MATERIALS AND METHODS

Santhosh S. “Investigation on the alpha taxonomy of bethylidae (hymenoptera: chrysidoidae) of southern western ghats” Thesis. Department of Zoology, University of Calicut, 2010
3.1 Study Area - Southern Western Ghats

The Western Ghats is a mountain range that runs approximately 1600 km along the western edge of the Deccan Plateau, India and separates the plateau from a narrow coastal plain along the Arabian Sea. It is home to four tropical and subtropical moist broadleaf forest ecoregions – lowland equatorial evergreen rain forests or tropical rainforests, moist deciduous and semi-evergreen seasonal forests, montane rainforests and freshwater swamp forests. The northern portion of the Ghats is generally drier than the southern parts. The southern ecoregions are generally wetter and more species-rich. Southern Western Ghats (Map.1) located between 8° N and 14° N latitude and 74° 40' E and 78° E falls within the biodiversity hotspot, Western Ghats and Sri Lanka.

Specimens were collected from the Southern Western Ghats and the adjoining regions falling in the three states viz. Kerala, Karnataka and Tamil Nadu. The survey was carried out in Kerala, Southern Karnataka and North Western Tamil Nadu, with a special emphasis to the wet western slope of the mountain range falling in Kerala. This narrow strip of land extends north-south between Lakshadweep Sea and the Western Peninsular Indian Hill range, the Western Ghats. The state is about 850 kms. in length north-south and its average width varies from 32 kms to over 120 kms with an area of 38,863 sq. kms. The hill range demarcating this strip of land from the rest of the peninsula, rises abruptly to an average elevation of 1500m above sea level with many sections ascending to 2000 or even 2500m. More than half the geographical area of the state is above 200m. in elevation with a complex topography of rocky ridges, high plateau and narrow valleys.
Map 1.
Collection localities in Western Ghats and adjoining areas

- Collection Localities
  - Coffee
  - Savanna-woodland
  - Scrub-woodland
  - Shrub savanna
  - Shrub savanna to grassland
  - Tea
  - Evergreen to semi-evergreen
  - Riparian type
  - Dry deciduous
  - Evergreen
  - Grassland
  - Moist deciduous
  - Swamp
  - Western Ghats

State Boundary

Kilometers

0 20 40 80 120 160
According to the geographical features, Kerala can be divided into three regions; highlands above 76m, midlands 7.6m to 76m and low lands below 7.6m. With an average height of 900m and a number of peaks well over 1800 m in height, the highlands of the Western Ghats comprise of forest areas, with major important hotspot areas of biodiversity of flora and fauna. The midlands with extreme undulating hills and valleys form an excellent area for the cultivation of cashew, coconut, arecanut, tapioca, banana, rice and different kinds of vegetables. The deposition of the sediments brought down by the rivers of Western Ghats and the sand deposited by the tidal inflow constitutes the lowlands of the coastal areas. The area also includes lagoons and backwaters formed by the excess flood waters accumulating in shallow basins during the rainy season, often separated from the sea by only a narrow strip of sand.

3.2 Climate

The annual temperature range is between a maximum of 35.9°C and a minimum of 23.8°C. Day temperatures are more or less uniform over the plains throughout the year except during the monsoon months, when the temperature drops by about 3°–5°C. The mean relative humidity value ranges from 60 – 90%, the maximum during the rainy season of June-September. The total annual rainfall is excessive with over 3200 mm, with even more than 70% of it received in the three month period of south west monsoon, during June to August. About 20% of the annual rainfall is received during the months, October to December, i.e. during the south east monsoon and the remaining 10% is obtained as occasional showers and pre-monsoon showers in April and May.

3.3 Collecting methods

The specimens for this systematic investigation were collected dead or alive from the field. Collecting is preferred in disparate habitats such as mountain meadows, grasslands, dense forests, agricultural plains, forest canopy etc. Sometimes the best spot to collect is not in some exotic, distant locale but rather locally. Local areas are advantageous in that they can be sampled consistently over longer periods of time and do not require extensive outlays of cash and time as would in extended trips.

Santhosh, S.
Successful collections were made from what appeared to be "crummy" habitat like bushes along roadsides and overgrown, weedy lots. Undoubtedly undisturbed sites can provide a wealth of interesting taxa.

