and many *Asteraceae* are echinate. Pollen grains with echinate exine sculpture appear to have dots on the surface.

iv) ***Clavate and Bacculate***: Exines with rods which have thicker ends are clavate and those with stick-like rods are bacculate. Pollen grains with clavate or bacculate exine sculptures are very rare. On examining the surface, the pollen grains with this kind of exine sculpture appear granular (they have larger grains than scabrates, verrucates, and gennuates).

v) ***Rugulate and Striate***: Exine sculptures with striations, e.g., species of *Acer, Prunus, Datura,* and *Fragaria,* belong to these categories. In optical section, the exines appear to have close, thin rods. On examining the surface, the pollen grains also appear striated.

vi) ***Reticulate***: This term is used when the exine sculpture is reticulate or net-like. This category is divided into fine reticulate, or beaded, or coarse reticulate. Examples are *Lilium* spp, *Hedera helix,* and *Ligustrum* spp. In optical sections, reticulate exines appear to have either thin or coarse rods, or they appear to be beaded. Pollen grains with reticulate exine sculpture appear reticulate on the surface also.

vii) ***Fenestrate***: Exines in which there are window-like holes between the ribs of the exine belong to this category. Important examples include *Taraxacum officinale* and members of *Amaranthaceae*. On the surface, the pollen grains appear to have window-like holes.

Other Features: Pollen grains of some plant species have other diagnostic features. For example, the pollen grains of many plant species in the families *Ericaceae* and *Leguminosae* (sub-family, *Mimosoidae*) are polyads, i.e., compound. In *Ericaceae*, four grains are aggregated to form a tetrad, e.g., *Rhododendron* spp, whereas in the *Leguminosae,* eight, twelve, or sixteen pollen grains are aggregated to form a polyad, e.g., *Acacia* spp, *Albizia* spp, *Calliandra* spp, and *Mimosa* spp (Figure 3.6). Aggregation of pollen grains is one of the important features in identifying pollen grains.

Still other features important for identifying pollen include the presence of air sacs in the pollen grains of plant species belonging to the family *Pinaceae*, e.g., *Pinus* spp; thickened or projecting edges to apertures, e.g., *Polygonum* spp; a cap or streak on the apertures, e.g., *Chenopodium* spp and *Cucurbita* spp; granules or projections scattered on the apertures, e.g., *Convolvulus* spp; intine swollen below the apertures, e.g., *Hippophae;* intines thick or very thick, e.g., *Viola;* and cell contents granular, e.g., *Rumex* spp (Figure 3.6).

