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A Review on the Collection, Preservation and Systematic Techniques in Phlebotominae

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Abstract: The aim of present study is to discuss the taxonomic and biological history and significance of Phlebotominae sand flies giving emphasis on their collection, preservation and identification of new species as well as differentiation of male and female. Several species of Phlebotominae sand flies are known throughout the world including India. These Phlebotominae sand flies are economically important natural enemies of human beings and cause Kala-azar and visceral leishmaniasis. This causes a lot of deaths. Taxonomy plays an important role in identifying these tiny insects accurately. The accurate identification is important to apply relevant control measures. A wrong identification may lead to misleading results and may upset the entire control strategy. From the start of sand fly research various methods for collection and identification were used. In the early twentieth century, the use of internal structures particularly, cibarium, pharynx and spermathecae was introduced as taxonomic characters. The identification of female sand flies was always found to be difficult and hence scanty work on females are available. Recently female external as well as internal genital structures are used for their identification. Another important character is the genital filament/spermathecal duct ratio which could be a good point for taxonomic differentiation of male and female Phlebotominae sand flies. The detailed study of some biologically important structures of sand flies e.g., mouthparts and reproductive organs both internal as well as external have led to the introduction of few new taxonomic characters.

Key words: Phlebotominae, leishmaniasis, collection, preservation, identification, taxonomy

INTRODUCTION

Phlebotomids were first studied by Bonnani (1961) in Rome. He described them as species of mosquito, recounted its nocturnal biting habits and attributed its mysterious origin to spontaneous generation from salt and water. In 1744, Admiral Anson reached the Brazilian Island of Santa Catarina during his voyage round the world and an encounter with these flies introduced the literature Sand flies, a term now widely used for phlebotomines. Later in literature, generic name *Phlebotomus* or vein cutter indicating blood-sucking habit was suggested by Rondani (1840). Walker (1851) is the first to coin the term Phlebotomidae for the group. Adler and Theodor (1957) suggested that Phlebotominae sand flies are important vectors of many human diseases including leishmaniasis. Loew (1862) used the family name Psychodidae with two sections, the Psychodina and the Phlebotomina.

Morphological description of sand flies has remained a neglected area. However the description of sand flies in India started in 1908 when Annandale described *P. argentipes* followed by a series of work done by Sinton (1923, 1924, 1925 and 1927) which contributed towards the importance of the morphological descriptions of the Indian sand flies. After 1942, few more reports on the taxonomic description and distribution of the sand fly in India came up (Mitra and Roy, 1953; Mitra, 1959; Kaul *et al*., 1973). In contrast, taxonomic studies of the New World sand flies were carried out by Young and Perkins (1984) and Young and Dunkan (1994). Recently, Ilango (2000) emphasised upon the use of antennal flagellum and its sensilla chaetica for morphological identification of *Phlebotomus argentipes*. During the last few decades, research on the taxonomy of Phlebotominae sand flies has changed from an activity principally based on the morphology of dead specimens to an eclectic pursuit involving several diverse techniques and incorporating a rapidly growing body of biological and field data. Since the early days, most advances in the taxonomy of sand flies have resulted from studies into their role in the transmission of disease. This has been and still is, the driving force behind both faunistic studies (review of the fauna of a country, districts or focus of leishmaniasis) and studies of intra-specific variation of vector or supposed vector species. Taxonomy of sand flies was considered indispensable basis for all work on their biology and their role as vectors by a World Health

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Organization expert group (WHO, 1977). Thus, with many zoogeographic regions either monographed or their faunas broadly understood an increasing emphasis was placed on studying intra and inter specific variations.

