CHAPTER II.

Materials & Method
MATERIALS AND METHODS

The present investigation was carried out at the seven reserved forests of Cachar district for about five years. Laboratory work was done both in the Department of Biotechnology laboratory, Gauhati University, Guwahati - 14, and the Department of Life Science laboratory, Assam University, Silchar. Field trips were made on alternate weeks during the first year, once a month in the second year and bimonthly in the third year, so as to study the entire district throughout the season. During field trips, observation on habit, habitat, abundant ecological features and other macroscopic features and characters of the selected species were recorded. Locations of the plants that were in vegetative condition were carefully marked to relocate them in subsequent trips for collecting flowering specimens. Data were collected on the following lines:

1. Geographical feature of Cachar district:

   It is well known that the types of vegetation depends on climatic, edaphic and biotic factors. Thus rainfall, relative humidity, temperature data for the year 1996, 1997, 1998 were collected from the office of the Senior Meteorological Officer, Met Section, Air Force Station, Kumbirgram, Cachar. From these data three years monthly rainfall, temperature and relative humidity were calculated.

2. Selection of species:

   Selection of species for the present study was made on the basis of available reports, records and literature. Following five commercially important Orchids, well distributed in different parts of Cachar district, which deserve special protection to save them from
extinction (Varma and Sahni, 1976; Hegde and Thapliyal, 1992; Hegde, 1996) were selected.

(1) Aerides odorata, Lour.

(2) Acampe papillosa, Lindl.

(3) Cymbidium aloifolium, Swartz.

(4) Dendrobium aphyllum Roxb. &

(5) Papilionanthe teres. Lindl.

Botanical description of the selected species are given below:

(1) Aerides odorata, Lour.

Epiphytes. Stem long, branched, erect, monopodial and stout, leafy towards apex. Leaves sessile flat, coriaceous, base sheathing, distinct midrib, apex obliquely two lobed. Inflorescence lateral, pendulous, raceme spike. Flowers pedicillate, light pink coloured, densely arranged on spike, scented; sepals and petals subequal, spreading; Labellum 3-lobed, side lobes large, midlobes ovate, acute, spurred, spur short, curved; column shorter than the lip; rostellum short, bifid, pollinia two, globose. Fruit capsule. (Plate 2 & 3).
(2) *Acampe papillosa*, Lindl.
   (Syn. *Saccolabium papillosum*, Lindl.)
   Epiphytes. Stem erect, monopodial and stout, simple or branched, multinodal, gradually tapering upwards. Leaves sessile, varying in size, linear-oblong, apex obliquely 2-lobed, distinct midrib, coriaceous, leathary, curved, conduplicate, base slightly broader and sheathing. Inflorescence raceme, spike lateral. Flowers pedicillate with small triangular bract, generally yellow coloured with different reddish brown shades. Sepals subequal, oblong, subacute, spreading; petals narrower than the sepals, subspathulate. Labellum slightly larger than the sepals, adnate to the base of the column, its base with a cylindrical, slightly tapering spur half as long as the ovary and subparallel to it. Column short. Anther broadly conical, pollinia two, deeply bipartite, subovoid, caudicle, elongate. Fruit capsule, fusiform and ridged (Plate 4 & 5).

(3) *Cymbidium aloifolium*, Swartz.
   Epiphytes. Stem sympodial, pseudobulbous, short, sheathed, many-leafed. Leaves long varying in size, sessile, linear-oblong, fleshy, apex obliquely two-lobed, midrib distinct, base slightly broader and sheathing. Inflorescence raceme-spike, arises at the base of the stem, lateral, shorter than the leaves, pendulous, sheathed at the base; sheaths tubular, inflated, acute. Flower yellowish-purple, pedicelled, sepals and petals ob lanceolate, subacute, subequal, spreading, creamy yellow coloured with pinkish tinges.
Plate 4.
Plant habit: *Acampe papillosa*.
Plate 5.
Floral parts: *Acampe papillosa*.
1. Flower (Front view), 2. Flower (side view)
3. Bract, 4. Column with labellum,
at the base; sepals oblong-lanceolate, subequal, acute; petals slightly shorter and broader, oblong acute; Labellum sessile at the base of the column, erect slightly saccate with pink veins, three-lobed, side embracing the column, mid lobes ovate, acute with a brownish heart, reflexed; column erect, stout cleavate, footless, puberulous; Anther bithecous, papillate, pollinia two, deeply grooved, subglobose, sessile with truncate base. Fruit capsule, obovoid, ridged, large grayish green coloured, pendulous (Plate 6A & 7).