Productive areas within a particular habitat may include blooming plants, spring or seepage areas with lush vegetative growth, or plants infested with insects. These areas have a common factor that is something that the bethylids want, possibly the host to parasitize. They were collected from underside of stones and crevices in the tree trunks. Various collection techniques were used. Each method has its own advantages and special uses and in general all methods were used, whenever possible.

Bethylids were collected by using two broadly defined methods:

1) Active collecting
2) Passive collecting

3.3.1 Active Collecting

3.3.1.1 Sweeping

Sweeping is found to be the most rewarding method for collection in clear windless weather. The early hours of morning and evening were found good for collecting. The net was swept across vegetation from low-growing grasses up to tree canopies.

The type of net most suitable for sweeping one with a triangular frame. While sweeping, the triangular shape of the frame allows a larger area of vegetation to get in contact with the opening of the net. The results obtained using this type of net show that the insects collected is roughly ten times more than the insects obtained by the conventional type of round nets (NOYES, 1982). The present investigation used a modified model designed by Noyes (fig.1). The sides of the frame measure 48 cm X 22 cm X 46 cm. The handle measures about 106 - 122 cm long and 3.5 cm girth. The long handle increases the proximity to underneath of long hanging bushes and extends the area of each sweep. The frame can be fitted to one end of the handle and can be easily separated, while not in use. The net bag is made

Santhosh, S.
Materials and Methods

up of 60 cm long durable white cotton cloth or terelene cloth, which have fine mesh that will permit the easy passage of air, but at the same time this prevents the escape of small insects of less than even 1 mm in size. To withstand the potential damage while sweeping, the rim of the bag is reinforced with thick material, preferably canvas. This canvas is either folded or sewn over the frame or is tied in position, through the small round holes of the frame.

As the materials accumulate after sweeping over the vegetation, a bolus will form at the bottom of the net bag. This bolus is pinched off and small to minute wasps were aspirated. Aspirator was used to collect the wasps from the net bag. It consists of a glass or perspex vial, with a stopper by two flexible tubes. The end of one of the tubes is covered by a small piece of gauze to prevent specimens from being drawn into the operator's mouth. It is operated by sucking at the end of the gauze covered pipe and holding the end of the other tube close to the specimen. Parasitic wasps appear as small, often black or brown specks walking and flying about. Since they are attracted to light, it is best to position the net bag so that sunlight shines through the top of the net rather than into the net opening. Alternatively, the bolus may be placed into an alcohol container (whirl-pak bag) for later processing.

3.3.1.2 Rearing

This is typically the most labor-intensive (but also potentially the most rewarding) form of obtaining bethylids. It is advantageous not only in that host associations and habitat requirements of the parasitoid can be identified, but also male and female associations can be ascertained, which is something difficult to do definitively with sexually dimorphic taxa which are collected by sweeping. Obvious things to collect for rearing are galls, leaf mines, larvae, pupae, etc. Any type of clear container will enable daily checking of materials for emergence. For large host samples such as plant materials containing larvae, leaf mines, etc. emergence cages were used. The emerging insects were attracted towards light and collected into a glass tube placed over an inverted glass filter funnel stuck on the roof or sides of the cage to prevent the return of the insects into the cage itself.

Santhosh, S.
3.3.2 Passive Collecting

3.3.2.1 Malaise Trapping

The most common passive flight-intercept trap is an open-sided tent-like structure called a Malaise trap (fig.3). Insects strike the central panel of the trap and either drop to the ground, or fly/crawl to the highest point. Those flying upwards are contained by the roof and concentrated in the collecting head of the trap where they are killed. Generally the trap is dark in colour with a light roof which reinforces any positive response to light in diurnal insects. The collecting head is attached to the higher end of the trap to take advantage of insects moving to the highest and sunlit area. Insects caught in the collecting head are killed by drowning in a solution of preservative. Ground Malaise traps catch flying insects up to one meter above ground level, in or above surface vegetation. They are most effective at sampling arthropods along forest edges and can also be used in forest interiors, and in associated habitats such as forest wetlands. This trapping has two advantages. Beyond the advantage of a passive sampling method, it can be emptied once in a week even by a non-entomologist.