Perfil'ev (1968) suggested that the development of sand fly taxonomy may be divided into two distinct phases according to the type of characters used to differentiate and classify species. In the first stage only details of the external morphology were studied with particular emphasis on measurements and ratios, a method known as phlebometery, with little attention given to the biology of the insects. The second stage of sand fly systematics began when two parasitologists Adler and Theodor (1927), investigating sand flies as vectors of disease, introduced the use of internal structures as taxonomic characters, particularly the cibarium, pharynx and spermathecae. The discovery no doubt followed the dissection of the insects in a quest for leishmania. Recently in females also external morphology and internal genital structures are used for their identification. Detailed studies of some biologically important structures of sand flies (e.g., mouthparts) have led to the introduction of new taxonomic characters and the rejection of some phlebometric (quantitative) characters (Kirk and Lewis, 1951). Consequently, there is a much closer integration of taxonomy with the biology of the fly and closer ties between taxonomy and epidemiological studies (Ascione *et al*., 1996). Considerable geographic, local morphological, behavioral, etc. variations have been found in some species indicating that they may be composed of species complexes, e.g., *Lutzomyia longipalpis* (Ward *et al*., 1983), *Phlebotomus argentipes* and *P. orientalis*. Singh and Ipe (2005) stated the usefulness of sub-basal hairy process on coxite as well as the shapes of spermatheca in the taxonomic descriptions of new species of phlebotominae sand flies from India. By dissections and microscopical examinations of several specimens of *Chinius junlianensis*, Leng (1987) has revealed the antennal and palpal formulae of both males and females. The majority of taxonomic studies have dealt with the importance of the male genetalia for identification and in some cases a few features of the female mouthparts as well as internal genital structures have also been considered (Sinton, 1927; Depaquit *et al*., 2007). Due to the discovery of the female genital armature in separating the females of *P. papatasi* and *P. duboscqi*, two very closely related Old World species that were difficult to separate, has now been well established (Pesson *et al*., 1994).

Methods of collection and preservation of sand flies: On account of the seasonality shown by the sand flies, field trips to various regions of faunal limits in appropriate seasons in various parts of India have given the authors varied experience regarding the collection of adult sand flies by Pooters or Aspirators. This was found to be a very convenient method (Singh, 1993). This method was found successful as collections could be made from various places like ceilings of godowns, crevices, cracks of boulders and caves etc. Aspirators were also found to be very handy, simple equipments to carry in the collection kit to the fields as they can be easily replaced or assembled, if broken. An ordinary 4.5 ×1.0 inch glass tube could be used as a replacement with a rubber cork with two small glass tubes fitted in holes of 0.5 cm each. One of the glass tubes was attached to a rubber sucking tube and the other converted into a short collecting end. The flies sucked in could easily be transferred into a killing tube of the same size with a cotton pad moistened with chloroform or ethyl acetate, which are used as killing agents. A fine cotton or plastic sieve is advisable to be attached at the end of the sucking tube and inside the aspirator so that the flies sucked in should not end up in the collectors mouth (Fig. 1A). Sometimes collection is easily done directly by alcohol rinsed brush (Fig. 1B).

Light traps are another important tools for the collection of adult phlebotomines. A tracing paper of 6×1 square coated with castor oil stapled on hard cardboard along with a light source can be hung in the field at night. The flies are attracted to the light and ultimately will get struck on to the oiled sheet of paper, which could be collected into alcohol with a soft brush. The size and mode of placement can be improvised according to the area or niche from where collections are to be made. For the collection, from the resting sites of sand flies, it was found effective to disturb them first by duct or smoke or twigs. The adults thus collected were transferred into alcohol with a few drops of glycerol (Kaul *et al*., 1994; CDA, 2006).

Another important trap which is known as soil emergence trap is the main direct method for collection as well as identification of the natural breeding sites of sand flies. Available trap designs vary in features such as size, shape, material and technique of trapping the emerging sand flies (Rutledge and Ellenwood, 1995; Arias and Frietas, 1982; Bettini *et al*., 1986; Vieira *et al*., 1999). The trapping techniques used in most of these traps require a high frequency of visits (1-3 days) to remove the insects. Casanova (2001) has described a trap in which the removal of flies is quite simple. The main advantage of this trap is that it needs to be examined only every 15 days. The trap is made up of a dark round plastic basin measuring 45 cm diameter and 15 cm height. A 6 cm diameter hole is cut at the base of the basin where a PVC

Fig. 1: (A) Collecting phlebotomid with the pooter from the crevices of the tree (B) from the mud wall of a house (C) and on the side of river bank

connection is filled. This connection is 2 cm tall and 7.5 cm in diameter. A 7 cm tall PVC tube is inserted in this connection and covered on the top with a fine mesh gauze to prevent the escape of flies. The inner surface of this tube is covered with a 3×22 adhesive paper which acts as a sticky trap.

