

NUMERICAL TAXONOMIC ANALYSIS OF SAND FLIES (DIPTERA; PSYCHODIDAE) FROM DIR DISTRICTS, KPK PAKISTAN

KHURSHAID KHAN¹, SOBIA WAHID¹, NAZMA HABIB KHAN¹, FARRAH ZAIDI¹,
BASIT RASHEED¹ AND NAHEED ALI¹

¹Department of Zoology, University of Peshawar, 25120, Peshawar, Pakistan

Abstract: The present study investigated phylogeny of sand fly species from Lower and Upper Dir districts, Khyber Pakhtunkhwa, Pakistan using numerical taxonomy. A total of 60 morphological characters were analyzed for 20 species using Hierarchical Cluster Analysis (HCA). The results successfully differentiated the two genera i.e. *Phlebotomus* and *Sergentomyia* in the sub family Phlebotominae. HCA dendrogram further distinctly clustered the 5 subgenera of genus *Phlebotomus* (*Phlebotomus*, *Paraphlebotomus*, *Larrousius*, *Adleirus* and *Symphlebotomus*) and 5 subgenera of genus *Sergentomyia* (*Sergentomyia*, *Paraotmyia*, *Sintonus*, *Rondanomyia* and *Grossomyia*). Overall, these results were in accordance to the classical taxonomy of Phlebotomine sand flies and emphasized the value of Phenetics as a sound and reliable method for ascertaining the phylogeny of this group.

Keywords: Phlebotomine sand flies, Dir Districts, Hierarchical Cluster Analysis.

Introduction

Sand flies are haematophagous insects of great medical and veterinary significance, due to their ability to transmit protozoal, bacterial and viral diseases to humans and other animals (Rangel and Lainson, 2009). Three genera: *Phlebotomus*, *Sergentomyia* and *Lutzomyia* are important disease vectors in tropics and temperate regions of the world (Lewis, 1971; Lane, 1993). Among them, *Lutzomyia* species is distributed in North and South America while the other two are vectors in the Old world.

In Pakistan, a total of 37 (Kakar, 2004) sand fly species are reported belonging to two genera; *Phlebotomus* and *Sergentomyia*. However, more species are expected to exist since many regions of the country are to date unexplored (Shakila *et al.*,

2006). A few pre-partition studies were carried out in this region of Pakistan. To date, no phylogenetic studies have been carried out on this medically important group of insects from Pakistan (Kakar, 2008).

The present study was a preliminary effort to describe the phylogeny of Phlebotomines from four Tehsils of Lower and Upper Dir using numerical taxonomy. It aimed at resolving the relationships and taxonomy of the species from this region of Pakistan.

Materials and Methods

Study design and collection procedure

The present study was conducted in 40 villages comprising four Tehsils (Timergara, Balambat, Khall and Warae) of Dir district (Lower and Upper). Sand flies

were collected using three methods; sticky traps, flit method and aspiration. During the day times sand flies were collected through insecticide spray and aspirator while night time sampling was performed through the use of sticky papers. Fly sampling was carried out at 572 indoor (bathrooms and bedrooms) and outdoor sites (cattle corrals, dry cattle dung, caves, tree holes etc.). For identification purposes taxonomic keys as described by Lewis (1967) and Artemiev (1978) were used.

Numerical taxonomy

A list of 80 morphological characters was made for both sexes including antennae, palpi, cibarium, setae (thorax and abdomen) and genital structures (male and female). Based on these characters a matrix was constructed (Table 1) as described by Sun *et al.*, (2009).

The matrix composed of taxa and characters was analysed for HCA using Statistical Package for the Social Sciences (SPSS) version 20.0.

Table 1 Sand fly species taxonomic characters and their state value used for the study.

