ASSESSMENT OF SAND FLIES (DIPTERA: PSYCHODIDAE) DIVERSITY, SEASONAL ABUNDANCE AND LEISHAMNIASIS RISK FACTORS IN DISTRICTS DIR, KHYBER PAKHTUNKHWA, PAKISTAN

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A THESIS SUBMITTED TO THE DEPARTMENT OF ZOOLOGY, UNIVERSITY OF PESHAWAR, PESHAWAR, PAKISTAN IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

ENTOMOLOGY

DEPARTMENT OF ZOOLOGY
UNIVERSITY OF PESHAWAR, KHYBER PAKHTUNKHWA, PAKISTAN

DECLARATION

The present research work is an original work conducted in four tehsils of Dir districts and have not been previously submitted to this or any other university.

Khurshaid Khan

CERTIFICATE BY THE RESEARCH SUPERVISOR

It is to certify that the present research work, presented by Mr Khurshaid Khan as a Ph.D. thesis is an original work carried out in four Tehsils; Timergara, Khall, Balambat and Warae, of Lower and Upper Dir districts. The study was designed to address the important issue regarding an emerging disease leishmaniasis in various parts of Pakistan including the studied region. Sand flies are the sole vectors responsible for the transmission of leishmaniasis in many parts of the world including Pakistan. Control efforts for any vector born disease require bio ecological information about the vectors, the present study was aimed to contribute in this aspect.

Collection of sand flies along with other relevant information was made from 499 houses in 40 villages of four tehsils. Indoor and outdoor collection of flies was made using different methods including sticky traps, flit and mouth aspirator. Environmental and altitudinal data was also recorded. House hold risk factor data was obtained and association of cutaneous leishmaniasis with various behavioural and environmental data was analysed for the studied area. Collection of field data and taxonomic identification of sand flies was difficult, time consuming and tedious job.

I do approve, recommend and forward this Ph. D thesis for onward submission to the examiners for final evaluation.

Research Supervisor

Date

Professor Dr. Naheed Ali
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Dedicated to my parents and siblings for their prodigious patience and constant praise throughout this study

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ABSTRACT

The aim of the current study was to determined sand flies species composition, diversity, seasonal variations, spatial distribution and leishmaniasis associated risk factors in Dir districts. The fieldwork was carried out in 40 villages from January to December, 2014 in Timergara, Balambat, Khall and Warae. A total of 7292 sand flies comprising 20 species of two genera; *Phlebotomus* and *Sergentomyia* were recorded through sticky traps, insecticide spray and mouth aspiration. *Phlebotomus* was presented by nine species while *Sergentomyia* was shown by eleven species. Peak number (6229) of flies were collected through sticky traps followed by insecticide spray (978) and mouth aspirator (85). Overall, male to female ratio was 3:1.

A total of 15 species were recorded in Tehsil Timergara; 13(Balambat); 10 (Khall); and 7 (Warae). Diversity of sand flies was high in Timergara followed by Balambat, Khall and Warae. *P. salengensis* was the most abundant species and was collected from three Tehsils (Timergara, Balambat and Khall) followed by *P. sergenti.* Similarity of species was high between Khall and Timergara as well as in Balambat and Warae. *P. sergenti, S. babu, S. baghdadis, S. dentata and S. hospittii* were reported from four tehsils. The remaining fifteen (15) species showed restricted distribution to one, two or three tehsils. *P. salengensis, P. hindustanicus, P. andrejevi, P. kazeroni, P. bergeroti, P. ansari, S. turkistanica and S. grekovi* were reported for the first time from Khyber Pakhtunkhwa. Among them *P. ansari* was recorded for this first time from Pakistan. A total of 88 specimens were not identified up to species level.

Regarding seasonal variation in the studied area, sand flies showed their appearance in May and presented peak aggregation in August and were disappeared from

November to April. Density of sand flies was positively associated with climatic factors (temperature and relative humidity). Flies presented greater abundance in domestic habitats (6535) as compared to peridomestic sites (621). Males to female ratio inside houses were 3:1 and outside houses 6:1. In domestic sites sand flies were collected from different designed houses; mud made, concreted and stone made houses. High flies abundance were found in mud made houses followed by concreted and stone made houses.

During the survey, immature males of three species (*P. salengensis, P. sergenti* and *P. andrejevi*) were collected from cattle sheds most particularly from combined dwellings and cattle corrals. Which were the probable breeding sites of these sand fly species. Similarly, a total of seven species were recorded from twelve plant species and greater number was recorded from plants belonging to Cucurbitaceae family. Moreover, eleven caves were visited and flies were sampled in which *S. bailyi* was the most abundant species.

Species specific spatial distribution of sand flies in relation to related physical factor (elevation) probably attributed to a variable land escape.

In univariate analysis, factors such as villages, number of rooms, family size, level of education, eaves, cooling equipment used, family members residing outside country, mosquito bed nets and vegetation were positively associated with the Leishmaniasis in the study area. In bivariate analysis, villages, low level of education, eaves and family members residing outside country were found to increase the risk of the disease in the region.

ABBREVIATION

AJ&K	Azad Jammu& Kashmir
CL	cutaneous leishmaniasis
MCL	muco-cutaneous leishmaniasis
DCL	diffused cutaneous leishmaniasis
NWA	North Waziristan Agency
Fig.	figure
VL	visceral leishmaniasis
CnL	canine leishmaniasis
G. T.	grand total
M	male/s
F	female/s
GIS	geographic information system
RS	remote Sensing
SJIC	small jackal inhabited caves
BSC	big sized cave
MSC	middle sized cave
VSC	very small cave
No.	number
KPK	Khyber Pakhtunkhwa

WHO	World Health Organization
RH	relative humidity
Pak	Pakistan

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CHAPTER - 01
INTRODUCTION

1.1 INTRODUCTION

Phlebotomine sand flies (Diptera: Psycodidae: Phlebotomine) are small hematophagous insects (Moncaz *et al*., 2014) with a total of 1000 known species and subspecies which inhabit various parts of the world (Killick-Kendrick, 1999; Kakar, 2004; Munstermann, 2004). Three genera of sand flies, *Phlebotomus*, *Sergentomyia* and *Lutzomyia* are medically very important for public health. The former two genera are present in Old World while the latter one is the principal genus in New World (Lewis, 1971). Sand flies are generally restricted to tropic and temperate regions of the globe and their preferable breeding sites ranging from semi desert to rain forest foci. Species belonging to *Phlebotomus* and *Sergentomyia* are present in some endemic parts of Pakistan, including Federally Administered Tribal Area (FATA), scattered regions in Sindh, Punjab, Baluchistan and Khyber Pakhtunkhwa (KPK) (Shakila *et al*., 2006). In Pakistan sand flies are reported as suspected vectors of cutaneous leishmaniasis and visceral leishmaniasis (Rowland, 1999).

Phlebotomine sand flies are responsible for transmission of several human diseases, including bartonellosis, leishmaniasis and sand fly fever viruses in many parts of the world. In terms of the geographical distribution and diversity of vectors the most important among these diseases is leishmaniasis, a vector-born zoonotic infection of human and animals which is endemic in 88 countries throughout Africa, Asia, Europe, North and South America (Lainson, 1982). Leishmaniasis is considered second to malaria in terms of number of people infected (Alexander and Maroli, 2003; WHO, 2010; Siddiki *et al*., 2010), where twelve million people are reported infected and 1.5 to 2 million new cases each year are estimated (Desjeux, 2001).

Leishmaniasis infection appear in various forms which is classified as VL (Visceral leishmaniasis), CL (cutaneous leishmaniasis), MCL (muco-cutaneous leishmaniasis) and DCL (diffused cutaneous leishmaniasis) globally (Ashford, 2000; Desjeux, 2001). Among all these disease forms, cutaneous leishmaniasis (CL) is endemic in Pak-Afghan region and is prevalent in Baluchistan, Khyber Pakhtunkhwa (KPK), Punjab and Sindh Provinces (Kakar, 2011). While Visceral Leishmaniasis (VL) one of the fatal form of leishmaniasis is largely confined to the north-east part of the country, notably in Azad Jammu and Kashmir (AJ&K) and Baltistan (Burney *et al*., 1981). The disease is observed to be spreading continuously from endemic areas to non- endemic areas (Kakar, 2011). Rowland *et al*., (1999) studied more than 1000 cases of Leishmaniasis in an Afghan refugee settlement at Timergara, Lower Dir (KPK) and confirmed that CL is due to *L. tropica* an emerging disease in the camp.

Currently, the distribution of leishmaniasis is on increase across the world as compared to previously reported incidence of the disease (Desjeux, 2001). This increase in the disease incidence was linked with the environmental and demographic factors such as, rising temperatures, the increased movement of infected individuals and transport of animals (could act as reservoirs) from endemic areas are suggested as the main risk factors that contribute to the spread of the disease (Desjeux, 2001).

The main strategy for the disease control and prevention are mainly focused on the chemotherapy to reduce the disease burden and vector control to reduce its transmission (Handman, 2001). Additionally, public health awareness could play an essential role in the control programs but less attention is paid to this area. To date, no effective vaccine is available despite substantial efforts by many laboratories (Parra *et al*., 2007).