The dissections are made on the cavity slides under the binocular. The dissected head, wings, legs, abdomen and genitalia are passed through the ascending series of alcohol and mounted in Canada balsom under microcoverslip of 12 mm size. It was found convenient to have all the four parts, namely head, wings, genitalia and legs of one species on a single slide. The slides thus mounted are kept in oven at 40°C to remove any residual moisture (Ipe and Singh, 1994). Killick-Kendrick *et al.* (1994) described a technique for the female abdomen. With the insects in Berlese's fluid, they separated the terminal parts using entomological pins (size 00) attached to small wooden sticks and covered it with cover slips. With this technique it is very difficult to see the spermathecae and ducts during the dissection, because they are fragile, transparent and frequently shrink. Marcondes (1998) suggested that excellent results were obtained by passing the insects in phenol (85%-24 h), potassium hydroxide (10%-12 h), acetic alcohol (10%-15 min), acid fucsin (8-10 min), 70% GL, 90% GL, 95% GL and absolute alcohol (15 min each), oil of clove (24 h) and dissection and mounting in NC medium (Cerqueira, 1943).

Temporary mounts in glycerine are found to be very useful for the study of the spermatheca, as they become transparent in Canada balsom mounts. After the study the material should be passed through a mixture of alcohol, glycerin and water in 1:1:1 ratio and then through alcoholic series and mounted in Canada balsom for permanent preparation.

Majority of the diagrams of the mouthparts, head, wings, legs and genitalia are generally made with the help of Camera Lucida or directly by computers attached to the microscopes. Morphometric measurements are generally done by micrometry using ocular and stage micrometers and finally calculated according to the magnifications (Singh, 1993). Dvorak *et al.* (2006) has mentioned the use of geometric morphometric analysis, two-dimensional Cartesian coordinates of 16 landmarks from the wings which were digitized and analyzed. Significant shape differences were found between colonies but not between sexes within each colony.

Taxonomic studies: Study of the mouth parts of modern species (Lewis, 1975) enables us to class them, according to the structure of the maxilla, as ridge tips and hook tips, which are largely associated with reptiles and mammals, respectively. In the Old World ridge tips are now typified by the genus Sergentomyia which comprises about 70% of the species and occurs mainly in the tropics where reptiles abound. The hook tip genus *Phlebotomus* is wide spread but now flourishes mainly in the North.

The diagnostic characters used in the early taxonomical work are also based on the pharyngeal armature. The character of male genitalia as is being done presently in the study of other Dipteran families. Other diagnostic characters used are presence of spines on the hind femora (Forattini, 1971), the proportional length of the palpal segments, ratios of wings and veins, legs and leg segments, ascoids, shape and structure of the labrum.

For the identification of Indian species including the newly discovered species new keys at generic as well as specific levels have been formulated, taking into account the structures of the genitalia, characters of legs, head,

