MATERIALS AND METHODS

The investigations in this thesis have been carried out in two parts. The first one was the field work for collecting the material and the second one was the laboratory work.

1. Collection of Specimens

The material for the present cytogenetic investigations has been provided by the bugs belonging to the family Pentatomidae. The adults range in size from very small to rather bigger ones. They are characterized by the possession of stink glands and a prominent scutellum. Most of them are phytophagus, feeding on both cultivated and wild plants while few species are predators which mostly feed on lepidopteran larvae and are important agents of biological control (Sheryl et al., 2002). Pentatomidae comprises eight subfamilies viz., Pentatominae, Asopinae, Podopinae, Discocephalinae, Edessinae, Phyllocephalinae Serbaninae, Cyrtocorinae (Schuh and Slater, 1995). Collection attempts were made all the year around. However, results were obtained from those bugs collected during the period from April to October as the bugs are sexually mature at this period. As many as twenty three species referable to three subfamilies (Pentatominae, Asopinae and Podopinae) and nineteen genera were collected from different localities of Punjab, Himachal Pradesh and Uttrakhand.

Out of the 4112 described species of Pentatomidae, 2771 species belong to the subfamily Pentatominae (Schuh and Slater, 1995). Pentatominae is a diverse subfamily of phytophagus bugs and were collected from different plant hosts (both cultivated and wild). Nineteen species of Pentatominae were collected in the present study. Most of them were collected during day time by hand picking. Small-sized bugs of Aeliomorpha were collected with the help of collection net from weeds and grasses. Few of the species, mainly of Eysarcoris, were collected during night time.
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using light trap.

The Asopinae comprises all predatory bugs of the family Pentatomidae. They are moderate to large size ranging in length from 7 to 25 mm, most with long and spiny prenatal angles. All the three species were collected by hand picking. In most of the cases, they were collected while feeding on larvae.

Podopinae is a cosmopolitan phytophagus subfamily. Only one species was collected by using light trap.

Collection localities, period of collection, host plants, if any, mode of collection and techniques used are shown in Table-1.

2. Laboratory Work

2.1. Identification

A few specimens of each collected species were killed using ethyl-acetate and were stretched, pinned and dried. The pinned specimens were mounted on a thermocol sheet placed in a wooden box provided with naphthalene balls. Information about localities of collection, collection date and host plant, if any, of each species were labeled. Specimens were identified in the department with the help of relevant literature and through comparison with reference collections lying in FRI museum, Dehradun. Some of the species were identified by Prof. (Dr.) Harbhajan Kaur by comparing with specimens lying in the Natural History Museum, London.

2.2. Preparation of Slides

Live adult male specimens were dissected in 0.67% saline solution and the gonads were extracted. The testes are elongated (in most Pentatominae species investigated) or round (in Asopinae species) and always colored as red, orange or yellow or rarely white (in Podops inuncta). The testes were fixed in freshly prepared Carnoy’s fixative (3 absolute alcohol: 1 glacial acetic acid) for 20 minutes followed
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by a second change of fresh Carnoy’s fixative for another 20 minutes. The fixed material was tapped on slides with the help of forceps and then the slides were air dried. The prepared slides were kept in refrigerator and were used for different parameters as and when required. The slides were processed differently for various methods of chromosome preparation as given below:

2.3. Conventional Staining

Air-dried slides were stained with carbol-fuchsin for two to three hours according to the methodology suggested by Carr and Walker (1961), followed by differentiation in N-butyl alcohol for 10-15 minutes. The slides were allowed to air dry and were finally mounted in DPX.

Preparation of Stain

A. Stock Solution A:

- Basic Fuchsin = 3 g
- 70% Ethanol = 100 ml

B. Stock solution B:

- Stock solution A = 10 ml
- 5% phenol in Distilled Water = 90 ml

C. Working solution:

- Stock solution B = 95 ml
- Glacial acetic acid = 12 ml
- 40% Formaldehyde = 12 ml

2.4. C-banding

To study constitutive heterochromatic regions, the C-banding technique suggested by Sumner (1972) was used with slight modifications as
follows:

Air-dried slides (two weeks old) were treated with 0.2N HCl for one hour at room temperature and were then washed with deionized water to remove acid completely. Then the slides were kept in freshly prepared aqueous solution of Ba (OH)$_2$ at 70ºC for 5-7 minutes. After rinsing in deionized water by giving several changes, the slides were incubated for 1 hour in 2x SSC. Then slides were rinsed briefly with deionized water followed by staining in 5% Giemsa for 10 minutes and finally the slides were rinsed in water, blotted, allowed to dry, soaked in Xylene and mounted in DPX.