This tent like trap was originally designed by R. Malaise (MALAISE, 1937) and later modified by Townes (TOWNES, 1972) and several others. Many Malaise trap designs are available. The latest model made by M/s Marris House nets, England has been used in the present investigation.

Factors such as likely flight paths, prevailing winds and Malaise head position can affect both the numerical and taxonomic composition of the catch. The following points should be adhered to when setting up Malaise traps. (1) The collecting head (high end of the trap) should be oriented consistently with the head pointing to the sunniest part of the forest habitat facing the open area (clearing or water) when placed in a forest edge. (2) Shrub vegetation directly beneath the trap should be cut back to a standard height of several centimeters above the surface. (3) Perturbations by large mammals (e.g., dogs) can be a problem with Malaise traps.
In order to fully sample any site or habitat, trapping should be continued throughout the active season. Such an extended trapping period is required to accommodate the different phenologies exhibited by the various taxa. In the last two years of the survey (2007 and 2008), extended trapping of the family was done using Malaise traps fixed at two different sites in the study area; the scrub jungle of Panayathanparamba near Kannur and Arboretum in the botanical garden of Calicut university campus. No malaise trapping was done during the monsoon season (June to August) as the heavy and continuous rains kept the trap netting wet and sticky.

3.3.2.2 Pitfall Trapping (unbaited)

Pitfall traps (fig. 4) are deep, small containers (cups) that are sunk into the ground to sample surface-active arthropods. They function in a manner similar to pan traps by collecting arthropods that fall into the container and drown in the collecting fluid at the bottom of the trap. Pitfall traps collect smaller samples of arthropods than pan traps because of the smaller trap perimeter of the pitfall trap. They are easier to install and service because of the smaller volume of collecting fluid needed to operate the trap, less expensive in materials to construct the trap, and less expensive in labour to process the smaller samples.

Isopropyl alcohol of 70% or propylene glycol mixture is placed in the trap and acts as the collecting medium and preservative. Pitfall traps can be used in any ground level substrate except water and rock. They can be used to sample arthropods in a variety of microhabitats such as rotting wood, surface litter, or peat lands, but the most common application is in soil substrate for surface-active arthropod fauna, like bethylids.

3.3.2.3 Pan Trapping (Moericke trap)

Pan traps are shallow, relatively large containers (compared to pitfall traps) that are placed on or sunk into the ground to sample arthropods. The most common modification to pan traps is the colour. Insects, particularly of low-flying nature are differentially attracted to a variety of colours, but most are attracted to bright yellow Moericke traps (fig. 5). In their simplest form, they function in nearly the same manner as pitfall traps, collecting arthropods that fall into the container and drown in the collecting fluid. Pan traps not only collect larger samples of arthropods than
pitfall traps, they also differ with respect to the light response of the diurnal (day-active) arthropod species that are captured. Pan traps collect more specimens than pitfall traps because of the increased surface perimeter of the trap. Although both pitfall and pan traps are neutral with respect to the light response of nocturnal (night-active) species, they differ in the light response of the diurnal species captured.

Yellow pans are placed in areas of the habitat which are likely to be productive for Bethylidae (insect infestations, under shrubs or near seeps, etc.). The shallow tray, about 60-75 mm deep and about 30 sq. cms area, painted bright yellow inside and some neutral colour such as black on the outside are then filled with a saturated salt solution or water (salt solution when emptying every 3-4 days or water when emptying 1-2 days) and 1-2 drops of liquid soap is mixed in to reduce the surface tension of the water. Many small insects will be attracted to the yellow colour and collect in the bowl. A small aquarium net is perfect for straining the catch, which can then be easily rinsed with fresh water to remove salt/soap residue and transferred to the vial containing ethanol.

Pan traps can be used in just about any terrestrial habitat or suspended in tree canopies, but the most common application is in a soil substrate for surface-active fauna. Traps should be placed where they are likely to remain undisturbed by humans or weather phenomena (rising water, flooding, etc.). Pan traps collect a diverse sample of ground-dwelling arthropods (similar to pitfall traps) but also collect a diverse sample of low-flying, agile arthropods.