Epidemiology of Leishmaniasis and their reservoir hosts play an essential role to reduce the transmission of the disease. Similarly, studies about vector ecology and its behaviour are thought to be the main prerequisites for the prevention of an infection (WHO, 1984). Detail record about sand flies are relatively lacking in Khyber Pakhtunkhwa (KPK) province of Pakistan including districts Dir. This study will increase the data pool on the epidemiology of Phlebotomine sand flies, their current distribution and abundance in Dir districts. Thereby, this study will be an important contribution to the knowledge of sand fly ecology and its distribution and determining the potential risk factors of leishmaniasis in the study area.

Sand flies are small (3-5 mm), delicate, hairy flies with long slender legs (Lane, 1993). The thorax is convex and wings are lanceolate and covered with dense hairs. When at rest the wings are raised in V fashion (Lewis, 1971). Taxonomically all sand flies are placed within the subfamily Phlebotominae, due to its distinguished characters from other so called sand flies of Simulidae and Certopogonidae. This subfamily is differentiated from other families of Psychodidae by the presence of 16-segmented antennae, 5- segmented palps, sucking mouth parts, small size and hopping movement (Triplehorn and Johnson, 2005).

1.2 PHLEBOTOMINE SAND FLY GENERA

Nearly, 1000 species of sand flies are recorded from different regions of the world (Kakar, 2004; Munstermann, 2004). All sand flies have the ability to develop local races and verities consequent to their limited power of flight and fragility (Morison, 1993; Andrade and Reginaldo, 2003). Due to medical importance as disease vectors, sand flies classification is renewed and updated in every country of the World (Forattini, 1973). A total of five sand flies genera are recognized three of which

including *Brumptomyia*, *Warileya* and *Lutzomyia* are prevalent in New World (Young and Duncan, 1994). Researchers demonstrated that the species in the former two genera take blood from humans but do not play any role in disease transmission. However, species belonging to the genus *Lutzomyia* are medically very important, as they contain vectors of diseases particularly leishmaniasis (Young and Duncan, 1994). In the Old World, *Phlebotomus* and *Sergentomyia* are the principal genera (Lewis, 1971). They are distinguished from each other on the basis of pharynx, ciberial teeth and spermatheca. *Sergentomyia* feed mostly on reptiles and is of key importance in Saurian Leishmaniasis. Sometimes they also take blood from mammals including humans. On the other hand *Phlebotomus* species feed on mammals and birds. Many important vectors of leishmaniasis belong to this genus (Lewis, 1982).

1.3 HISTORY OF SAND FLIES CLASSIFICATION

In Old World, Scopoli (1786) described *Phlebotomus* sand flies for the first time and the genus *Phlebotomus* was created by Rondani (1840) in Italy. The actual classification was started soon after their medical importance as disease vectors was realized (Forattini, 1973). Newstead (1911) classified sand flies into two groups on the basis of abdominal hairs while Parrot (1917) classified sand flies as *Phlebotomus* and *Newsteadia* on the basis of external genitalia of male flies. After careful identification of sand flies species, Franca and Parrot (1921) suggested five genera including *Phlebotomus*, *Prophlebotomus*, *Brumptomyia*, *Lutzomyia* and *Sergentomyia* for both Old and New World flies. This classification had many shortcomings but provided basis for further classification (Parrot, 1934).

In (1926) Alder and Theodor studied some taxonomic structures of sand flies such as abdominal hairs, buccal cavity, pharynx and spermatheca, on the basis of which

Sinton (1929), placed sand flies into two groups *Phlebotomus* (erect hairs and segmented spermatheca) and *Sergentomyia* (recombinant hairs and smooth spermatheca). Using morphological structures like buccal cavity, pharynx and spermatheca, Nitzulescu (1931) arranged all sand flies in to five subgenera (*Sintonus, Brumptomyia, Larrusis, Phlebotomus* and *Adleirus)*. Parrot (1934) rejected Nitzulescu classification because of no consideration of male taxonomic characters. Theodor (1958) classified sand flies into three genera. The first two genera exist in the Old World and the third one in the New World. Later on sand fly species and subspecies in all zoogeographical regions are placed in six genera i.e. *Phlebotomus*, *Sergentomyia* and *Chinius* in Old World and *Brumptomyia*, *Lutzomyia* and *Warileya* in New World (WHO, 1990). *Lutzomyia* is not classified into subgenera but actually there exist a great diversity in the genus (Lewis, 1971).

1.4 EVOLUTIONARY HISTORY (ORIGIN OF SAND FLIES)

Systematic and evolutionary history suggests that sand flies were evolved 38 million years ago in Oligocene. They were also reported from the Triassic period the time when continents were not separated (Andrade and Reginaldo, 2003). Family Psycodidae species are present in all continents except Antarctica, strongly support the view that their evolutionary history was started in Mesozoic area (Ansorg, 1994). The oldest specimen of sand flies was recovered from Virginia, USA (Fraser *et al*., 1996) and Germany (Ansorg, 1994). Later on, a number of sand flies species which are trapped in amber have been reported from Baltic region (Haenni, 2001), Lebanon (Azar *et al.,* 1999), Mexico (Rasnitsyn and Ross, 2000) and Myanmar (Morales and Pimentel, 2001).

A total of four sand flies ancient genera (*Phlebotomilla*, *Phlebotomite*, *Measphlebotominella* and *Libanophlebotomes*) have been recorded from Cretaceous

period but they are not living genera today. *Phlebotomus* and *Sergentomyia* are the only living genera of the ancestral members most probably from Holocene and Eocene. Galati (1995) grouped the oldest four genera in the tribe *Hertigini* on the basis of their primitive characters while Phlebotomine was designed for present day genera. According to Keer (2000) the ancestors of Old World sand flies were evolved in Palearctic region during Cretaceous period. Noyes *et al*., (2005) suggested that sand flies were evolved in Cretaceous period when the continents were still in contact.

Sand flies have limited capacity of flight due to which they are restricted to local geographical areas (Chaniotis and Coura, 1974, Morrison, 1993). This limitation shows that there is a small gene flow and a little phenotypic variation in the species (Andrade and Reginaldo, 2003). Recent evidences explain that they were evolved in Triassic period when Pangaea was still in intact. When continents become separated sand flies also dispersed to specific regions which produced the modern genera and species (Andrade and Reginaldo, 2003).

1.5 BIOLOGY OF SAND FLIES

Sand flies inhabiting various parts of the world have some common features but they are different to some extant in their morphology and biology (WHO, 1990).

1.5.1 Life cycle

Like other Dipterans all sand flies have a holometabolous life cycle (eggs, larvae, pupae and adults) and the first three stages are found in soil which are rich in organic and other nitrogenous matter with a pH of about 9.0 (Moncaz *et al*., 2012).

1.5.1.1 Immature stages

Sand flies eggs are usually dark brown or black and ovoid in shape. The eggs are patterned that can be used for species identification. Female sand flies lay 40 to 70

eggs singly which varies greatly by species and other factors such as type of host blood meal and ambient temperature. Although eggs are not laid in water but their eggs require high humidity and moist habitats. Under optimum conditions hatching occur after about 6-17d but in cooler areas hatching may be delayed up to 30 days. Their oviposition sites mainly depends on species but generally deposit their eggs in cracks, termites mounds, stables floors, poultry, birds nets, caves, crevices in houses and tree holes in forests generally in darkness as they cannot withstand desiccation (Feliciangeli, 2004; Sing *et al.*, 2008).

Like mosquitoes sand flies have four larval instars. Morphologically mature larvae (3-6 mm in length) is greyish or yellowish in colour, with 12- segmented body, well developed head with a pair of small prominent mandibles. Phlebotomine sand flies larvae can easily be identified due to the presence of matchstick (thick bristle) on the entire body. In addition, the first seven abdominal segments have pseudopodia like structures. In most species there are two caudal setae in the last two abdominal segments while first larval instars have a single bristle. Larvae are scavengers and mainly feed on decaying vegetables, fungi, animal faeces decomposing bodies of other animals especially insects. Larvae take 19-60d for its complete development but generally depending on species, temperature and availability of food. Before pupation, larva changes its structure and become erect in position followed by the split opening of larval skin and pupa coming out. The larval skin is still attached with the last portion of the pupa which is the recognition of sand flies pupae. Emergence of adults from the pupae takes 5-10d (Killick- Kendrick, 1999).

The life cycle lasts from 1 to 4 months depending on species and environmental factors especially temperature, humidity and availability of food (Ready, 1979).

Similarly the life cycle extend in cooler areas while in temperate region adults die in late summer or autumn. Some sand flies lay their first batch of eggs without any take of blood, the phenomenon known as “autogeny”. Some sand flies take single blood for eggs development while others need it several times. In temperate region of the Old World sand flies species are abundant in summer season, while in tropical areas they may be present throughout the year (Alder and Theodor, 1957).

Little information about the early stages of sand flies particularly the larval habitats of a handful species are available. Mostly they are taken from a suspected soil from inside houses or cattle sheds. Sand flies are recovered from rodent burrows, leaf litter of forest floor and termite’s mounds (Mutinga *et al*., 1989). *Longipalpis* species of New World are found in cattle sheds (Forattini, 1954; Deane and Deane, 1957). Reportedly, sand flies breeding sites have been identified in various ways; such as incubation of the soil or by placing emerging traps on the suspected sites but still detailed information about the sand fly breeding related aspects are not sufficient. Searching for developmental stage of sand flies in their natural biotopes is tedious and has proven to be unproductive (Killick-Kendrick, 1986, 1999).