(4) *Dendrobium aphyllum*, Roxb.
(Syn. *D. amoenum*, Wall.)

Epiphytes, stem slender, monopodial, pendulous, slightly thickened at the nodes, multinodal, gradually tapering downwards, sheathed, branched, many leaved. Leaves sessile, oblong lanceolate, acute with distinct midrib. Inflorescence pendulous, raceme spike. Flowers two or three from very short bracteate peduncles springing from nodes of leafless stems, light purple coloured, pedicelled; Bracts triangular, membranous, persistent and brown coloured; Sepals subequal, oblong lanceolate acute; lateral sepals adnate to the foot of the column forming a sac or mentum. Petals larger than the sepals, subequal, oblong, obtuse. Labellum shortly clawed, lower half incurved, upper half spreading, margin entire, a small notch at the apex, pink coloured veins are present on the both sides of lower half of lip. Pollinia four, oblong, adnate. Fruit capsule. (Plate 6B & 8).
(5) *Papilionanthe teres*, Lindl.

Epiphytes. Stem long terete, sub-scandent, slender, branched, smooth, naked. Leaves fleshy terete, tapering to the sub-obtuse apex, smooth, slender, 2 to 4 flowered, peduncle extra-ascillary, slender, larger than the leaves, suberect, bearing a few short bracts. Flower bracteate, pedicelled, light pink coloured. Sepals subequal, dorsal sepal broadly elliptic, oblong, blunt smaller than the lateral sepals; lateral pair broadly obovate, falcate, obtuse, somewhat undulate. Petals subequal, subrotund; Labellum larger than the sepals and petals, adnate to the lateral sepals, lower half spreading and yellow coloured, upper half purple coloured with brown dots on both the lower and upper half, bilobed, small notch at the apex of two lobes; spur infundibuliform, puberulous inside. Column short with a long apical beak. Pollinia two, hard yellow in colour, broadly ovoid, bifid, ridges present at the ventral surface. Fruit capsule, large, pendulous. (Kataki, 1971; King and Pantling, 1898) (Plate 9 & 10).
Plate 9.
Plant habit: *Papilionanthe teres*.
Plate 10.
Floral parts: *Papilionanthe teres*.
1. Flower (Front view), 2. Flower (side view)
3. Bract, 4. Columnn with labellum,
Plate 11.
Photograph: A. *Aerides odorata* (wild).

"B. *Aerides odorata* (cultivated).
Plate 12.
Photograph: A. *Acampe papillosa* (wild).

B. *Acampe papillosa* (cultivated).
Plate 13.
Photograph: A. Cymbidium aloifolium (wild).

"B. Cymbidium aloifolium (cultivated)."
Plate 14.
Photograph: A. & B. Inflorescence of *Cymbidium aloifolium* (wild).
Plate 15.
Photograph: A. Dendrobium aphyllum (wild).
   B. Inflorescence of Dendrobium aphyllum.
Plate 16.
Photograph: A. & B. Inflorescence of Dendrobium aphyllum.
(under cultivation)
Plate 17.
Photograph: A. *Papilionanthe teres* (wild).

B. *Papilionanthe teres* (cultivated).
3. Frequency distribution studies of Selected Orchid Species:

The frequency distribution of selected orchid species in seven Reserved Forests (RF) of Cachar district were studied by following method as mentioned below:

Data for all the seven reserved forests were collected from D.F.O. office, Silchar, Cachar. For convenience of present study, the seven reserved forests (RF) viz., Upper Jiri RF, Lower Jiri RF, Sonai RF, Barak RF, North Cachar RF, Barail RF and Innerline RF (Part) were marked respectively as RF 1, RF 2, RF 3, RF 4, RF 5, RF 6 and RF 7. Each reserved forest was divided by drawing 10 horizontal and 10 vertical imaginary lines (Plates 18-24). Total number of divisions or sectors inside the boundary of each reserved forest was recorded. Then out of total number of sectors present inside the forest, 10 sectors were chosen at random. Frequency distribution of selected Orchids were studied from those 10 sectors for each forest. Same procedure were followed to study the frequency distribution for selected Orchid species in all the reserved forests.

In each sector of a reserved forest, frequency of selected Orchid species was scored and recorded. Then the frequency distribution of Orchid species per sq. km for each species were calculated. The distribution pattern in seven reserved forests were computed by dividing average frequency of species from 10 sector by the area of the sector. The area of the individual sector was calculated by dividing the area of each reserved forest by the number of sectors present in the reserved forest after drawing 10 vertical and 10 horizontal lines. Again from each sector 10 plants of an Orchid species having same growth (mature) were selected and tagged for taking observations. Ten observations obtained from each RFs were used for computing Mean and Variance and standard error.