S. No.	Characters	Character state and their values		
1	Hairs on body	Densely covered(0)	Not present(1)	-
2	Wing shape	V-shaped wings(0)	Not V-shaped(0)	-
3	Colour of the eyes	Black eyes(0)	Not black(1)	-
4	Wings	Broad (0)	Narrow(1)	Flatted(2)
5	Abdominal sockets	Large and round(0)	Dropped shaped(1)	Absent(2)
6	Antennal formula	2/3-5	1/3-15	Absent(2)
7	Papilla formula	1/3-5	1/3-4	Absent(2)
8	Spines on Style	5 or 6	4	3 (2)
9	Cibarium morphology	With pigment patch(0)	Without(1)	-
10	Mesaneuristernum	Without postspiracular hairs(0)	Present(1)	-
11	Spermatheca morphology	Segmented or incompletely segments(0)	Smooth(1)	Spindle(2)
12	Terminalia of males	Long(0)	Short(1)	-
13	Paramere morphology	Long dorsal process(0)	Short(1)	-
14	Style	Long(0)	Short(1)	-
15	Spines	Short(0)	Long(1)	-
16	Spines on surstyle	Short(0)	Long(1)	-
17	No of spines	4(0)	5(1)	6(2)
18	Coxite	With basal process (0)	Without(1)	-
19	No. of ventral process	1-2(0)	Tubercal(1)	Absent(2)
20	Genital filament length	6-11(0)	3-3.5(1)	short(2)
21	Spermatheca	Regular segmentation(0)	Spindle shapes(1)	smooth(2)
22	Head of the spermatheca	distinct neck(0)	Without(1)	Absent(2)
23	Size of neck	Long(0)	Short(1)	Absent(2)
24	Cibarium structure	Unarmed(0)	Distinct teeth(1)	Absent(2)

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Continued

25	Chitinous arch	Not developed(0)	Well developed(1)	Absent(2)
26	Apical segment of spermatheca	Enlarged(0)	Usual(1)	Absent(2)
27	No. of spermatheca segmentation	2-9(0)	15(1)	Absent(2)
28	Head size	Wide(0)	Narrow(1)	Oval(2)
29	Paramere distal part	Curved(0)	Straight(1)	Slightly curved(2)
30	Basal spines	Closer to each other(0)	Apart(1)	-
31	Pharynx	Blunt teeth(0)	Rough squamae(1)	-
32	Style size	Short(0)	Long(1)	-
33	Basal process	Asymmetrical thin head(0)	Symmetrical(1)	-
34	Aedeagus shape	Wide(0)	Narrow(1)	Jugged ventral surface(2)
35	Antennal segment-8	With 2 ascoid(0)	With 1 ascoid(1)	Absent(2)
36	Antennal segment 6 and 7	2 long ascoid(0)	One long and one short(1)	-
37	Hairs on Coxite	65-95(0)	100-115(1)	-
38	Position of hairs on Coxite	basal half(0)	Distal half(1)	-
39	Shape of Coxite	Broad (0)	Not broad(1)	-
40	No. of hairs on Coxite	125-210(0)	42-86(1)	-
41	Spermatheca segmentation	2- segmented(0)	4-9 segmented(1)	6-9 segmented(2)
42	Pharyngeal armature	Projecting Sharpe angle(0)	Blunt angles(1)	-
43	Ascoid-4	Long(0)	Short(1)	-
44	Pharyngeal armature	Long squamae(0)	Irregular network(1)	-
45	Shape of aedeagus	Finger shaped(0)	Gradually trapping(1)	-
46	Paramere structure	Hooked shaped(0)	Slightly hooked(1)	-
47	A3	Without ascoid(0)	One ascoid(1)	-
48	Ends od aedeagus	Blunt(0)	Sharp(1)	-
49	Genital filament	Transverse striations(0)	Without(1)	-
50	Paramere structure	Hairy tubercle(0)	Without(1)	-
51	Spermatheca	Regular segmentation(0)	Without(1)	-
52	Shape of spermatheca	Capsule(0)	Tubular(1)	-
53	Head of spermatheca	Numerous spicules(0)	Smooth(1)	Striated(2)
54	Paramere	rounded(0)	Slightly hooked(1)	-
55	Aedeagus shape	Almost straight(0)	Little curved(1)	-
56	Ciberial teeth	12-25(0)	16-20(1)	-
57	Genital filament	2.2-3.1(0)	3.2-4.4(1)	-
58	Pigment pitch	Big(0)	Small(1)	-
59	No. ciberial of teeth	horizontal teeth 14-24 (0)	Hardly visible 20(1)	-
60	Anterior femora	Short spines(0)	Without(1)	-

Results and Discussion

A total of 7292 sand flies were sampled comprising of 20 species, belonging to two genera. Genus *Sergentomyia* was represented by 11 species while 9 species constituted genus *Phlebotomus*. Morphological character matrix of 60 characters was developed for 20 species. The matrix utilized synanomorphic and other characters within the sub family Phlebotominae such as shape of wings (character 1), colour of eyes (character 1) and occurrence of body hairs (character 3). Phylogenetic investigation of the sand flies using (HCA) exhibited many informative

characters. These included the arrangement of hairs on abdominal tergites (2-6) and antennal formula of males (2/3-5, 1/3-15) for genus level differentiation (Table 1).