1.5.2 Adult behaviors

1.5.2.1 Resting sites (indoor and outdoor)

Sand flies habitats are usually associated with ecosystem having stable temperature, high relative humidity and organic matters presenting suitable breeding and better resting sites during day times (Feliciangeli *et al*., 2004). Sand flies endophilic and exophilic studies are more important from the control point of view (Gebresilassie *et al*., 2015) because they are the determining factors that where transmission of infection take place. All sand flies may be exophilic (outdoor resting) or endophilic

(indoor resting). During day times they are usually found in dark bathrooms, humid corners, behind clothes, pictures, caves, crevices, tree holes, stone walls, animal habituations, termite mounds and around human houses (Samir, 2000). Gebresilassie *et al.,* (2015) found *P. orientalis* males are more abundant in outdoor habitats (agriculture field) while females of this species have low endophilic behaviour in Northern Ethiopia.

In southern Iran *P. papatasi* are frequently present in indoor sites while *P. major* presented their greater distribution in peridomestic places. *P. sergenti* and *P. bergeroti*-in Saudi Arabia prefer domestic pattern of behaviour.-Domestic or peridomestic sand flies commonly feed on humans are endophilic e.g. *P. papatasi* in Old World and *L. Longipalpis* in New World.

Due to their hopping movement (short flight with many landings) sand flies disperse with a short range from their breeding sites. However, female sand flies during feeding activities may travel up to 2200m (WHO, 1984). Sand flies relationships with cattle sheds and corrals

Close association among sand flies, cattle sheds and corrals in both old and new worlds are found. These are the preferred breeding sites for various species of sand flies. *P. argentipes* the proven vector of VL in India preferentially feed on a variety of domestic animals inside and around human houses which are the risk factors of the disease development in humans (Palit *et al.,* 2005). Poche *et al.,* (2011) found in three villages of Bihar (India) that sand flies prefer cattle's shed because they observed that a large number of sand flies were emerged from these shed. In during summer season farmers ties their livestock in cattle corrals (few meters away from the human

sleeping sites) which may be treated as sand flies breeding sites because sufficient organic manure are present in these places.

Elaagip *et al*., (2010) isolated seven species of sand flies from three types of cattle shelters (goat, cow and sheep) in two villages of Eastern Sudan with *Sergentomyia* species found in more abundance as compared to *Phlebotomus* in these sites. They (loc. cit.) sampled females of all species in high ratio from the shelters of sheep followed by that of goat and cows indicating each specie's specific ecological requirements around human dwellings or domestic animals. These sites may be treated as resting, feeding, breeding or larval developmental places for various sand flies species. In central Morocco, inhabitants are living in shanty towns where cattle's droppings and junk are present in open environment without any proper control measures, providing suitable sites for sand fly oviposition (Lahouiti *et al.*, 2013).

1.5.2.2 Sand flies species existence in caves

Among the breeding and resting sites, caves have received little attention. Sand flies of caves are very important medically, because tourists visiting these sites are at great risk of infection and transmission of Leishmaniasis (Vilela *et al*., 2011). Historically, a few research works are available on caves revealing that caves provide suitable breeding sites for sand flies (Galati *et al*., 2006). Alves *et al*., (2011) isolated 15 species of sand flies from different limestone caves in Amazon and concluded that the presence of sand flies in caves are directly related with the presence of organic matters (guano) and the presence of birds, rodents, and reptiles which may provide blood source for their life activities.

Similarly in Brazil, Galati *et al*., (2010) recovered 11 species of sand flies from different caves. Alves *et al*., 2011 examined the diversity of caves flies and suggested

that guano inside caves may provide breeding sites for sand flies. The distribution of sand flies varies from caves to caves on the basis of temperature, humidity and food availability (Auler and Zogbi, 2005; White and Culver, 2005). Carvalho *et al*., (2013), demonstrated that in Brazil caves are sometimes opened for public before any biological or geological studies. This might loss the biodiversity and geological records of these sites. Reportedly, caves have unique ecosystems as compared to external environment. Commonly three types of faunal adaption are found in caves; troglobites (animals having unique modification to caves), troglophiles (animals with no modification but adopt caves life), troglopoxenes (animals using caves for shelter and refuge). Caves have a geographical barriers having distinct ecosystem. Such sites are very important for exploring new genera and species of sand flies (Galati *et al*., 2003).

Both in Old and New Worlds caves provide suitable environment and shelter for sand flies because of permanent darkness. Cave sand flies have the ability to re-colonize villages once they were cleared from the stated sites. In Central Asia and Brazil cave sand flies are involved in the transmission of Leishmaniasis to the locals and tourists (Lewis, 1971). *P. martini* is the main vector of termite Kala Azar in Kenya which take rest in microhabitats of termites hills (Minter, 1963).

1.5.3 Feeding habits and vector sand flies

Both sexes feed on specific plant's sugary secretion, which mainly provides energy for their life activities and-such plants restrict its distribution. Their sugar meal usually contains sap of plants and honeydew of aphids. They are usually nocturnal in their activities but some may bite during day times in darks rooms, forests and some have peak hours of biting during night times. Generally they bite humans on their

exposed parts such as face, hands, arms, legs, ankles and the parts which are not covered properly during sleeping or when sitting outdoors (Rozendaal, 1997; Wijers and Ngoka, 1962).

In addition, female sand flies take blood from a variety of vertebrates for their eggs development. Each species of sand flies have specific blood hosts. Sand flies on the basis of mandibles and other structures in buccal armature are divided into two groups; first group (*Phlebotomus* and *Lutzomyia*) takes blood from mammals and the second group with one genus (*Sergentomyia*) feeds on reptiles (Alder and Theodor, 1957). Females of some species were the vectors of many human and other animal diseases. *S. babu* in India transmit reptilian leishmaniasis (*L. tarentolae*) and *P. argentipes* is main vector of VL in the whole country (Mukherjee *et at.*, 1997). *P. papatasi* distributed in old World (Africa, Asia and Europe) is the confirmed vector of *L. major* and many other *Phleboviruses* (Ashford, 2001). *P. alexandri* (anthrophilic) is the confirmed vector of *L. donovani* and suspected vector of *L. infantum* (Azizi *et al*., 2006).

In Northwest Iran, *P. kandelakii* and *P. perfilewi* were examined to transmit VL while other two species *P. major* and *P. keshiskeani* were considered as important VL vectors (Sahabi *et al*., 1992; Azizi *et al*., 2008). In New world, *L. longipalpis* is the proven vector of *L. infantum* (Oliveria *et al*., 2008). In Turkey *P. sergenti* and *P. tobbi* are the proven vectors of *L. tropica* while VL parasites were also isolated from *P. tobbi* in the region (Svobodova *et al*., 2009).

In Italy, four species of *Phlebotomus* genus (*P. pernicious, P. perfilewi, P. ariasi* and *P. neglectus*) are the proven or suspected vectors VL (Ready *et al*., 2010). In Northern Ethiopia, *P. orientalis* is the proven vector of VL in endemic areas (Elnaiem *et al*.,

1997). In Israel, *P. sergenti* was shown to be the vector of *L. tropica* (Schnur *et al*., 2004).

Sand flies like other Dipterans require sugar meal for life activities. Muller and Schliden (2004) reported that sand flies take plant juices (sugar meals) for survival and other life activities in open environment where they live. It has been searched out that flies prefer different parts (leaves, stem and flowers) of plants for taking food. Both sexes require plant juices but slight differences in order of preference do occur in different regions of the world. In general sand flies attraction to plants is based on CO_2 emission or other plants chemicals that affect olfactory receptors (Schliden and Jacobson, 2008). Junnila *et al*., (2011) reported that sand flies show more attraction to fruit plants.

1.5.4 Courtship behavior in sand flies

A number of behavioural patterns are involved during mating such as male wing fanning to a female or female wing fanning to an approaching male. In the natural environment mating occur mostly on or near the host where they get together. According to Kelly and Dye (1997), sand flies pheromones and host kairomones play important role in this attraction. It has been suggested that first the males aggregate near the host which is followed by females. Male often form leks which attract females for blood meal and increase their chances of mating (Jarvis and Rutledge, 1992). Lawyer and Perkins, (2000) and Lane *et al*., (1985) observed trans –beta-farnesene (aphid alarm pheromone) in the tergal spots of male sand flies which are involved in sand flies aggregation. Hall (1994) investigated that male sand flies produce 'Love song' prior to mating. Males produced these sounds when they vibrate their wings which are different in species of the same population.

1.6 SAND FLIES ECOLOGY

1.6.1 Distribution

Sand flies are mainly distributed in warm and temperate regions of the world. All sand flies are terrestrial in nature therefore exist in vast areas of the world specially in dry and open environment with a few species inhabiting forests (*Lutzomyia*). They are not uniformly distributed in the forests but occupy specific habitats (microhabitats). Some species of sand flies have a unique adoption to live in cold environment for example Kirgizia (USSR), Beauvais (north of Paris), Kamloops (Canada) and Isle of Jersey (Lewis, 1971). Wind speed is observed to be the major limiting factor in sand flies distribution, like in Uzbekistan, wind blows most of the times so sand flies prefer to settle inside instead in the open environment. They take protection in rodent burrows when in wind, even in low speeded wind, sand flies could not bite humans in open environment (Hoogstraal *et al.*, 1962).