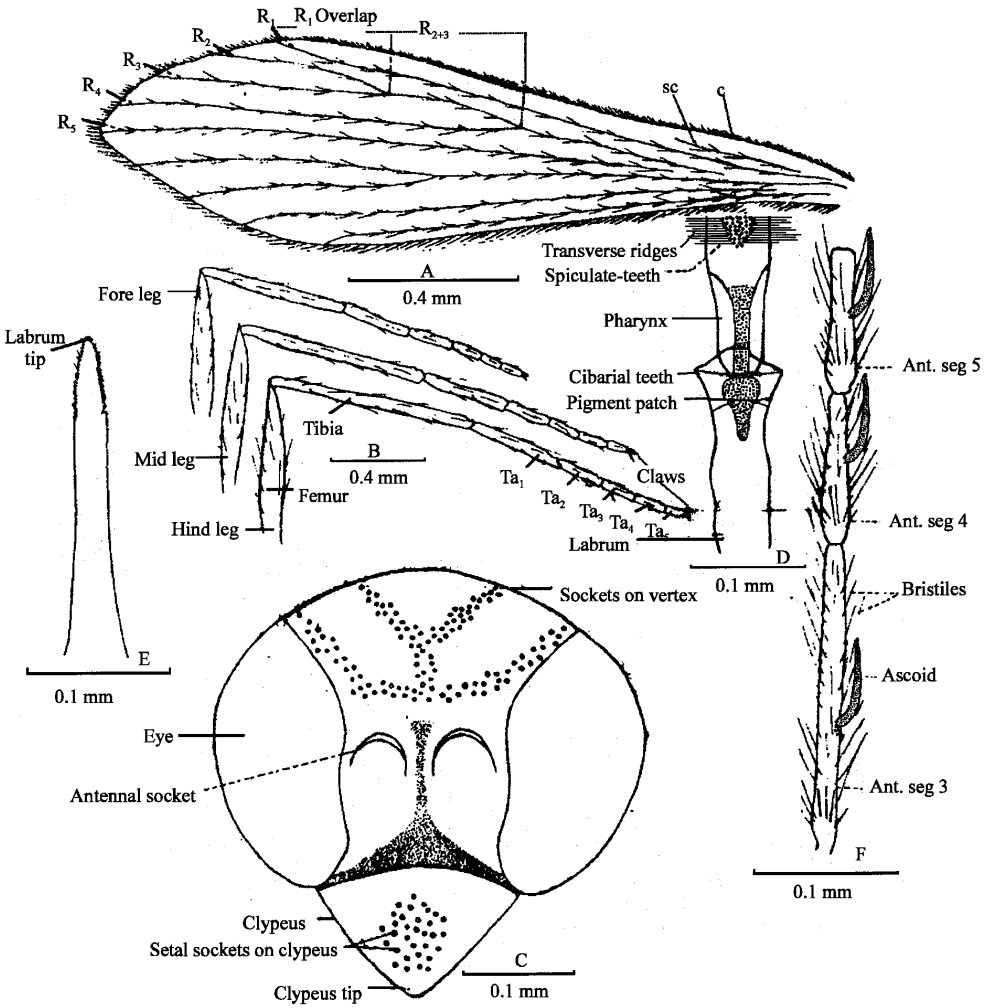

Fig. 2: Morphological characters of taxonomic importance (A) Wing, (B) Legs, (C) Head, (D) Pharynx, (E) Labrum and (F) Antenna

wings, palpi etc. and other characters of taxonomic importance used by Lewis (1978), Abonnenc and Minter (1965), Abonnenc (1972) and Duckhouse (1973).

The differences between new world and old world species is real according to Lewis (1978) but on the basis of the present studies, specially comparative studies of the genitalia it is difficult to draw clear lines as overlapping characters are much more than differentiating ones.

Morphological characters of taxonomic importance: Many morphological characters of male as well as female flies are used as aids in establishing specific identity of the species concerned. Also some of them provide valuable points for the formulation of keys for the separation of one species from another. Terminologies used by different authors often cause confusion as same terms are also used to denote different characters. Hence, for the sake of clarity, it is essential to explain here, the terminologies and characters used in the present study.

Abbreviations and definitions: Abbreviations L: Labrum; Antenna 3 or A 3: Antennal segment 3; Ascoid/4, length of an ascoid on antennal segment 4/length of segment; W, wing length; Index: length of vein R_2/that of R_{2+3} and R_1 overlap/R_2: Overlap of R_1/length of R_2.

In the description of the head the terms often used in the text and what they denote are given (Fig. 2).

- **Height of the head:** Distance between upper margin of vertex and lower margin of the clypeus of the head in the frontal view.
- **Height of the eye:** Distance between the vertical and the genal end of the eye in profile.

- **Interocular space:** Distance between the two eyes in frontal view.
- **Setal sockets of frons and clypeus:** Setal placement marks of permanent nature on the vertex and clypeus in slide preparations after the setae are fallen off.
- **Antenna 3:** Length of antennal segment 3.
- **Antenna 4+5:** Combined length of antennal segment 4 and 5.
- **Pharyngeal length and width:** Length and width of pharynx in frontal view in slide preparations.
- **Spiculate teeth and ridges:** Teeth and ridges on the posterior region of pharynx in frontal view seen in slide preparations.

In the case of wing the following characters are of importance (Fig. 2a).