**Preparation of Solutions**

A. 0.2 N HCl

- Concentrated hydrochloric acid = 2 ml
- Distilled water = 98 ml

B. Barium hydroxide solution

- Barium hydroxide = 5 g
- Distilled water = 100 ml

C. 2 x SSC

- Sodium citrate = 0.882 g
- Sodium chloride = 1.755 g
- Distilled water = 100 ml

D. Sorrensen's buffer

- Stock solution A
  - Disodium hydrogen phosphate = 1.876 g
  - Distilled water = 100 ml
- Stock Solution B
  - Potassium dihydrogen phosphate = 0.907 g
  - Distilled water = 100 ml

- Working solution (pH 6.8)
  - Stock solution = 50.8 ml
  - Stock solution B = 49.2 ml

E. Giemsa Stain

- Giemsa stock
  - Giemsa = 0.380 g
  - Glycerol = 25 ml
  - Methanol = 25 ml

- Working Giemsa Stain
  - Giemsa stock = 10 ml
  - Sorrensen's buffer (pH 6.8) = 90 ml

2.5. Fluorescent banding

Sequence-specific fluorochromes 4’-6-Diamidino-2-Phenylindole (DAPI) and Chromomycin A₃ (CMA₃) were used to investigate the distribution of AT and GC rich regions of DNA respectively according to the methodology suggested by Schweizer (1976) with slight modifications as follows:

The refrigerated slides were brought to the room temperature and then were stained with Methyl-Green solution for 20 minutes. Then the slides were treated with DAPI solution for 15 minutes, rinsed sequentially with distilled water, McIlvaine buffer and distilled water, and were air dried. Afterwards, one drop of CMA₃ solution was poured on each slide which was covered with a cover slip and was kept for half an hour at room temperature. The slides were then rinsed sequentially with distilled
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water, McIlvaine buffer and distilled water, and were air dried. Finally the slides were mounted in a solution consisting of 9 ml of glycerol mixed with 1 ml of McIlvaine buffer pH 6.8 containing 10 mM MgCl$_2$. Slides were kept in incubator at 37$^0$ C for 72 hours before being examined under the microscope.

Preparation of Solutions

A. McIlvaine buffer Stock

- Solution A
  - Disodium hydrogen phosphate = 3.5 g
  - Distilled water = 125 ml

- Solution B
  - Citric acid = 2.4 g
  - Distilled water = 125 ml

B. Working McIlvaine buffer (pH 6.8)

- Solution A = 90 ml
- Solution B = 10 ml

C. 10 mM Mg Cl$_2$

- Magnesium chloride = 5.07 g
- Distilled water = 25 ml

D. Methyl Green

- Methyl Green = 1 g
- Distilled water = 99 ml distilled water

E. DAPI

- DAPI = 4 µg
- McIlvaine buffer = 1 ml

F. CMA$_3$
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- CMA_3 = 0.4 mg
- Distilled water = 1 ml

2.6. Silver nitrate (AgNO_3) staining

To study the localization of Nucleolar Organizer Regions (NORs), silver nitrate staining was used. The methodology as suggested by Howell and Black (1980) was employed on air-dried slides as follows:

Two drops of colloidal developer and four drops of aqueous silver nitrate were dropped onto the surface of air-dried slides containing chromosomal preparations. The solutions were mixed and covered with cover glass. The slides were placed onto the surface of a slide warmer stabilized at 70°C and were removed from the slide warmer the moment the solution turned golden brown. The slides were then washed under running deionised water, blot dried and mounted in DPX.

Preparation of Solutions

A. Colloidal Developer

- Gelatine powder = 2 g
- Deionized water = 100 ml
- Formic acid = 1 ml

B. Aqueous silver nitrate solution

- Silver Nitrate = 4 g
- Deionized water = 8 ml

2.7. Study of Slides:

Prepared slides were scanned under the microscope. Initial scanning was done under 40X objective and the readings of selected stages were noted down. Already studied stages were re-observed under the immersion oil (100X) to study the details of chromosomal behavior during division and the localization of bands in differential
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staining. Well spread stages were photographed under the microscope (Nikon-Optiphot-2) equipped with digital camera. Slides stained with fluorochromes were studied and photographed under Nikon fluorescent microscope using UV filter for DAPI and BV filter for CMA3.
### Table 1: Details of collected species and parameters studied