Reportedly sand flies are more active between 25-28C^0 and below this temperature they are not able to bite human beings. Similarly, type of soil and height from sea level are very important for sand flies distribution. In Old World sand flies species are found at great altitude, while in South America sand flies exist at an altitude of 2800m. Moreover, deforestation and urbanization also play a key role in the distribution of sand flies (Minter, 1964). There are no scientific evidences for sand flies transportation except *S. babu* in India where they are seen in trains and river steamers (Lewis, 1971).

Aslamkhan (1998) described the distribution of Pakistani sand flies which are listed below.

Table 1.1: Area wise distribution of sand flies in Pakistan

Species	Baluchistan	Sindh	Punjab	Khyber Pakhtunkhwa	Northern Areas	Kashmir
Genus *Phlebotomus*						
P. alexandri	-	+	-	-	+	-
P. argentipes	+	-	+	-	+	-
P. colabaensis	+	-	+	-	-	-
P. eleanorae	+	-	-	-	-	-
P. kandelakii burneyi	-	-	-	+	+	-
P. keshishiani	-	-	+	-	+	-
P. papatasi	+	+	+	+	+	+
P. longiductus	-	-	+	-	+	+
P. major	-	-	+	+	-	+
P. nuri	-	-	+	+	-	-
P. salehi	+	-	-	-	-	-
P. sergenti	+	+	+	+	+	+
Genus *Sergentomyia*						
S. africana magna form asiastica	-	+	-	-	-	-
S. babu	-	-	+	+	+	-
S. baghdadis	+	+	+	+	-	+
S. bailyi	-	+	+	+	.-	-
S. christophersi	-	-	+	+	-	-
S. clydei	-	+	+	+	-	-

S. dentate	+	-	+	+	+	-
S. grekovi	-	-	-	-	+	-
S. hodgsoni	-	-	+	+	+	-
S. hospitii	-	-	+	+	+	+
S. indica	+	+	+	+	-	+
S. montana	-	-	+	+	+	+
S. palestinensis	-	-	-	+	-	-
S. punjabensis	-	-	+	+	-	+
S. shorttii	-	-	+	+	-	-
S. theodori pashtunica	+	+	+	+	-	-
S. tiberidis pakistatiica	-	-	-	+	-	-
29 species	10	9	21	21	13	8

1.6.2 Sand flies sampling from their resting and breeding Sites

A variety of collection methods are used to capture of sand flies with further and more new techniques are developing for collecting various stages of sand flies. Generally adult sand flies in both Worlds are collected through sticky traps, CDC (Centre for Disease Control) light traps, CO_2 traps, insecticide spray and mouth aspirators. All traps are divided into two broad categories; interceptive traps and attractive traps which are used in the field of sand flies (Alexander, 2000). Interceptive traps like sticky traps randomly collect flies in an area and are used for determining sand flies species composition of a selected area (Kasap *et al*., 2009). On the other hand CDC and CO_2 are attractive traps for sand flies, including phototropic flies and are used for species composition and population estimation of sand flies (Kasap *et al.,* 2009). Shannon traps are used for reporting anthropophagic species of sand flies (Perez *et*

al., 1988). Flit method (insecticide spray) are used for sand flies collection in closed places while aspiration is applied for individual fly (Sawalha, 2000).

1.6.3 Seasonality in sand flies densities

The information about abiotic factors on sand flies densities could be used in designing most effective control strategies together with the determination of expected period of disease transmission. The environmental changes vary according to the geographical regions of the world. In temperate regions flies abundance are associated with temperature showing main activity in summer season and generally with a single generation period while in tropical zones sand flies are found in both dry and wet seasons of the year (Lewis, 1974). In Midwest Brazil, *L. longipalpis* have a bimodal (appears in two seasons; dry and wet) pattern of behaviour but usually form aggregate population in wet season of the year and have a negative correlation with wind speed (Oliveria *et al*., 2011). Quate (1964) divided sand flies of the Upper Nile into two groups; seasonal species (appear in dry season) and non-seasonal species (present throughout the year).

Elnaiem (2011) reviewed the ecology of *P. orientalis* in East Africa and found that this is seasonal species (active in dry season) and have close relationship with climatic factors. Balen and Alten (2011) found that *P. tobbi* in Turkey start life activities in May, form peak aggregation in August and disappear in October. In Marrakesh, Boussaa *et al.,* (2005) investigated that *P. sergenti shows* mono-model annual pattern of behaviour (appears in summer season) and active from May to October while *P. papatasi* have bi-model distribution (present throughout the year). *P. papatasi* collected indoor form aggregation in May while *P. major* shows peak activities in October and November in Southern Iran (Kassiri *et al*., 2013). In Israel, sand flies

abundance were found from May to October and usually form peak aggregation in June and August (Kravchenko *et al.*, 2004).

In Oriental region sand flies are abundant in hottest months of the year and shows two generation period. Lahuitii *et al.*, (2013) found a positive correlation between sand flies density and annual temperature and negative association with humidity. (Lewis, 1978; Dhanda *et al.*, 1983). Negative association of sand flies with wind speed was reported in Brazil (Oliveria *et al.*, 2013).

1.6.4 Sand flies habitat mapping and the role of GIS (Geographic Information System)

Studies about RS, GIS and spatial analysis were started recently particularly in the last 25 years (Thomson and Connor, 2000; Hay *et al.*, 2002). Both epidemiologists and health workers are working to design maps which are excellent means of communication, spatial dynamics of disease pattern and their transmission. Remote sensing (RS), geographical information system (GIS) and spatial analysis (spatial statistics, geo statistics and spatial distribution processes models) are important tools which are used for the epidemiological studies. Such technologies play an important role in disease mapping, eco-environmental analysis, and parasites occurrence as well as to highlight surveillance of different parasitic diseases (Cringoli *et al.*, 2005). Such approaches stress to find out the link between disease development and their possible explanatory variables (Buhina, 2011).

Currently, ecological and remote sensing techniques have been used for a number of diseases such as Dengue (Carbajo *et al.*, 2001), Onchocerciosis (Thomson *et al.*, 2000). Modern approaches such as RS, GIS and bioinformatics are very important for vector ecology and other environmental factors (Bhunia *et al.*, 2011). In

epidemiology, environmental factors (temperature, humidity), vegetation and land surface are very important factors which can easily be investigated from remotely sensed data (Graham *et al.*, 2004). Sometimes seasonal environmental changes alter transmission risk pattern of a disease, in such a situation RS provide accurate data to map and project these transmission pattern (Graham, 2004).

Close association between sand flies and other environmental factors have been found e.g. in Kenya and Columbia sand flies were abundant closed to the canopy forests (Basimike *et al*., 1992; Travi *et al*., 2002). The availability of land cover data can be used at regional level to identify sand fly habitats where public is at higher risk of exposure (Claborn *et al.*, 2008). With the help of RS and GIS the breeding sites of *P. argentipes* can be observed directly from space (Buhina *et al*., 2011). In Egypt, GIS based predictive maps were designed for sand flies species distribution, which analysed and found some environmental factors (climatic factors and elevation) were directly related to the presence or absence of flies distribution and disease risk (Kassem *et al*., 2012)

1.7 MEDICAL IMPORTANCE

Some female Phlebotomine sand flies are biological vectors of many human diseases important one are visceral Leishmaniasis (VL), cutaneous leishmaniasis (CL), Bartonelosis (Oroya fever) and sand fly fever (three–day fever) (Lane, 1993).

1.7.1 Bartonellosis

It is a type of bacterial disease caused by gram negative facultative bacterium *Bartonella basilifornis*. Sand flies are the important vectors of this bacteria, while sometimes ticks, fleas as well as mosquitoes are also involved in their transmission. Barton discovered this bacterium in 1905 for the first time and described its original

structure in 1909. Historically, they infect human beings from thousands of years. Reportedly there are eight sub species of this bacterium. Epidemiologically, this species is distributed in Peru, Ecuador as well as in Colombia where their main vectors are sand flies species belonging to the genus *Lutzomyia* (Wikipedia, 2015).

Until 1993, there was only one species in the genus *Bartonlla*, but now more than 23 species are reported for this genus (Maroli *et al*., 2012). Medically there are two phases of the disease; acute phase (Carrion´s disease or Oroya fever) and chronic phase (Verruga peruana or Peruvian wart). In the former case, the bacterium invades the human red blood cells due to which haemolysis and fever occur. If the infection is left untreated, fatality rate is 40 to 85% patients in this phase. The infection can be more complicated by overwhelming infections primarily by *Salmonella* species, *Toxoplasma gondii* and *Pneumocystis jirovecci*. The latter, chronic phase is characterized by eruption of lesions malaise and osteoarticular pain and finally bleeding. *Bartonella* can be isolated from blood cultures and secretion of the lesions in people from endemic areas (Maroli *et al.*, 2012).

1.7.2 Sand fly fever

Sand flies also transmit various *Phleboviruses* which are responsible for febrile illness as well as severe neuroinvasive disease. The virus with its different forms has been studied in humans and domestic animals in sand fly endemic areas. The disease is mostly present in travellers, soldiers and people in endemic areas. Sand flies born viruses belongs to families Bunyavaridae, Rhebdoviridae and Reoviridae. These viruses are distributed in Asia, Middle East, Central Asia, Indian Subcontinent and Mediterranean regions (Alkan *et al*., 2013). An accurate estimation of infection rates

in the world is not possible because of insufficient data. A total of nine viruses have been reported in the world (King *et al.,* 2011).