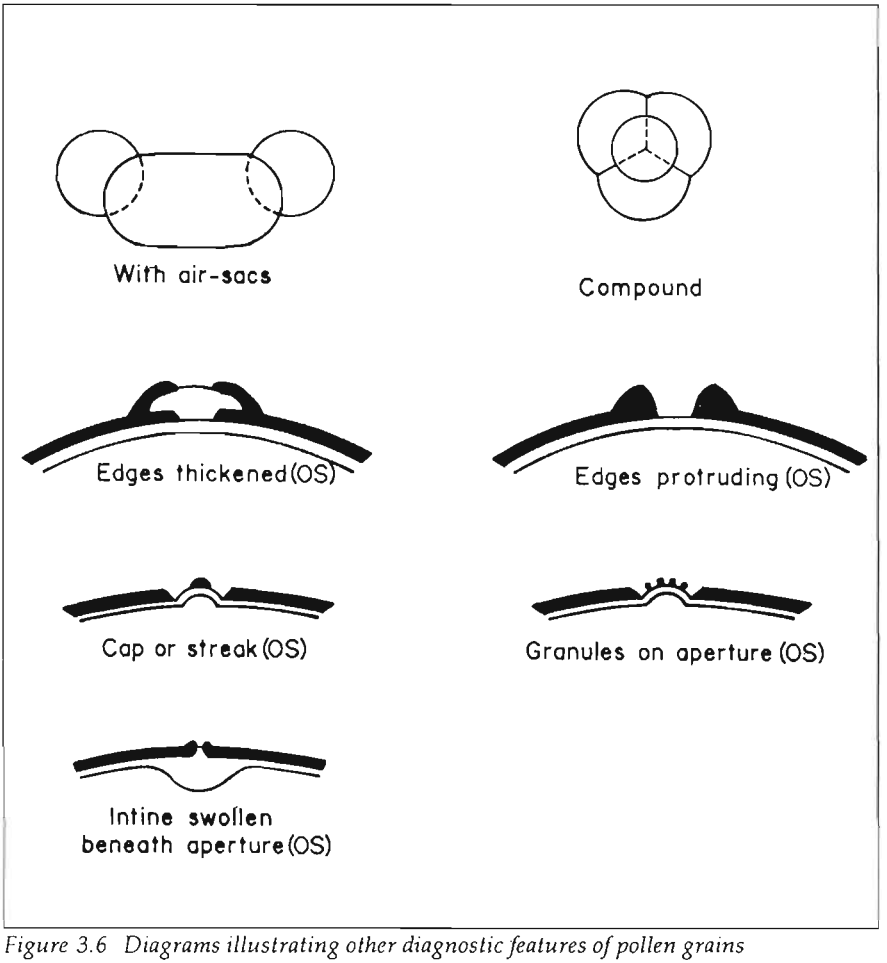

Figure 3.6 Diagrams illustrating other diagnostic features of pollen grains (Source: Sawyer 1981)

Colour: The colours of dry bulk pollen from the anthers or pollen loads of bees vary considerably and may sometimes provide a valuable clue to the identification of pollen (Sawyer 1981).

How to Examine Pollen Grains in Honey and Bee Pollen Load Samples

From whatever source the pollen is obtained, it must be mounted in a standard manner so that a uniform description and comparison can be made. The simplest and most informative presentation can be obtained from expanded and lightly-stained pollen grains. Pollen can be prepared either by the acetolysis method or without this method. The **acetolysis method** was introduced by Erdtman in 1934 as a way of preparing

pollen grains from organic deposits. It relies on the fact that the exine of most pollen grains is highly resistant to both strong acids and bases; so treating a sample thus would dissolve the extraneous materials while not affecting the pollen grain. This technique can be used with herbarium specimens, or with fresh samples in order to prepare reference collections. This method leaves the exine very clean, which makes the pollen grains well-suited for studying sculpturing by light microscopy or scanning electron microscopy.

Erdtman (1969), however, reported that the pollen grains of some taxa are often destroyed or badly damaged by acetolysis, e.g., the genus *Populus* (Salicaceae) and the families *Lauraceae, Ceratophylaceae, Juncaceae, Thruniaceae, Rapateaceae, Cannaceae, Musaceae, Zinziberaceae,* and *Zannichelliaceae*. Therefore, pollen from the plants of these families should be prepared **without acetolysis** (i.e., it should not be treated with the acetolysis mixture).

Whether prepared by acetolysis or without acetolysis, pollen is mounted in glycerine jelly. Erdtman (1960; 1969) developed this technique for use with samples prepared by acetolysis, but it also works well with untreated samples. Methods of preparing pollen, and glycerine jelly and mounting the pollen are described in the following text.

Preparing Reference Slides of Pollen

1. Acetolysis Method

Anthers from known flowers are mashed with a glass rod in a centrifuge tube containing glacial acetic acid, the excess acid is then decanted. Five to10ml of the acetolysis mixture is poured on to the mashed anthers and heated in a water bath at 70°C, stirred thoroughly with a glass rod (one separate glass rod is used for each tube throughout, to prevent mixing the pollen between samples). Acetolysis mixture is prepared by adding one part of concentrated sulphuric acid, drop by drop, to nine parts of acetic anhydride. A fresh mixture is prepared every time it is needed.

The contents are allowed to cool and then centrifuged at 2,500rpm for five minutes. The supernatant liquid and the large pieces of tissue are decanted. The pollen left in the centrifuge tube is washed in glacial acetic acid and centrifuged, and the supernatant liquid is decanted. Distilled water is then added to the pollen, which is thoroughly shaken

before it is recentrifuged. After centrifugation, the supernatant liquid is decanted. A few drops of a mixture of equal parts of glycerine and distilled water are added to the pollen and then allowed to stand for 10 minutes before the excess liquid is decanted. This sediment is transferred to the microscope slide and placed on a warm plate at 40°C so that the excess water evaporates. It is then mounted in glycerine jelly.