3.3.2.4 Canopy collection

Since collection permission in the Nilgiri Biosphere reserve was difficult to obtain, the present investigation sought help from the UK Government funded Darwin Initiative project on Bees, Biodiversity and Forest Livelihoods conducted by Keystone Foundation, Kothagiri, Tamil Nadu for materials from the core forest areas within the study region. During the project, a year long survey was conducted in 2007 at 16 sites within the Nilgiri Biosphere reserve. Specimens were collected from the forest canopy in elevated yellow, white and blue pan traps. The traps were hung near the flowers as it was mainly meant for collecting anthophilous insects.
3.3.3 Basic Collecting Equipments

*Sweep net:* The triangular framed sweep net with bag made out of durable white cotton cloth or terelene cloth, fitted to the long handle.

*Aspirators:* Aspirators are used to suck up the specimens from the net.

*Vials of alcohol:* 70% ethyl alcohol (ethanol) is preferred for dispatching aspirated bethylids, although isopropyl alcohol can be used in a pinch. Leak-proof, screw top glass vials are best for storing individual samples.

*Nalgene bottle:* Small, screw-top Nalgene bottles are convenient for bringing along additional alcohol or ethyl acetate.

*Killing tubes:* Ethyl acetate or acetone is probably the safest and easiest to acquire killing agent for charging tubes.

*Fine tip forceps & Brushes:* Useful for manipulating small insects that become lodged in tight spaces during transfers to and from killing to storage receptacles or re-capturing stunned escapees.

*Writing implements:* Pens and pencils are essential for recording field observations, host identifications and collecting locality information.

*Pan traps:* Yellow pan traps are an inexpensive and simple means of passively sampling micro hymenoptera in an area.

*Glassine envelopes and brown paper bags:* The small-medium sized bags are of great use while collecting potentially parasitized host materials for rearing or host plant samples for identification. Placing plant samples or live insect host samples inside the paper/glassine bags retards sample desiccation and the condensation inside the plastic bag. Samples so packaged can be maintained for 3-4 days on ice or in the refrigerator.

*Field notebook:* A simple field notebook to record the collection details is compulsory.
Magnifying lens: A small magnifying glass is optional and allows one to examine specimens in the field.

3.3.4 Processing

Processing the collected wasps involves sorting, relaxing, mounting, labeling, registering and preserving the mounted and un-mounted materials. Specimens from the field should be stored in a cool dark place (refrigerate, if possible), but not in direct sunlight or near a heat source. Replace alcohol with fresh 80% alcohol after one to two weeks. The sooner the catch is processed the better in terms of quality specimens and reduced sample backlog. For long-term storage (several months to years) it is essential to replace alcohol (to prevent ice crystal formation) and to keep the catch below 0°C. Sample processing consists of sorting, mounting, and labeling specimens in preparation for identification.

3.3.4.1 Un-mounted material

The un-mounted specimens were stored in 70% alcohol (ethanol) in small vials and kept in a refrigerator. The preservative was periodically changed and replenished to prevent damage to the stored specimens. Specimens are sorted using a sorting tray (fig. 6).

3.3.4.2 Relaxing

For relaxing, the specimens are kept in an atmosphere of acetic acid for at least 6 – 8 hours. This method was found very suitable for the specimens, which had been killed using ethyl acetate or other killing agent. Relaxing helped to prevent breakage of specimens when they were being card mounted. In order to achieve best results, a clear plastic sandwich box with a tight fitting lid was taken and the bottom was covered with a thick layer of cotton wool. Specimens to be relaxed were placed on top of the piece of tissue in a glass dish and the whole dish, kept in the box, which was then closed.
3.3.4.3 Card Mounting

The method followed in the present work is that adopted by Boucek and Noyes (NOYES, 1982). The specimen was mounted on a triangular or rectangular card, measuring 14 mm in length and 4 mm in width, with properties of medium absorption. The specimen was mounted on the card, tilted slightly on its sides (at about 45° to the plane of the card) in such a way that the face and mandibles were clearly visible.

The materials used for the card mounting were;

i. A fine zero point brush

ii. A pair of fine needles or pins

iii. A pair of fine forceps

iv. Water soluble glue

v. Mounting cards measuring 14 mm X 4 mm

Before mounting, it was made sure that the glue is cold water soluble, when dry as well as when wet and also that it had the right consistency. For large specimens thick glue was used.