For species diagnosis the analysis revealed the utility of several characters such as head structure and male/female genital terminalia. Characters associated with these structures were observed to be indispensable for taxonomic identification of sand flies. About 88.4 % characters in our matrix were based on the shape and form of these morphologically important structures (15 head: 27 male genitalia: 11female genitalia).

Table 2 The characters state matrix composed of species with codes, 2015.

Species	Characters																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
<i>P. papatasi</i>	0	0	0	1	0	0	0	0	0	0	2	0	2	1	2	2	2	1	2	0
<i>P. bergeroti</i>	0	0	0	1	0	0	0	0	0	0	2	0	2	1	2	2	2	1	2	0
<i>P. sergenti</i>	0	2	2	0	1	1	1	1	1	1	2	0	2	1	2	2	2	0	0	2
<i>P. andrejevi</i>	0	2	2	0	1	1	1	1	1	1	2	0	2	1	2	2	2	0	0	2
<i>P. kazeroni</i>	0	2	2	0	1	1	1	1	1	1	2	0	2	1	2	2	2	0	0	2
<i>P. ansari</i>	0	2	2	0	1	1	1	1	1	1	2	0	2	1	2	2	2	1	2	1
<i>P. hindustanicus</i>	0	2	2	0	1	1	1	1	1	1	2	1	2	2	0	2	2	2	2	2
<i>P. salengensis</i>	0	2	2	0	1	1	1	1	1	1	2	1	2	2	2	2	2	2	2	2
<i>P. major</i>	0	2	2	0	1	1	1	1	1	1	2	0	2	0	0	2	1	2	2	2
<i>S. babu</i>	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
<i>S. baghdadis</i>	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
<i>S. bailyi</i>	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
<i>S. grekovi</i>	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
<i>S. montana</i>	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
<i>S. dentata</i>	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
<i>S. theodori</i>	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
<i>S. hospittii</i>	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

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<i>S. tiberidis</i>	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	
<i>S. hodgsoni</i>	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	
<i>S.turkistanica</i>	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	
	Characters																			
Species	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
<i>P. papatasi</i>	0	0	0	0	1	1	0	0	0	0	2	2	2	2	2	2	2	2	2	2
<i>P. bergeroti</i>	0	0	0	0	1	1	0	0	0	0	2	2	2	2	2	2	2	2	2	2
<i>P. sergenti</i>	0	0	0	0	1	1	0	0	0	0	1	2	2	2	0	2	1	0	0	1
<i>P. andrejevi</i>	0	0	0	0	1	1	0	0	0	0	1	2	2	2	1	0	0	2	2	2
<i>P. kazoni</i>	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	2	2	2	2	2
<i>P. ansari</i>	0	0	0	0	1	1	0	0	0	0	2	2	2	2	2	2	2	2	2	2
<i>P. hindustanicus</i>	0	0	0	0	1	1	0	0	0	0	2	2	2	2	2	2	2	2	2	2
<i>P. salengensis</i>	0	0	0	0	1	1	0	0	0	0	2	2	2	2	2	2	2	2	2	2
<i>P. major</i>	0	0	0	0	1	1	0	0	0	0	2	2	2	2	2	2	2	2	2	2
<i>S. babu</i>	2	2	2	2	0	0	0	1	1	1	2	2	2	2	2	2	2	2	2	2
<i>S. baghdadis</i>	2	2	2	2	0	0	0	1	1	1	2	2	2	2	2	2	2	2	2	2
<i>S. bailyi</i>	2	2	2	2	0	0	0	1	1	1	2	2	2	2	2	2	2	2	2	2
<i>S. grekovi</i>	2	2	2	2	0	0	0	1	1	1	2	2	2	2	2	2	2	2	2	2
<i>S. montana</i>	2	2	2	2	0	0	0	1	1	1	2	2	2	2	2	2	2	2	2	2
<i>S. dentata</i>	2	2	2	2	0	0	0	1	1	1	2	2	2	2	2	2	2	2	2	2
<i>S. theodori</i>	2	2	2	2	0	0	0	1	1	1	2	2	2	2	2	2	2	2	2	2
<i>S. hospittii</i>	2	2	2	2	0	0	0	1	1	1	2	2	2	2	2	2	2	2	2	2
<i>S. tiberidis</i>	2	2	2	2	0	0	0	1	1	1	2	2	2	2	2	2	2	2	2	2
<i>S. hodgsoni</i>	2	2	2	2	0	0	0	1	1	1	2	2	2	2	2	2	2	2	2	2
<i>S. turkistanica</i>	2	2	2	2	0	0	0	1	1	1	2	2	2	2	2	2	2	2	2	2