Mean, variance and standard error were calculated on the basis of individual observation as follows:

\[
\text{Mean (} \bar{x} \text{)} = \frac{\Sigma x}{N}, \quad \text{Variance (} \delta^2 \text{)} = \left( \frac{\Sigma x^2}{N} \right) - (\bar{x})^2 \text{ and}
\]

Standard error (SE) of mean = \( \frac{\delta^2}{N} \).

where, 
\[
X = \text{individual observation}, N = \text{Total number of observations}, \text{and} \\
\delta^2 = \text{Variance.}
\]
MAP OF UPPER JIRI RESERVED FOREST.
Total Area = 62.56 sq.km.
Area of each sector = 1.08 sq.km. (approx).
Scale: 1 cm = 0.62 km.
MAP OF LOWER JIRI RESERVED FOREST.
Total Area = 36 sq.km.
Area of each sector = 1.08 sq.km. (approx).
Scale: 1 cm = 0.72 km.

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MAP OF SONAI RESERVED FOREST.
Total Area = 35.53 sq.km.
Area of each sector = 0.69 sq.km. (approx).
Scale: 1cm = 0.62 km.
Plate: 21.

MAP OF BARAK RESERVED FOREST.
Total Area = 202.01 sq.km.
Area of each sector = 3.482 sq.km. (approx).
Scale: 1cm = 1.2 km.
MAP OF NORTH CACHAR RESERVED FOREST.
Total Area = 252.38 sq.km.
Area of each sector = 4.137 sq.km. (approx).
Scale: 1cm = 1.2 km.
MAP OF BARAIL RESERVED FOREST.
Total Area = 73.02 sq.km.
Area of each sector = 1.1 sq.km. (approx).
Scale: 1 cm = 0.62 sq km.
Plate: 24.

MAP OF INNER LINE (Part) RESERVED FOREST.
Total Area = 559.2 sq.km.
Area of each sector = 9.47 sq.km. (approx).
Scale: 1cm = 15.02 sq km.
4. Phenological studies:
A detailed study of the five species of Cachar district of Assam was attempted in the present study to estimate the variation of different vegetative, floral and commercially important phenophases of the selected species. For the present study, phenological observations were carried out both in the field trips (viz., vegetative and floral characters etc) and in the laboratory (viz., stomatal character, vase life studies etc.). A detailed statistical analysis of the following 18 phenological characters were recorded.

4.1. Estimation of Genetic Variation in vegetative and floral characters of selected species:
(1) Leaf length: The leaf length was measured (in cm) from base to the tip of the leaf by taking mature leaf from an individual plant.
(2) Leaf breadth: The leaf breadth was measured (in cm) by taking maximum expanded portion of the same leaf.
(3) Bract length: Length of the bract was measured (in cm) from the base to the tip.
(4) Bract breadth: Breadth of the bract was measured (in cm) at maximum width of the same bract.
(5) Dorsal sepal length: Length of the dorsal sepal was measured (in cm) from the base to the tip.
(6) Dorsal sepal breadth: Breadth of the dorsal sepal was measured (in cm) at maximum width.
(7) Ventral sepal length: Length of the ventral sepal was measured (in cm) from the base to the tip.
(8) Ventral sepal breadth: Breadth of the ventral sepal was measured (in cm) at maximum width.
(9) Lip length: Length of the lip was measured (in cm) by taking the length from the base to the tip of the labellum.
(10) **Lip breadth:** Maximum breadth of the labellum was taken as the measurement of the pouch (in cm).

(11) **Lateral petal length:** Length of the lateral petal was measured (in cm) from the base to the tip.

(12) **Lateral petal breadth:** Breadth of the lateral petal was measured (in cm) at maximum width.

4.2. **Estimation of Genetic variation in commercially important characters of selected orchids:**

(1) **No. of flowers:** Number of flowers were scored from full bloomed inflorescence of individual plant.

(2) **Flower size:** The size of fully mature flower from inflorescence was measured (in cm) by taking the maximum spread out of the lateral petals.

(3) **Longevity of flowers:** Longevity of flowers was measured (in days) from the full bloom condition of the flower to the dehiscence of the petals.

(4) **Shelf life (Test of vase life):** Test of vase life viz., shelf life including biochemical analysis and dry storage studies were carried out for each of the selected five species. Data were recorded from 10 inflorescence (approximately of same size) of 10 individual plants from each of the 10 sectors of the seven reserved forests. (Thangaraj et al., 1984; Accati & Jona, 1989).