These disease causing pathogens are transmitted by sand flies in both Old and New Worlds. All Phleboviruses have a tri-segmented (L, M and S segments) single strand genome. Recently a number of sand fly viruses have been isolated from humans and sand flies (Alkan *et al*., 2013). In Italy these viruses are also reported from bats (Verani *et al*., 1988). In the active season (May- October) of sand flies these viruses are transmitted to humans and other animals. The reservoir hosts are not fully identified yet. Generally sand flies have dead-end hosts. They are found in both sexes and their tranoverial (vertical) transmission has not been fully studied. There maintenance and transmission mainly depends on sand flies vectors species.

Pick (1886) described these viruses for the first time in Balkans region, where the disease was prevalent in local population posing a great risk for visitors to the region. The disease was named Papatasi fever or three day fever. They circulate in sand flies each year in endemic areas where they infect a large number of human populations. During WWII a large number of allied troops of British and Germany were infected with these viruses (Sabin, 1951; Hertig and Sabin, 1964).

1.7.3 Leishmaniasis

Currently leishmaniasis is present in 88 countries of the world including Pakistan (Alexandar and Maroli, 2003). Morphologically, *Leishmania* parasite exist in two distinct forms; extra-cellular promastigotes having a prominent flagella found in the gut of the vectors and amstigotes which lack flagella, are present in the phagocytosomes of host macrophages. Cultural form of *Leishmania* parasites are morphologically identical to that present in the alimentary canal of sand flies (Mazumdar *et al*., 1993). In the tropical, subtropical and temperate regions the disease

spreading largely depends on sand flies distribution (Chang *et al.*, 1985). Sand flies species in two genera, *Phlebotomus* in OW and *Lutzomyia* in NW are involved in the spreading of the disease. Currently 98 species are proven or suspected vectors which exist in various parts of the world (Maroli *et al.*, 2012).

1.7.3.1 Visceral Leishmaniasis (VL)

VL is one of the fatal form of leishmaniasis if left untreated. Their major signs include spleen enlargement, irregular fever, loss of weight, enlarged liver and lymph nodes, anaemia, cough and diarrhoea. Sometimes the disease mimics the sings of other important diseases like malaria, TB and splenomegaly (WHO, 1996).

Dogs are the reservoir hosts of VL in Pakistan. Rab *et al.*, (1995), examined domesticated dogs and found anti-*Leishmania* antibodies. They also isolated parasites from infected dogs and identified *L. infantum* through molecular techniques. Therefore, the role of dogs as the reservoir of VL in these endemic foci in northern areas of Pakistan, AJ&K and KP areas are confirmed.

In neighbour country India the *P. argentipes* is the proven vector of VL. Currently there is no authentic confirmation of the sand fly vector of this disease in the country (Kakar, 2010). *P. kandelakii burneyi, P. keshishiani, P. major* are suspected vectors in Pakistan. *P. hindustanicus* may be a probable vector because their females were found infected with *L. infantum* in Azad Jammu and Kashmir (Munir, 1994).

1.7.3.2 Cutaneous Leishmaniasis

Cutaneous Leishmaniasis locally known as ‘kaal Dana’ permanently disfiguring the exposed parts especially face (Kassi *et al.*, 2008). The disease is wide spread present throughout the Pakistan. Epidemiologists and health workers investigated that the infection is most frequent in Baluchistan KPK, Punjab and Sindh provinces of

Pakistan (Shakila *et al*., 2006). Rowland *et al.,* (1999) suggested that women and children being particularly affected by this disease in Pakistan and Afghanistan. Reportedly, socioeconomic development of a society has a direct association with this neglected tropical diseases (Kassi *et al*., 2004).

Keeping in view CL is presumed to be anthroponotic with no animal reservoir. Burney and Lari (1986) suggested that rodents in Pakistan are the reservoir of CL. In Balochistan, amstigotes of CL have been identified (Rab *et al.,* 1986; Kakar, 2004).

Up till now no authentic information are available about the vectors of CL in Pakistan. Rowland *et al.,* (1999) suggested that *P. sergenti* and *P. papatasi* are the suspected vectors of CL in Pakistan. There may be possibility that some other species of genus *Phlebotomus* are involved in the transmission of CL in the country (Kakar, 2008).

1.8 STUDIES ON SAND FLIES IN PAKISTAN

Pakistan has a diverse fauna of sand flies because parts of two zoogeographical regions of the world are included in it. Sand flies of Pakistan are predominantly Palearctic and Oriental however some species may have enter from Ethiopian region (Lewis, 1967). Pakistan comprised great plains of Indus and its tributaries in Sindh and West Punjab, high mountains of the North, hills and plateau in the South-West of Peshawar as well as northern Kashmir. This may suggest that the country provide a wide range of habitats for a variety of species of sand flies (Lewis, 1974).

Sinton (1924; 1927) and Anderson (1939) were perhaps the first ones who initiated study on sand flies of sub-continent including areas later on included in Pakistan. After 1947 (when Pakistan was created) quite a few attempts were made to describe sand fly fauna of different areas of Pakistan, particularly those from where frequent

cases of leishmaniasis were reported (Qutubuddin, 1951; Nasir 1958; Barnett and Suyemoto, 1961; Ahmad and Burney, 1962; Lewis, 1967, Burney *et al.,* 1979). Among them Lewis (1967) study is the most comprehensive study of sand fly fauna collected from various parts of Pakistan sand fly vector relation to human diseases are also included.

Aslamkhan, (1996) worked on the Biodiversity of sand flies of Pakistan and also reviewed the literature on 29 recorded sand flies species of Pakistan in 1998. Since 1996 to 2001 extensive studies on sand flies of Baluchistan (South West Pakistan) was conducted by Kakar (2004a and 2004b) who reported 37 species of sand flies from various parts of the country. More species may be expected to occur because of the untreated areas of the country especially, FATA, KPK, some parts of Sindh (Shakila *et al.,* 2006). Ali *et al.*, (2015) recorded 14 species of sand flies from North Waziristan Agency (Pakistan).

Dir sandflies are unexplored despite the frequent cases of cutaneous leishmaniasis. This is the first detailed study about the species composition, relative abundance and seasonal variation of sand fly species in districts of Dir.

1.9 OBJECTIVES OF THE STUDY

The aims of our study were to determine

1) Species composition and distribution of sand flies in Dir districts
2) Seasonal variation of sand flies
3) Sand flies association with abiotic factors of environment (temperature and relative humidity)
4) Density and diversity of flies
5) Flies Habitat analysis
6) Breeding (Domestic and Peridomestic) sites
7) Sand flies species relationship with different plants families
8) Risk factors associated with Leishmaniasis and flies habitats mapping
9) Determining most suitable sticky oil for sand flies collection in field using sticky traps.

CHAPTER - 02
MATERIALS AND METHODS

2.1 STUDY AREA

Dir districts lies in the Khyber Pakhtunkhwa province of Pakistan and are located in the northern area of the country. It is in the South of district Chitral between 35° 50' and 34° 22' N and 71° 2' and 72° 3' E, taking its name from the village of Dir, the headquarter of the former rulers. Initially it was a state, ruled by Nawabi- Dir and in 1970 it was merged in Pakistan. In 1996 district Dir was bifurcated into two separate entities i.e., district Lower and Upper Dir. In the North West of the districts is the district of Chitral, in the South is the Malakand Agency and in the East is the district of Swat, while in the West adjoins Afghanistan and Bajwar Agency. The total area of both districts is of about 5282km$^{2.}$ (3698 km^2 Upper Dir and 1584 km^2 Lower Dir). There are limited facilities of education, health, agriculture, transportation and communication.

Main tribes of the area are Yousafzai Pathan (Utman Khael, Akhund AIyas and Painda Khael) and Afghan refugees inhabiting different parts of the districts. There is a general trend of people going out for work within or outside the country (mostly to Middle East). Topographically, Dir (Upper and Lower) is divided into six parts; Dir valley Jandool Valley, Maidan Valley, Passes (Daray), several plain areas and mountains. Mostly the districts area are hilly and surrounded on all sides by high mountains of Hindu Kush Range. Panjkora is the leading river in the region. There are distinct summer and winter seasons but in winter many areas of the both districts covered with heavy snowfalls. Maximum of 241.23mm of rainfalls occur in winter and spring. Temperature ranges between 34.45^0 C in summer and -2.39^0 C in winter.

2.2 STUDY DESIGN

The present entomological survey was carried out in four tehsils where three (Timergara, Balambat, Khall) are in Lower Dir and One (Warae) is in Upper Dir.

2.2.1 Tehsil Timergara

It is the main tehsil of Lower Dir situated in the Centre of district and on right side of the river Panjkura which is the business hub of Dir. It shares its Northern part with Khall, West with Balambat, East with Adenzae and South with Arang (Bajwar Agency) separated by river Panjkora. Surrounding areas of Timergara Bazaar is densely populated including inhabitants migrated from other parts of districts. People living in this area have mostly concrete made houses. While in outskirts (rural area) people have predominantly mud made or iron sheet houses. The flora and fauna of the area are rich but no organized data is available about the number and type of species.