Species	Characters																			
	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
<i>P. papatasi</i>	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
<i>P. bergeroti</i>	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
<i>P. sergenti</i>	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
<i>P. andrejevi</i>	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
<i>P. kazeroni</i>	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
<i>P. ansari</i>	2	2	2	2	2	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2
<i>P.hindustanicus</i>	2	2	2	2	2	2	1	0	0	0	2	2	1	2	2	2	2	2	2	2
<i>P. salengensis</i>	2	2	2	2	2	2	1	1	1	1	1	1	2	2	2	2	2	2	2	2
<i>P. major</i>	1	1	1	0	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
<i>S. babu</i>	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	0	1	0	2
<i>S. baghdadis</i>	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	0	1	0	2
<i>S. bailyi</i>	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	0	1	0	2
<i>S. grekovi</i>	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	0	1	0	2
<i>S. montana</i>	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	0	1	0	2
<i>S. dentata</i>	2	2	2	2	2	2	2	2	2	2	2	2	2	0	0	2	2	2	2	2
<i>S. theodori</i>	2	2	2	2	2	2	2	2	2	2	2	2	2	0	0	2	2	2	2	1
<i>S. hospittii</i>	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	2	2	2
<i>S. tiberidis</i>	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	2	2	2
<i>S. hodgsoni</i>	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	0	2	2
<i>S. turkistanica</i>	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	0	2	2	2	2

The HCA dendrogram was consistent with traditional taxonomy of Phlebotomine sand fly species (Figure 1). The analysis grouped together the species data in two generic clusters namely *Phlebotomus* and *Sergentomyia* with a λ (divergence distance) of 10. The tree places all species in their specific genera. The taxa are well defined within sub-genera *Phlebotomus*, *Adleirus*, *Paraphlebotomus*, *Larrousius*, and *Symphlebotomus* of Genus *Phlebotomus*. Similarly Genus *Sergentomyia* demonstrates clearly differentiated taxa in five sub-genera *Sergentomyia*, *Paraotomyia*, *Sintonus*, *Rondanomyia* and *Grossomyia*. The present analysis therefore describes Phlebotomine flies as a distinct clad of the family Psychodidae.

Family Psychodidae is distributed in all zoogeographical regions of the world (Andrade and Brazil, 2003). To date, the classificatory arrangement and phylogenetic relationships to other families and within the family remains a matter of debate (Wagner, 2006; Wagner and Ibáñez-Bernal, 2009; Stebner *et al.*, 2015). Reportedly, the specialists recognized six subfamilies (Horiaellinae, Sycoracinae, Trichomyiinae, Bruchomyiinae, Phlebotominae and Psychodinae) within the family Psychodidae (Stebner *et al.*, 2015). Among these six subfamilies, Phlebotomine are the principal vectors of *Leishmania*, *Bartonella bacilliformis*, and some arboviruses (Ready, 2013).

Sand fly species are distributed throughout Pakistan but correct identification of sand fly species is doubtful as no authentic taxonomic keys are available to identify specimens up to species level. Considering their importance as vectors of visceral and cutaneous leishmaniasis, their morphological

and morphometric characteristics may serve as vital diagnostic tools.