The experiment for shelf life data was conducted with the selected five species during the flowering season of respective species. The inflorescence per replication were cut along with peduncle during marketable stage and kept in test tube with 25 ml distilled water. Then the shelf life viz. the days taken for the flowers of inflorescence to show petal necrosis were recorded. (Burdett, 1970; Meetem, 1978 & 1979; Ashwath, 1994).

After completion of the floral shelf life the exudate was taken out for Biochemical analysis.
**Biochemical analysis:** Biochemical analysis viz., the estimation of reducing sugar and estimation of total phenol were done in the inflorescence of each of the five selected species. The experiment was conducted during flowering season of five selected orchid species to study the metabolites released by the stem in the vase. One inflorescence from each species was harvested at marketable stage and kept in test tube containing 25 ml of distilled water. After completion of floral shelf life the exudate was taken out for biochemical analysis viz., estimation of reducing sugar and phenol.

**i) Estimation of reducing sugar:**

Reducing sugar was estimated by following the method as described by Nelson (1944). In this method, amount of reducing sugar was determined by cuprous oxide which was produced by heating sugar with alkaline copper and reacting it with arsenu-molybdate reagent.

**Preparation of Reagents:**

**Copper reagent A:** 25 g. anhydrous sodium carbonate, 25 g. Sodium Potassium tartrate (Rochelle's salt), 2 g. Sodium bicarbonate and 200 g. anhydrous Sodium sulphate were dissolved in 800 ml. of double distilled water and the volume made to 1000 ml.

**Copper reagent B:** 15 g. Copper sulphate in 100 ml. distilled water and 2 drops of conc. H$_2$SO$_4$ was added to it.

**Arsenomolybdate colour reagent:** 25 g. Ammonium molybdate was dissolved in 450 ml. distilled water and then 21 ml. of conc. H$_2$SO$_4$ was added and mixed. Later the solution where freshly prepared solution of 3 g. Sodium arsenate and 25 ml. distilled water was added to the above solution. The reagent was stored in glass stopped brown bottle.

**Estimation procedure:** 1 ml. of exudate was taken in a narrow graduated test tube and 1 ml. of mixture of 25 parts of reagent A and reagent B was added to it. The tube was placed in boiling water bath for 20 minutes and then cooled in running water.
Later 1ml of Arsenomolybdate reagent was added and the mixture was diluted to 25ml. After 15 minutes the absorbance was taken at 500 nm. The blank was maintained by using 1ml of distilled water instead of exudate.

The amount of reducing sugar was determined using a standard curve prepared from glucose and expressed in glucose equivalents.

ii) Estimation of phenol:

Phenol was estimated by Folin-Ciocalteu method (Mahadevan and Sridhar, 1986). This method is based on reaction between phenols and an oxidizing agent phosphomolybdate, which results in the blue colour and intensity of colour was measured by Spectrophotometer.

**Estimation procedure:** 1ml of exudate was pipetted to narrow graduated test tube to which 1ml of Folin-ciocalteu reagent was added. Then 2ml of 20% Sodium carbonate (20 gm. of Sodium carbonate in 100ml of distilled water) was added and the tube was shaken and heated in boiling water bath for one minute. Later the tube was cooled in running water and blue coloured solution was made upto 25ml by adding distilled water. The absorbance was read in spectrophotometer at 650nm. The amount of phenol was estimated by using the standard curve made from different concentration of catechol.

(5) **Bend recovery (Dry storage studies):** Bend recovery or Dry storage studies were conducted during flowering season of the respective species. The inflorescence per replication were taken at marketable stage and dry stored for 24 hours horizontally at room temperature in carton boxes to simulate the transportation conditions.

A common point to all the species were marked from the base of the involucre. After dry storage of 24 hours the basal end of the stem was held in vertical position (at marked point) and the curvature of the bend was measured with protector. The initial angle (in degree) was recorded immediately after harvest at the same point. The dry inflorescence were subsequently put in test tubes containing 25 ml of distilled
water and allowed to restore it's turgor. After 24 hours in water the recovery of the stem bend was measured at marked point to find out the difference (in degrees) it recovered (De Jong, 1978 & 1985) (Plates No.25 – 29).

(6). Stomatal index (Study of stomatal character): This character was studied from abaxial surface of the leaf with the help of light microscope (Stace, 1965 & 1966; Singh, 1981; Rasmussen, 1981, 1981a, 1981b). For light microscopic studies epidermal peels were used. The leaf to be peeled was detached, placed on glass plate and cut with a sharp scalpel into lamina strips of manageable width (5mm). Lamina strips so obtained were floated on water to avoid desiccation. The strip was then mounted on a thin layer of water on the slide. Techniques for obtaining epidermal strips were followed as discussed by Metcalfe (1960); Meidner and Mansfield (1968); Weyer and Travis (1981).