2. Glycerine Jelly Method

Pollen from the known plant is shaken on to a microscope slide, or the anthers are placed on a slide, and a drop of ether is added to disperse the pollen. Any visible particles that are larger than the pollen grains should be removed. Drops of ether are then carefully run over the pollen from a pipette. This will dissolve any oil in the pollen and carry it to one side where it can be wiped off or where the solution can be absorbed by the tissue. Then two drops, one of warmed, stained jelly and another of unstained jelly, are placed on the pollen by means of a glass rod. A cover slip is carefully positioned on top, one edge lowered first to avoid trapping air bubbles. The slide is left on a warm plate (40°C) for about ten minutes. The jelly should be just sufficient to fill the space under the cover slip.

Glycerine jelly is prepared by dissolving seven grammes of gelatine in 42ml of cold distilled water. 50ml of glycerine is added, warmed gently, and stirred until it is dissolved; 0.5 grammes of phenol is then added to prevent the growth of mould. To prepare the stained glycerine jelly, 0.1 grammes of basic fuchsin is dissolved in 10ml of alcohol (methylated spirit). This stain is then added drop-by-drop to the glycerine jelly until a clear pink colour is produced.

A few hours later, when the jelly has finally set, any surplus should be cleaned off with cold water. The cover slip is then sealed along the edges with clear nail varnish or paraffin wax. Thus treated, the slides will last for many years.

Extraction of Pollen from Honey

Pollen is extracted from honey by mixing 10 grammes of honey with 20ml of hot, distilled water. After a thorough mixing, the solution is placed in two centrifuge tubes and centrifuged at 2,500rpm for 10 minutes. The supernatant liquid is decanted, and both tubes are filled with water and recentrifuged for five minutes. The liquid is again decanted, and the sediment is transferred to a microscope slide using a

Pasteur pipette, spread over a suitable area, and dried. De-greasing is not necessary. The rest of the procedure, i.e., staining, covering, and sealing is the same as that described for reference slides.

In the absence of a centrifuge, the **sedimentation method** can be employed. Ten grammes of honey are diluted with at least 100ml of water. This mixture is allowed to stand overnight; the pollen will settle to the bottom.

In order to use the **acetolysis method**, five to10ml of acetolysis mixture is added to the sediment obtained after centrifugation. This is then placed in a water bath at 70°C for 10 minutes and centrifuged after incubation for five minutes. The supernatant liquid is decanted, and the centrifuge tube is filled with distilled water and a drop of strong detergent (teepol) added. It is again centrifuged for five minutes, and a drop of glycerine and water mixture (1:1) is added to the sediment. The rest of the procedure is the same as that described for preparing reference pollen by acetolysis.

Pollen Loads Carried by Bees

The pollen load is placed on a flat plate. A few drops of water are added and mixed gently, using a glass rod, to form a thin slurry. The slurry is transferred to a microscope slide to form a smear. The smear is dried on a warm plate and treated exactly as the pollen obtained from the flower.

How to Identify, Count and Record Pollen Grains

The pollen grains recovered from the honey and bee pollen load samples are examined microscopically and identified with the help of reference pollen slides made from identified plants; and/or by recording the data on pollen grain features (listed in Table 3.1) and comparing this data with those from the reference slides.

The latest technique in honey pollen identification is a computer-assisted reference pollen data bank which greatly facilitates the procedure. The data on pollen grain features of reference pollen slides as described in Table 3.1 are entered into the computer. Similar data of unidentified pollen grains are also entered into the computer. The computer then displays the name of the plant to which that particular pollen grain belongs. Thus, the plant source of that particular pollen grain is identified.

The number of pollen grains is counted by using a hemocytometer (Louveaux et al. 1978; Seethalakshmi 1980; Suryanarayana et al. 1981). The honey sample is considered to be rich, poor, or extremely poor in pollen if the number of pollen grains per 10 grammes of honey is above 100,000, 20,000-100,000, or below 20,000 respectively (Maurizio 1975).

Similarly, a honey sample having 45 per cent or more pollen grains of a single pollen type is considered to be **unifloral honey** and one having several types of pollen grains in considerable percentages is called **multifloral honey** (Iwama and Melhem 1979; Chaturvedi 1983). Based on the frequencies of pollen grains in the honey, four frequency classes are identified (Louveaux et al. 1978). These are i) **predominant pollen** (when it is more than 45% of the total pollen count); ii) **secondary pollen** (16-45%); iii) **important minor pollen** (3-15%); and iv) **minor pollen** (less than 3%).

To close the gap in research on Himalayan bee flora, the author carried out monthly surveys of blossoming trees, shrubs, and annuals and collected flowering plants to create, **a computer-assisted reference pollen data bank** of more than 1,000 plant species. The important species are given in the Annex to this chapter.