The survey was carried out randomly in seven villages including two Afghan Refugees Camps (1 and 2), Yarkhan Banda, Danvah, Siar, Saddo and Sikaolae. A total of 15-20 houses were randomly selected for sand flies collection in each village in this tehsil. Leishmaniasis infection were observed in both Afghan Refugees camps, Danvah, Siar and Saddo respectively.

2.2.2 Tehsil Balambat

This tehsil comprises plain area on the river bank and high hills in the mountainous sites. It share its borders with Timergara in East, Summer Bagh in West, Maidan in North and Khazana (Summer Bagh) in South. Balambat, flora and fauna were similar with other two tehsil (Timergara and Khall). Richest peoples of the districts are residing in this area. All the three types of houses (mud, concrete and stone made) were included in the survey but mostly these were concreted.

A total of 17 villages (15-34 houses/ village) fulfilling our study criteria were visited. These includes Khima, Shatae, Palusoo, Haji Abad, Shzadi, Koto, Munji, Manzari Tangae, Baroon, Mandish, Malae, Rani, Dab, Kotkae, Shagae, Tangae and Nawagae respectively. Leishmaniasis cases were observed in Shagae, Kotkae, Palusoo, Nawagae, Dab, Haji Abad, Rani, Baroon and Munji. Fertile soil was present on the river bank.

2.2.3 Tehsil Khall

This tehsil was present on both sides of river. A total of thirteen villages (Kalone, Sacha, Mira, Watange, Khall Banda, Shalfalm, Sarmu Obu, Dwaba, Lower and Upper Lalo, Luqman Banda) were visited for flies sampling. Topographically, most villages were located in mountainous areas. A total of 10-20 houses/village were randomly surveyed. Houses in these villages were either concreted or mud made and in some cases iron sheeted.

There plants biodiversity varied in different locations having tall tree while in some parts dense forests were found. This site has the same animal fauna as in Timergara. On both sides of the river the land is fertile and some seasonal crops, vegetables and fruits were cultivated. Leishmaniasis cases were observed in eight villages (Mira, Watange, Shalfalm, Sarmu Obu, Dwaba, Lower and Upper Lalo and Kalone).

2.2.4 Tehsil Warae (Upper Dir)

This tehsil was the first part of Upper Dir while entering from Lower Dir. It is located on both sides of the river. Sand flies were captures from four villages (Warae Bala, Akhagram, Toor Mung kalone and Jugha Banj). Like in other villages different types of houses were found but concreted houses were dominant. The environmental

variables as well as flora and fauna of this tehsil were largely different from other three tehsils. Leishmaniasis disease cases were reported in Akhagram.

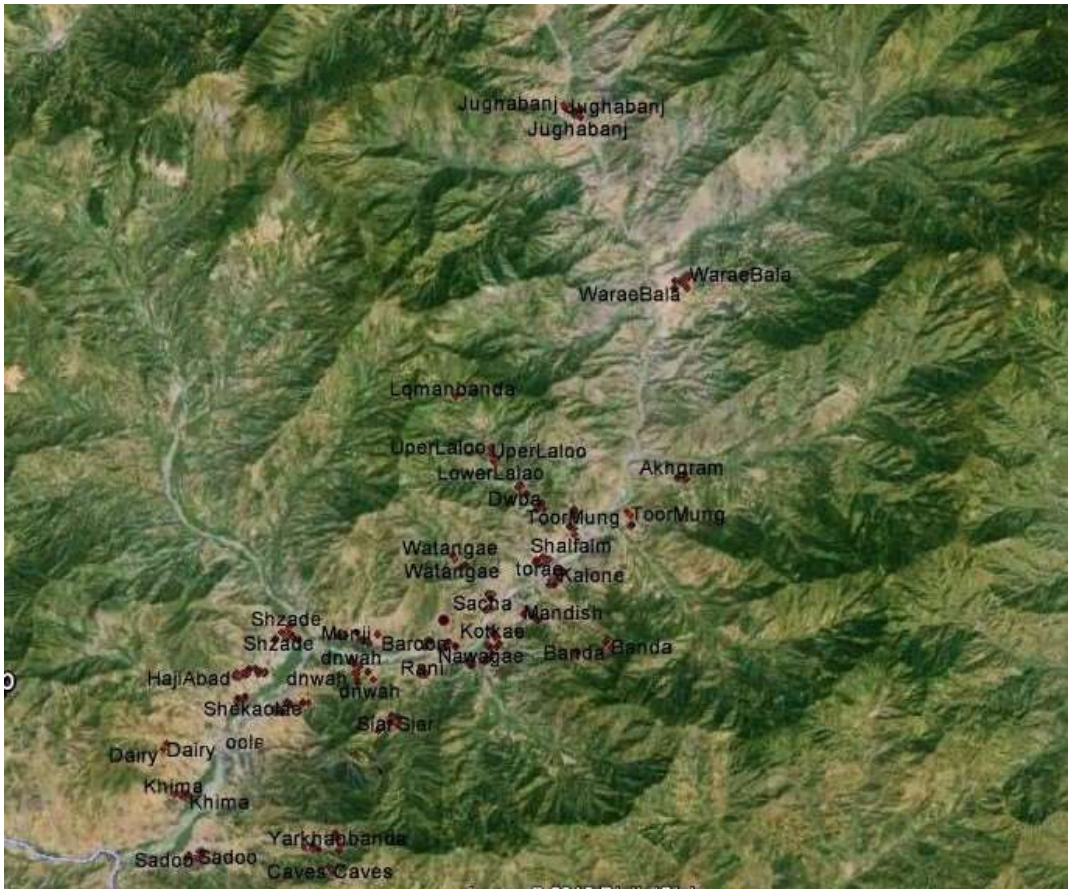

Fig. 2.1: Google earth map of the study sites

2.3 COLLECTION PROCEDURES

Sand flies were collected through different methods such as sticky traps, aspirator and flit method. During the survey two times collections (day and night) were made from indoor and outdoor sites. Sand flies from domestic habitats were sampled through sticky traps, flit method and aspirator. While peridomestic flies were trapped through sticky traps only.

2.3.1 Sticky traps

Ordinary A-4 white paper (20 × 30 cm) smeared on both sides with castor oil and fixed at a height of 30 cm above the ground. These traps were fixed before 7:00 PM in

the evening and were recovered before 7:00 AM in the following morning. A total of 3-4 traps/ house were distributed in various parts of inside the houses and variable number of trapping were spread in peridomestic environment. Trapping method was carried out in Khima, Manzari Tangae, Mandish, Malae, Afghan Refugees Camps (1 & 2), Khall Banda, Yarkhan Banda, Sacha, Mira, Watange, Shalfalm, Barkale, Dwaba, Sarmu Obu, Luqman Banda, Upper and Lower Lalo.

This method was mainly used for the collection of flies in mud made houses, combined dwellings (human and livestock living together), caves, cattle corrals, tree holes, plants, fresh and dry dung cattle dungs, drainage holes in walls and chicken sheds.

Sand flies were collected with the help of a fine hair brush and placed in methanol for a few minutes to remove oil and other debris from the flies before transferring to properly labelled vials containing 70% alcohol. After removing the flies and other insects, these traps were used for the next3-4 time's collections.

2.3.2 Flit Method

Bed rooms and bathrooms (in door sites) of the houses were selected for the collection of sand flies. Diurnal sand flies sampling were carried out through this method.

A total of 23 villages; Saddo, Sikaolae, Dhiry, Palusoo, Haji Abad, Koto, Shzadi, Munji, Baroon, Kalone (Khall), Kalone (Toor Mung), Akhagram, Warae Bala, Jugha Banj, Dab, Kotkae, Tangae, Shagae, Nawagae, Rani, Siar, Danvah were sampled for sand flies collection. White bed sheet were used for the collection on floor and beds etc. All the outlets including doors, windows and ventilators were closed and Pyrethroid insecticide spray with a formula (Tetra- methrin 0.30% w/w, d-Allethrin

0.12% w/w and cypermethrin 0.07% w/w with a trade name of BOP insecticide) was sprayed for a few seconds and the rooms were kept closed for 25 minutes.

The dead sand flies on the sheet were collected with the help of a fine brush and placed in a labelled collection tube, containing silica gel covered by cotton swab. Collection through this method was mostly concentrated to concrete made houses.

2.3.3 Collection through Aspirator

Live sand flies were collected from their resting sites through aspirator mostly during day times, a few times collection was also made in the evening with the help of torch. This method was used when flies number were low below 2-4 inside houses in day times.

2.4 RECORDING OF METEOROLOGICAL PARAMETERS

Day and night time's humidity and temperature were recorded with the help of a special device (Kestrel 4500NV Pocket Weather Tracker, USA). It was programmed to collect climatological data (temperature and relative humidity). Global Position System (GPS) co-ordinates of each points were taken at the same time to be used in the analyses of sand flies ecology and epidemiology of leishmaniasis disease.

2.5 COLLECTION OF RISK FACTORS ASSOCIATED WITH LEISHMANIASIS

A standard questionnaire (appendix) regarding information about the house hold, incidence of disease and type of control measures of sand flies if used was made for data collection. The data was recovered from the heads of the surveyed houses or help was sought from the school teachers, students and health workers of the area.