These peels were stained in acetocarmine, rinsed and mounted in glycerine for temporary mounts and observed under a light microscope (Van Cotthem, 1970). Camera lucida drawings of typical stomata from each of the five species were drawn at 10X45 magnification (Plate 30).

Stomatal index was calculated by using the formula put forwarded by Robert (1980).

Stomatal index (SI) = \( S / (E + S) \times 100 \).

where,

\[ S = \text{No. of stomata/cm}^2. \]

\[ E = \text{No. of epidermal cell/cm}^2. \]

At first, the area of microscopic field was determined at 10X45 mg. as below:

\[ \text{Area} = \frac{1}{4} \pi X d^2 \text{cm}^2 \]

(where, \( d = 92 \text{ occ. Divs.} \times 0.0044 = 0.040\text{cm} \))

\[ = \frac{1}{4} \times 3.14 \times (0.040)^2 = 0.001256 \text{ cm}^2. \]
Then the number of stomata and epidermal cells per cm$^2$ was computed by counting the number of stomata and epidermal cells within the microscopic field in epidermal peeling and dividing it by 0.001256 cm$^2$.

4.3. Statistical Analysis

For all the 18 characters mean values and variance were calculated using the following formulae (Panse & Sukhatme, 1967):

\[
\text{Mean} = \frac{\sum fx}{N}
\]

and the variance = \(\frac{\sum f d^2}{N}\)

where,

\(f = \) Frequency, \(x = \) Class value,

\(d = \) deviation, and \(N = \) Number of observation.

Further for analysis of variation (ANOVA), mean values from each sector consisting of 10 observations were used for computation. The first comparison for phenological characters were made among the 7 reserved forests and secondly among 10 sectors. The model may be written as-

\[
Y_{ij} = \mu + b_i + l_j + L_i
\]

where,

\(i = \) number of RF or replications 1,2,3,............(\(r = 7\))

\(j = \) number of sectors 1,2,3,..........................(\(s = 10\))

(Each line consists of 10 observations).

\(F\) - test was done between mean square of RF with (7 - 1) = 6 degree of freedom. The sectors (s) were compared to find whether the plants selected within the sector differs significantly from each other or not with (10 -1) = 9 degrees of freedom. From the ANOVA table using mean squares different genetic parameters like phenotypic, genotypic, environmental variance and heritability (in broad sense) were calculated (Mahalanobis, 1936).
The following genetic parameters were estimated for all the phenological (or quantitative traits) characters:

(a) **Genotypic variance** ($\delta^2 g$): Genotypic variance was calculated according to Burton (1952),

$$\delta^2 g = \frac{(MSe - MSg)}{r}.$$ 

(b) **Environmental variance** ($\delta^2 e$): Environmental variance was calculated by using the formula:

$$\delta^2 e = \frac{MSe}{r}.$$ 

(c) **Phenotypic variance** ($\delta^2 ph$): Phenotypic variance of each character was calculated by using the formula:

$$\delta^2 ph = \delta^2 g + \delta^2 e.$$ 

(d) **Heritability** ($h^2 b$): Heritability in broad sense is the ratio of genotypic variance to the phenotypic variance and expressed as percentage. It was calculated by using the formula as suggested by Johnson et al. (1955).

$$h^2 b = \frac{\delta^2 g}{\delta^2 ph} \times 100.$$ 

where,

- $MSg = \text{Mean square due to genotype,}$
- $MSe = \text{Mean square due to environment and}$
- $r = \text{Number of replications.}$

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Source of variation (r)</th>
<th>Degrees of freedom</th>
<th>Mean Square</th>
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<td>1.</td>
<td>Reserved forests (r)</td>
<td>(7 - 1) = 6</td>
<td>MSr.</td>
</tr>
<tr>
<td>2.</td>
<td>Sector (s)</td>
<td>(10 - 1) = 9</td>
<td>MSG.</td>
</tr>
<tr>
<td>3.</td>
<td>Error (e)</td>
<td>{ (r x g -1) - (6 + 9) }</td>
<td>MSe.</td>
</tr>
</tbody>
</table>
5. Ethnobotanical studies:

Ethnobotanical studies were carried out for the selected five species in different parts of Cachar district. To get the information on the medicinal utility of the Orchids personal interview with village wisepersons, group discussion and assistance of local informants were used.