In this study, 60 morphological characters of sand flies were included in HCA analysis based on numerical taxonomy. Previous studies have shown that taxonomy of the genus *Phlebotomus*, relies on a small number of adult characters (Rispaill and Léger, 1998a). Three groups of characters were found to be most informative with respect to species diagnosis. These included head, male and female terminalia. Many pioneer studies in the field have highlighted the significance of these characters especially the genitalia. Structure of the male genitalia (França, 1919; França and Parrot, 1920-21) aided in the formation of subgenera. Female characters such as the cibarial and pharyngeal armatures, and spermathecae introduced by Adler and Theodor (1926), and developed by Sinton (1927-28) and Nitzulescu (1931) further helped in sand fly taxonomy (Rispaill and Léger, 1998b).

Numerical polythetic taxonomy simultaneously takes into account numerous taxonomic characters in phenetic analysis and intergroup relationships are expressed by means of factorial graphs and dendrogram (Rispaill and Léger, 1998a).

The phenetic analysis performed in this study using HCA was in congruence with the classical taxonomy of sand flies. Our results identified five subgenera within genus *Phlebotomus*. The twenty taxa were each assigned correctly to their respective subgenera. The HCA dendrogram strictly adheres to the traditional taxonomy of the group (Figure 1).

In the present study we exclusively utilized adult morphological characters.

Previous studies based on morphological and morphometric characters of adults point out that phenetic and cladistic analyses could be further improved by utilizing larval characters (Vattier-Bernard, 1971), spermatozoa (Dallai

et al., 1984) and chromosomal structures (White and Killick-Kendrick, 1976) along with molecular data, to provide a more elaborate analysis of Phlebotomine (Rangel *et al.*, 1996, Dujardin, 1999).

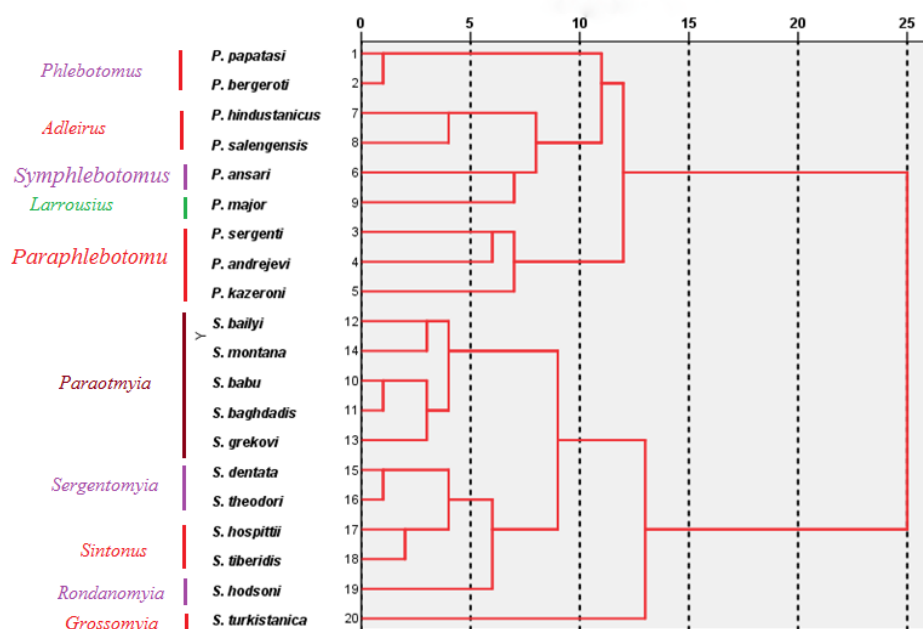


Fig.1. HCA dendrogram of sand fly species constructed using linkage between groups. Numbers on the broken vertical lines represent the divergence distance, λ .

Conclusion

Our analysis showed that integrative taxonomy can be vital in improving species identification and resolving issues in misidentification of certain species of sand flies. Sand flies identification based on morphological structures is widely accepted although such studies are limited in Pakistan. The present study, therefore, can be beneficial in classifying this group within Pakistan. However, a better understanding of such cluster analysis of sand flies species can be achieved when representatives of every species from all provinces of the country are utilized in a composite analysis.

Competing interests

The authors bear no financial or non-financial competing interests.

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