2.6 SAND FLIES PROCESSES

2.6.1 Preparation of mounting media

Gum Arabica…… 8gm

Chloral hydrate ……..70grams

Acetic acid ……...3ml

Glycerine ……..5ml

Distilled water …….....10ml

Gum Arabica was dissolved in distilled water and were left for one night. Next morning other ingredients were added in the order shown. The whole mixture was gently warmed on water bath till the entirely mixture become cleared and dissolved. The solution was filtered hot through the colloid filter paper followed by evaporation until it drops and spread easily.

2.6.2 Permanent slides preparation

Permanent slides of sand flies were made by using Berle's media. Fly head was separated on a clean slide and placed ventrally to expose the mouth parts (cibarium and pharynx) required in taxonomic identification. The abdomen part was laterally placed on the slide. The prepared specimen was mounted according to the technique of Aslamkhan *et al.,* (2000) for taxonomic identification.

2.7 TAXONOMIC IDENTIFICATION

Sand fly identification involves the study of internal organs, like the shape and structure of pharyngeal and buccal armature in both sexes. The shape and segmentation of spermatheca of the female fly, while male genitalia, spines on the styles, size and the number of spines of the lateral lobe, shape of aedeagus and

paramere are the major characters used in identification. In addition length of maxillary palps segments (papal formula) is also used in the identification process. Keys describe by Lewis (1974) were primarily used for identification purpose.

2.8 DATA ANALYSIS

Data were transferred to Microsoft excel and were analysed in IBM SPSS Statistics 20. Density of monthly collected sand flies through traps were calculated with the help of formula specimens/ night/ traps. Biodiversity of sand flies were estimated by Shannon-weaver and through species richness. Sand flies abundance and seasonal activity parameters were calculated on monthly basis for each tehsil. Global Position System (GPS) co-ordinates were transferred to ArcGIS software and were analysed accordingly.

Questionnaire data was entered into a Microsoft Access database. All explanatory variables were derived, including household members, number of rooms, prevalence of active or past cases of Leishmaniasis etc. collected during the study times. The data was analysed with Stata software, version 12 (Appendix).

CHAPTER - 03
SPECIES COMPOSITION AND SEASONAL VARIATION OF PHLEBOTOMINE SAND FLY SPECIES AND EFFICIANCY OF DIFFERENT TRAPS IN FOCI OF CUTANEOUS LEISHMANIASIS IN DIR DISTRICTS

3.1 INTRODUCTION

Phlebotomine sand flies, small haemotophagus insects of the subfamily Phlebotominae transmit leishmaniasis, a group of diseases, which is considered among the top emerging diseases (Shaw, 2007). Visceral leishmaniasis is the most serious form is sporadically reported in some parts of Pakistan (Lewis 1967; Aslamkhan and Rafiq, 1980), but the most frequent leishmaniasis in the country are skin forms, normally called cutaneous leishmaniasis (CL) (Rowland *et al*., 1999; Kakar, 2004). The dimorphic life cycle of *Leishmania* (protozoan parasites) involves the blood sucking female sand flies and the mammalian host including human, with three stages i.e. development in vector (sand fly), transmission into host and development within the host (Ghosh and Bandyopadhyay, 2004).

A total of 37 species of sand flies belonging to two genera, *Phlebotomus* and *Sergentomyia* are so for reported from Pakistan but quite a few species are yet to be described (Kakar, 2004; Shakila *et al*., 2006). There is no detailed study available to determine the sand fly vectors of leishmaniasis in Pakistan, however, two species *P. papatasi* and *P. sergenti*, could be considered as suspected vectors of leishmaniasis in Pakistan (Rowland *et al*., 1999; Kakar, 2004).

The present study aimed to find out sand flies species composition, their habitats (indoor and outdoor), comparison of various collection techniques, seasonal

abundance and its relationship with environmental factors (temperature and humidity) in Dir districts where CL are being frequently reported.

3.2 MATERIAL AND METHODS

3.2.1 Sand flies Collection

Sand flies were collected on monthly basis from May to October, 2104 in two Dir districts; Lower and Upper Dir. Collection was made both during day and night times from domestic and peridomestic habitats. A total of 40 villages were surveyed during the field study. Indoor collection was carried out in 498 houses; 278 houses in day times and 220 houses night times while outdoor collection in 72 sites were conducted in Lower Dir.

3.2.1.1 Day time samplings

Sand flies collection was carried out through aspirator and flit method (Pyrethroid insecticide spray). Aspirator was used occasionally for individual sand fly collection while flit method was used for collection on larger scale from indoor habitats during day times

3.2.1.2 Night time sampling

Night time collection from both outdoor and indoor sites was made on monthly basis using sticky traps (white papers 20 cm x 30 cm coated with castor oil on both sides). Outdoor sites comprised caves, tree holes, plants, fresh cattle corrals, chicken sheds, dry cattle dung, drainage holes in walls and indoor sites included bed rooms and combined dwellings, shared by both people and animals,. This method was mainly used for collection of sand flies from mud made houses (3-4 traps per house) in Lower Dir.

3.2.2 Data Analysis

Total density of sand flies collected through sticky traps was calculated with the help of formula specimens/ night/ traps. Sand flies abundance and seasonal activity parameters were calculated on monthly basis. Climatological factors (temperature and humidity) were recorded throughout the study period using Kestrel 4500NV Pocket Weather Tracker, USA.

3.3 RESULTS

Table 3.1: Comparison of different types of castor oil, mustard oil and glycerine used for the capturing of sand flies in more than 5% abundance in Dir Districts, 2014.

Species	**Different Brands of Castor oil**						**Mustard oil**		**Glycerine**		**Grand Total**
	Marhaba company		**Micko company**		**Rahmat Pharma**						
	M/F	**Total/ %**	**M/F**	**Total/ %**	**M/F**	**Total/ %**	**M/F**	**Total/ %**	**M/F**	**Total/ %**	
P. sergenti	1855	29.90	585	9.48	71	1.15	1/0	1/0.61	-	-	**2511**
P. salengensis	2652	42.98	232	3.76	210	210/ 3.40	-	-	1/0	1/0.61	**3095**
P. major	129	2.09	-	-	-	-	-	-	-	-	**129**
P. andrejevi	-	-	35	0.56	2	0.03	-	-	-	-	**37**
S. babu	89	1.41	24	0.37	8	0.12	-	-	-	-	**120**
S. baghdadis	50	0.81	11	0.17	7	0.11	-	-	-	-	**68**
S. dentata	75	1.21	92	1.49	6	0.09	-	-	-	-	**173**
S. bailyi	47	0.76	-	-	-	-	-	-	-	-	**47**
Total	**4897**		**929**		**304**		1		1		**6180**

Sticky traps were coated on both sides with three brands of castor oil, mustard oil and glycerine (Table 3.1). Marhaba brand showed best result by capturing maximum number of sand flies individuals and species. As compared to other oils and glycerine. Therefore, rest of the study was carried out using Marhaba brand castor oil. Castor oil of three different companies namely Marhaba, Rahmat Pharma and Micko were used. The former two are national and the latter one is an international company. Marhaba castor oil gave best results among all the three brands (Table 3.1).

3.3.1 Major taxonomic characters used for the identification of sand flies

Phlebotomine sand flies were differentiated from other flies of family Psychodidae, on the basis of dense hair on the body and V-shaped wings with peculiar venation (Fig. 3.1). Male sand flies of species belonging to both *Phlebotomus* and *Sergentomyia* genera were identified on the basis of number and spines on styles, aedeagus, basal process and paramere present in external genatilia as well as number of ascoids on the antennal segments (Fig. 3.2-3.3). Similarly, number, segmentation and head of the spermatheca as well in some cases pharyngeal armature in the head region were used for the identification of female sand flies up to species level in both genera (Fig. 3.4 and 3.6).