- **Length of wing:** Length of the wing from wing base to apex in permanent slide preparations.
- **Width of wing:** Width of wing at its widest part in permanent slide preparations.
- **R_1 overlap/R_2:** Ratio of length of R_1 overlap and R_2.
- **R_2/R_{2+3}:** Ratio of length of R_2 and R_{2+3}.
- **Radial fork:** First dividing point of vein radius from wing base.
- **Medial fork:** First dividing point of vein medius from wing base.

In the case of legs the respective length of leg segment in a tabular form is given in the text though leg ratios have not been used in the description or as diagnostic aid (Fig. 3).

- **Length of coxite:** Distance from the articulating margin with the genital segment to the distal margin of coxite.
- **Length of style:** Distance between the coxite articulating point to stylar apex of style.
- **Sub basal hairy process:** Patch of hairs on the sub basal region or coxite.
- **Spines and seta on style:** Arrangement of prominent spines and seta on style.
- **Ratio of ejaculatory pump with ejaculatory duct:** Ratio of full length of the ejaculatory pump with full length of ejaculatory duct.

Other characters used are the shape and type of aedeagal and parameral tips as well as lobes found on parameres. In the case of female, shape, segmentation and size of spermathecal segments are very valuable diagnostic features used in species determination and separations.

COMMENTS

From the perspective of leishmaniasis control, the study of the taxonomy and geographical distribution of species of Phlebotominae is of great importance. Leger *et al.* (1983) compared the morphology on the base of the spermathecal ducts. Rogo *et al.* (1988 and 1992) believed that the identification of sand flies appeared to be impossible but with well-mounted specimens it is possible to recognize males of sand flies solely by the morphology of the aedeagus. However, depending upon the way the aedeagus is lying, some specimens may be difficult to identify without remounting the fly. In these circumstances, the appearance, position and number of hairs on the inner surface of the coxite are good diagnostic characters (Lewis, 1982). Initially it was thought that there is no other simple and reliable morphological character to separate females of sand flies as in some cases females as well as males are so closely related that it is difficult to separate the females for e.g., *P. papatasi* and *P. duboscqi.* But, Pesson *et al.* (1994) have shown the importance of female genital armature in separating them. Still there was a necessity of an easily detectable and reliable female specific diagnostic character, which may also be very important in identification and determining the epidemiological role of the insect. Mukhopadhyay and Ghosh (1997) suggested that the female external genital structures in sand flies are less complicated and are limited to the genital plate. The genital plate is actually a modification of the 9th sternite and its modification probably is a functional need. They further suggested that in *P. argentipes*, *P. papatasi* and *P. major major* the genital plate is triangular in shape but this shape is quite different having different dimensions at the different sides of the plate. In all the above three cases as the orientation of the plates are different so is the space in between them. The external genital opening also varies in shape and size. Thus the external genital structures bear species specific diagnostic characters.

Killick-Kendrick *et al.* (1994) have shown that the morphology of the genital atria of six known Kenyan species of *Larroussius*, *P. aculeatus*, *P. elgonensis*, *P. guggisbergi*, *P. longipes*, *P. orientalis* and *P. pedifer* bear distinguishing armatures. This suggests that the taxonomic description of new species should include an illustration of the armature in the genital atrium.

In some cases, it is also established that spermathecae can be utilized for species identification. However, as spermathecae are soft and contractile, there is a high probability that the morphology of the structure may change. Spermathecae of certain species of sand flies