Before mounting, (both freshly killed and relaxed) specimens had to be thoroughly dried. For this, the specimens were placed with a drop of alcohol on an absorbent piece of card. The wings, legs and antennae were then correctly positioned. After drying, it was placed on the stage of the microscope; near-by a card triangle. Using a fine pointed pin, a tiny drop of glue (approximately 2/3 the volume of the thorax of the specimen to be mounted) was put on the card at the point where the imaginary lines bisecting the angles at the top corners of the card met. Then a fine pointed brush was picked taken and moistened by a minute quantity of saliva from the tongue, the specimen was thus picked up by touching the brush against the mesopleuron. It was then positioned with the midpoint of the thorax on the glue, the body lying length wise along the card and the head pointing towards the
far end. Then the body was tilted so that it lay on its sides at about 45° to the card. The specimen was pressed down firmly but gently with the brush for a good adhesion. Care was taken to avoid the glue sticking to head, antenna and wings etc. all body parts need to remain well spread.

3.3.4.4 Labeling and Registering

Temporary labels were written in the field at the time of collecting. After mounting, permanent labels indicating the name of the country, state, date of collection, etc. were added. Registering of the specimens was done after the specimens have been identified at least up to generic level. The registering of entries is as follows:

i. Collection number

ii. Scientific name

iii. Name of locality of collection

iv. Date of collection

v. Name of host

vi. Name of collector

vii. Identification

viii. Remarks

3.3.4.5 Storage and preservation

Serial numbers were also given to each specimen. The specimens mounted were held on entomological pins (Asta Insect pins No.3, 38 mm X 0.53 mm made by Newy Goodman & Co. England), labeling and kept in insect boxes, for detailed systematic studies. Naphthalene balls were placed in the boxes to protect from insect attacks. Thymol crystals were used as fungicides. 1,2- Dichlorobenzene can also be used to prevent fungal growth.
3.3.5 Observations and Illustrations

For sorting, mounting and dissecting, Olympus (Japan) microscope was used. Observation and description of the card mounted specimens were done using Leica MZ6 Stereozoom (Switzerland). The figures were drawn using the drawing tube (photo tube) of the Leica MZ6 Stereozoom (Switzerland). The digital images of the specimens were obtained using Cannon Powershot G5 digital camera attached to Carl Zeiss (Germany) Stereozoom microscope.

Since each frame of the digital images from microscope is in focus at a different depth into the specimen, all parts of the wasp were not in focus in any single frame. This is due to depth of field limitations. CombineZM is used to process the stacks of digital images. This is an open source image processing software package that helps to blend the focused areas of several partially focused digital images in order to create a composite image with an extended depth of field, created from the in-focus areas of each image. On an average 12 serial frames were taken for a single final composite photograph. In total, 2050 frames were used to make 173 composite images.

3.3.6 Specimens on Loan

Specimens already collected and well preserved in museums and depositories around the world were studied. Types and unidentified materials from the museums were also studied in this investigation. Many specimens collected in the past from the study area are deposited in foreign museum holdings. Several attempts were made to procure the Indo-Malayan materials and some of them met with success. Materials from natural history museums of Amsterdam, London and Genova were studied on loan. Holotype of *Sclerodermus bicolor* Smith deposited in Oxford University Natural History Museum (Hope Museum) was in a very bad condition and so no direct study was conducted, but photographs were made available for this investigation.
3.3.7 Distribution maps

The latitudinal and longitudinal co-ordinates and elevation of collection locality are marked with the help of Global Positioning System (GPS) by GARMIN etrex®H. The forest map layers are digitized and compiled from forest maps. They are generalized for forest types in GIS. Forest types are represented using different colors on Western Ghats boundary. The GIS software used is ArcGIS version 9.3. The GPS locations corresponding to the collection localities are converted into shape files and plotted on forest map layers. These automated layers together give the forest type in which a particular species was found.

3.3.8 Interactive keys

The DELTA (DEscription Language for TAxonomy) software (DALLWITZ, 1993) is used to record taxonomic descriptions for computer processing and generating interactive keys for identification. The program runs on Windows XP and later versions. The interactive keys (DELTA) to the species of four genera revised in this study is included in the Compact Disc (CD) attached to the back cover of the thesis.