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>No</th>
<th>Species</th>
<th>Area of collection</th>
<th>Period of collection</th>
<th>Host if any</th>
<th>Mode of collection</th>
<th>Techniques used</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pentatominae</strong></td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td></td>
<td>1</td>
<td><em>Aeliomorpha sheanensis</em></td>
<td>Hoshiarpur, Patiala</td>
<td>April</td>
<td><em>Cortaderia jubata</em></td>
<td>Hand picking, net</td>
<td>√</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td><em>Apodiphus pilipes</em></td>
<td>Ferozepur, Patiala</td>
<td>August-September</td>
<td>-</td>
<td>Hand picking</td>
<td>√</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td><em>Bagrada picta</em></td>
<td>Pathankot, Patiala</td>
<td>May-June</td>
<td><em>Brassica oleacea, Hibiscus esculentus</em></td>
<td>Hand picking</td>
<td>√</td>
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<tr>
<td></td>
<td>4</td>
<td><em>Carbula scutellata</em></td>
<td>Ferozepur</td>
<td>August-September</td>
<td>-</td>
<td>Hand picking</td>
<td>√</td>
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<td></td>
<td>5</td>
<td><em>Dolycoris baccarum</em></td>
<td>Pathankot, Ropar, Patiala</td>
<td>April-October</td>
<td><em>Parthenium hysterophorus</em></td>
<td>Hand picking</td>
<td>√</td>
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<tr>
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<td>6</td>
<td><em>Erthesina fullo</em></td>
<td>Amritsar</td>
<td>May-June</td>
<td><em>Fragaria virginiana</em></td>
<td>Hand picking</td>
<td>√</td>
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<td></td>
<td>7</td>
<td><em>Eysarcoris guttiger</em></td>
<td>Solan, Hoshiarpur</td>
<td>July - September</td>
<td><em>Cortaderia jubata</em></td>
<td>Hand picking, light trap</td>
<td>√</td>
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<tr>
<td></td>
<td>8</td>
<td><em>Eysarcoris inconspicuous</em></td>
<td>Pathankot, Patiala</td>
<td>September-October</td>
<td>-</td>
<td>Light trap, net</td>
<td>√</td>
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<tr>
<td></td>
<td>9</td>
<td><em>Eysarcoris rosaceus</em></td>
<td>Pathankot, Patiala</td>
<td>August-September</td>
<td>-</td>
<td>Hand picking</td>
<td>√</td>
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<td></td>
<td>10</td>
<td><em>Eurydema pulchrum</em></td>
<td>Pathankot, Patiala</td>
<td>July-September</td>
<td><em>Brassica oleacea, Hibiscus esculentus</em></td>
<td>Hand picking</td>
<td>√</td>
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<td>11</td>
<td><em>Halys seregera</em></td>
<td>Pathankot</td>
<td>August-September</td>
<td><em>Ficus cariaca</em></td>
<td>Hand picking</td>
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<tr>
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<td>12</td>
<td><em>Halys sulcata</em></td>
<td>Patiala, Pathankot</td>
<td>July-September</td>
<td><em>Ficus cariaca</em></td>
<td>Hand picking</td>
<td>x</td>
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<tr>
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<td>13</td>
<td><em>Halyomorpha murrea</em></td>
<td>Patiala</td>
<td>June</td>
<td>-</td>
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<td>14</td>
<td><em>Nezara graminea</em></td>
<td>Ferozepur, Patiala</td>
<td>August-September</td>
<td>-</td>
<td>Hand picking, light trap</td>
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<tr>
<td></td>
<td>15</td>
<td><em>Nezara viridula</em></td>
<td>Ferozepur, Ropar, Patiala</td>
<td>June-September</td>
<td>-</td>
<td>Hand picking</td>
<td>√</td>
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<tr>
<td></td>
<td>16</td>
<td><em>Piezodorus rubrofasciatus</em></td>
<td>Ropar, Patiala</td>
<td>April-July</td>
<td>-</td>
<td>Hand picking</td>
<td>√</td>
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<tr>
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<td>17</td>
<td><em>Plautia fimbriata</em></td>
<td>Ropar</td>
<td>April</td>
<td>-</td>
<td>Hand picking</td>
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<tr>
<td></td>
<td>18</td>
<td><em>Priassus exemptus</em></td>
<td>Bilaspur</td>
<td>August</td>
<td>-</td>
<td>Hand picking</td>
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<tr>
<td></td>
<td>19</td>
<td><em>Tropicoris punctipes</em></td>
<td>Bilaspur</td>
<td>August</td>
<td>-</td>
<td>Hand picking</td>
<td>√</td>
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<tr>
<td><strong>Asopinae</strong></td>
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<td>20</td>
<td><em>Andrallus spinidens</em></td>
<td>Patiala, Pathankot</td>
<td>July-September</td>
<td><em>Parthenium hysterophorus</em></td>
<td>Hand picking</td>
<td>√</td>
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<tr>
<td></td>
<td>21</td>
<td><em>Canthecona furcellata</em></td>
<td>Patiala, Pathankot</td>
<td>July-September</td>
<td><em>Parthenium hysterophorus</em></td>
<td>Hand picking</td>
<td>√</td>
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<tr>
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<td>22</td>
<td><em>Perillus bioculatus</em></td>
<td>Patiala, Ferozepur</td>
<td>April-October</td>
<td><em>Parthenium hysterophorus</em></td>
<td>Hand picking</td>
<td>√</td>
</tr>
<tr>
<td><strong>Podopinae</strong></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>23</td>
<td><em>Podops immacula</em></td>
<td>Patiala</td>
<td>July-September</td>
<td>-</td>
<td>Hand picking, light trap</td>
<td>√</td>
</tr>
</tbody>
</table>

N= Normal, C= C-banding, F= Fluorescent banding, S= Silver nitrate banding