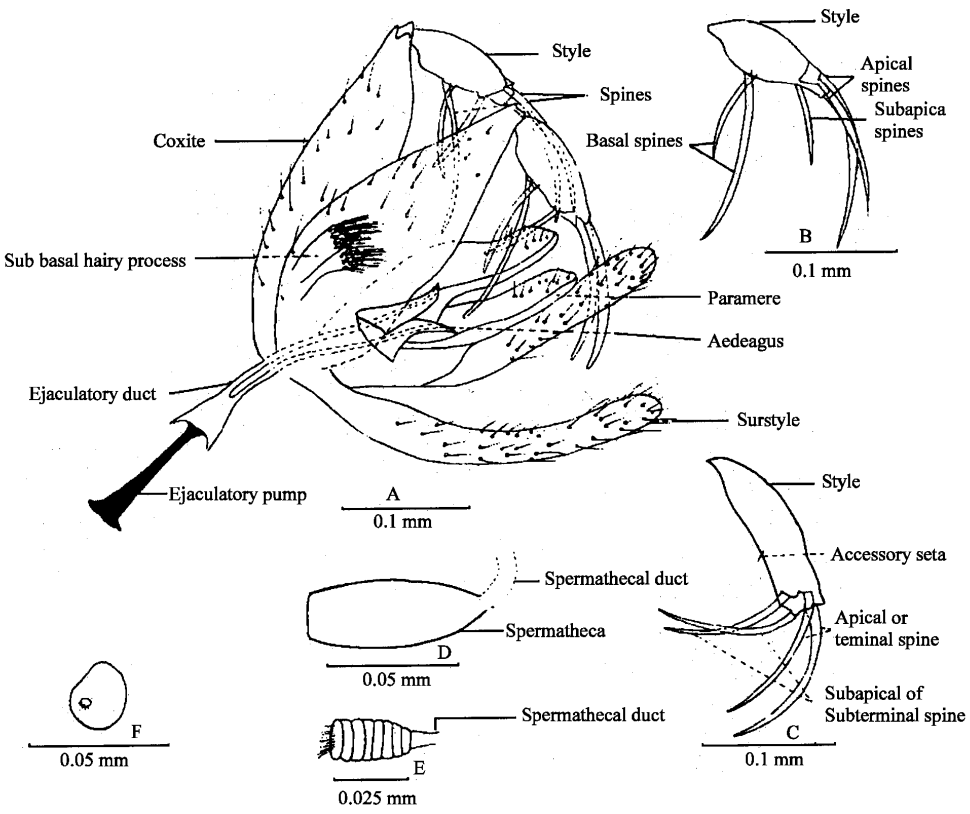

Fig. 3: Reproductive morphological characters of taxonomic importance (A) Genitalia, (B) Style without accessory seta, (C) Style with accessory seta, (D) Tubular spermatheca, (E) Segmented spermatheca and (F) rounded spermatheca

may vary considerably owing to contraction and also for the different mounting media (Quate, 1962; Lewis and Dyce, 1976). On the other hand, the comparatively hard cuticular parts are less vulnerable to changes and so they are more reliable for taxonomic identification.

Another important character is the length of the genital filament/spermathecal duct ratio which could be used as a character to differentiate between different intermedia (Marcondes, 1996; Marcondes *et al*., 1998 a-c; Marcondes and Borges, 2000). Geographical variations with respect to this ratio should also be studied. Initially, Williams (1988) demonstrated that the lengths of genital filaments and those of spermathecal ducts are similar in *Lutzomyia* sand flies. But, some recent studies contradicted this. Ilango and Lane (2000) noted that the common spermathecal duct of *P. argentipes* is very large and the male aedeagus correspondingly wide. There are differences in the common ducts in some species (Marcondes and Alexander, 2003). It would be interesting to study the shape and dimensions of aedeagi and their relationship with the common ducts. Few studies have also demonstrated the difference between the genital filaments and the spermathecal duct which could be a very good point for taxonomic differentiation of male and female phlebotominae sand flies (Ilango and Lane, 2000; Marcondes and Alexander, 2003). Very recently Depaquit *et al.* (2007) have reviewed the studies of Prof Leng (1987) and suggested that dissection and microscopical examination of several specimens of *Chinius junlianensis* have revealed the antennal and palpal formulae of both males and females of this species and the true morphology of the spermathecae in the females. The latter have smooth elongated reservoirs and a very long common duct which matches the long genital filaments of the males. There are no setae on the mesanepisternum, a feature shared with another primitive genus, *Warileya* and with *Sergentomyia*.

It is suggested that similar studies on other old and new world sand flies are necessary for a better understanding of the validity of the female external as well as internal genital structures as a possible identifying character. The results from the future work no doubt will strengthen the foundation that the female genital structures might be of significant importance in separating the females of very closely related species.

There is, however, a need for systematic surveys at different times of the year over much of the countries worldwide to map the distribution of the Phlebotominae sand flies species more accurately than is possible now.

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