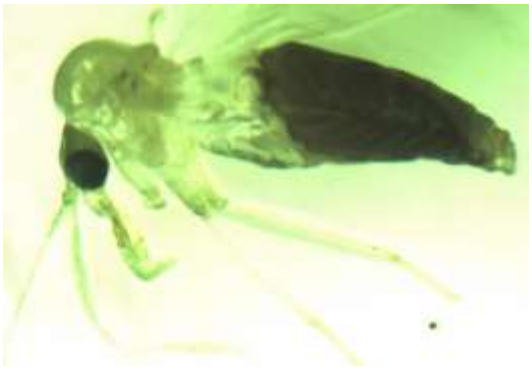

Fig. 3.1: Adult sand fly

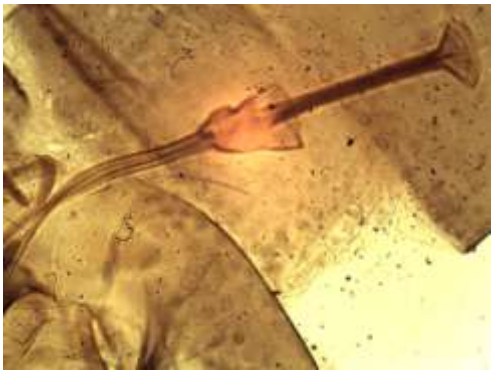

Fig. 3.2: Aedeagus of male sand fly

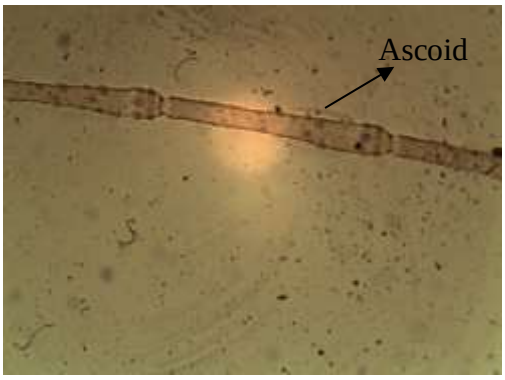

Fig. 3.3: Antennal segment with one ascoid

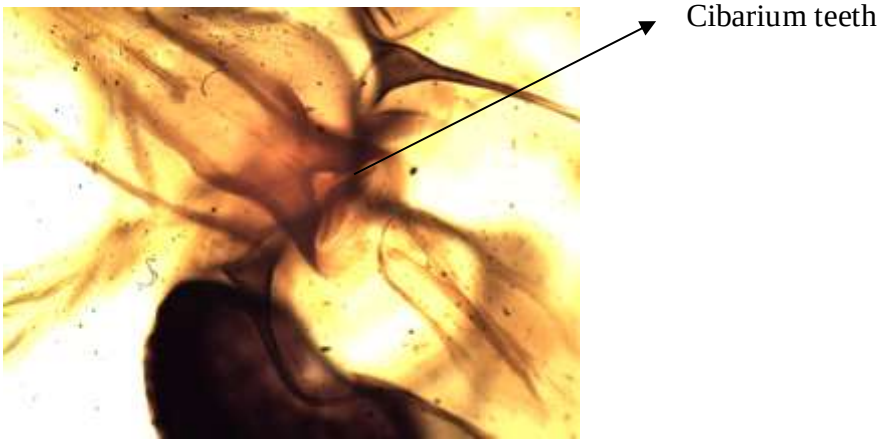

Fig.3.4: Female sand fly cibarium teeth in the head region

Fig. 3.5: Segmented spermatheca of female sand fly

Fig. 3.6: Smooth Spermathacae of sand fly

A total of 7292 sand flies were collected from domestic and peridomestic sites in Dir districts from May to October, 2014. Nine species of genus *Phlebotomus* and 11 species of *Sergentomyia* were collected. The most dominant species was *P. salengensis* (42.2%) followed by *P. Sergenti* (36.6%), *S. babu* (7.4%), *S. dentata* (4.7%), *S. baghdadis* (4.2%) and *P. major* (1.89 %). Rest of the 14 species were with less than 1% abundance (Table 3.2).

Table 3.2: Abundance and sex ratio of sand fly species collected through different collection methods, in two district of Dir, 2024

Species	Sticky traps		Insecticide spray		Aspiration		Grand Total/%	Over all M:F
	T/%	M:F	T/%	M:F	T/%	M:F		
P. salengensis	3095/99.8	3:1	6/0.19	-	-	-	3101/42.55	3:1
P. sergenti	2511/93.6	16:1	165/6.15	5:1	4/0.12	1:3	2680/36.66	22:5
S. babu	120/2.22	2:1	383/71.0	1:4	36/6.6	1:6	539/7.413	4:11
S. dentata	173/49.85	-	159/45.8	-	15/4.3	-	347/4.763	-
S. baghdadis	68/22.07	1:21	222/72.0	1:14	18/5.84	1:2	308/4.228	3:37
P. major	128/98.4	16:1	2/1.53	-	-	-	130/1.89	16:1
S. bailyi	47/92.15	2:1	4/7.84	1:1	-	-	51/0.700	3:1
P. andrejevi	37/100	-	-	-	-	-	37/0.507	-
S. hospittii	12/42.8	-	14/50	1:3	2/7.14	-	28/0.384	1:3
P. kazeroni	11/45.8	-	8/33.3	-	5/20.8	-	24/0.329	-
S. montana	1/5	-	14/70	-	5/25	-	20/0.274	-
P. papatasi	14/100	1:2	-	-	-	-	14/0.193	1:2
P. hindustanicus	4/100	-	-	-	-	-	4/0.054	-
P. ansari	2/100	-	-	-	-	-	2/0.027	-
P. bergeroti	2/100	-	-	-	-	-	2/0.027	-
S. grekovi	1/100	-	-	-	-	-	1/0.013	-
S. hodgsoni	1/100	-	-	-	-	-	1/0.013	-
S. turkistnica	1/100	-	-	-	-	-	1/0.013	-
S. tiberidis	1/100	-	-	-	-		1/0.013	-
S. theodori	-	-	1/100	-	-		1/0.013	-
Total	**6229**	**85.4**	**978**	**13.4**	**85**	**1.16**	**7292**	**3:1**

Male individuals appear to be more in number in *P. salengensis*, *P. sergenti*, *S. dentata* and *P. major* while female number exceed that of males in *S. babu* and *S. baghdadis.* However, the overall male to female ratio was 3:1 (5589/1703). Some species were represented by one sex only, like, *P. andrejevi*, *P. hindustanicus*, *S. dentata*, *S. grekovi*, *S. theodori* were represented by males while in case *P. ansari*, *P. bergeroti* , *P. kazeroni*, *S. hodgsoni*, *S. montana*, *S. tiberidis pakistanica*, *S. turkistnica* only females were found.

Sticky traps happened to be the better choice for collecting sand flies as highest number (85.4%) was collected using this technique. The other two methods were not very effective for sand flies collection. However, two species *S. babu* and *S. baghdadis* were collected in high proportion (71.0% and 72.0%) by insecticide method. This method was also, almost equally good for *S. dentata*. Moreover, more males were trapped in sticky traps, while using the other methods, though the total number was relatively low but female number exceed that of males. Results of multiple comparison (two way anova) test revealed a non-significant interaction ($p>0.05$) between the collection methods and species (Table 3.2).

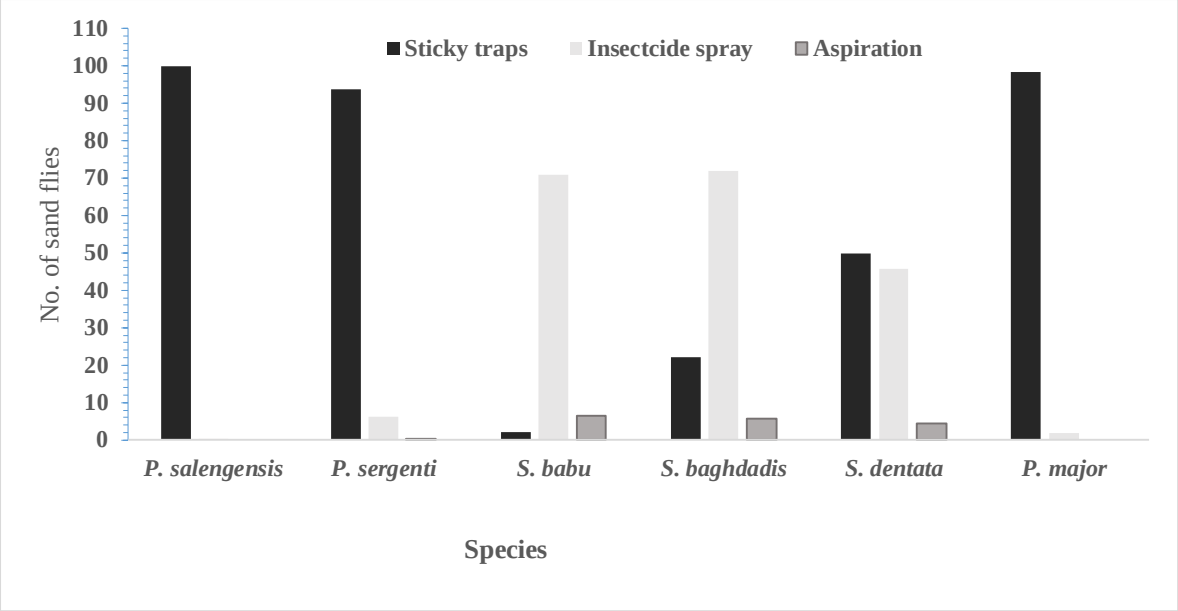

Fig. 3.7: Abundance (%) of sand fly species collected through different collection methods in Districts Dir

Table 3.3: Multiple comparison (two way Anova) testing the effect of species and methods in Dir districts, 2014

Source	Sum of Squares	df	Mean Square	F	Sig.
Intercept	2438492.190	1	2438492.190	1.585	0.335
Species	3336139.810	6	556023.302	0.908	0.521
Methods	3077932.667	2	1538966.333	2.513	0.123
Species * Methods	7349523.333	12	612460.278	-	-

Table 3.4: Abundance of sand flies species in indoor and outdoor habitats of Dir districts, 2014

Species	Indoor			Outdoor			Indoor: Outdoor
	Total/%	**M/F**	**M:F**	**Total/%**	**M/F**	**M:F**	
P. sergenti	2545/94.96	2381/164	14.5:1	135/5.03	124/11	11.2:1	19:1
P. salengensis	2774/89.45	2113/661	3.1:1	327/10.54	302/25	8.12:1	8:1
P. major	115/88.46	100/15	6.6:1	15/11.53	13/2	6.5:1	8:1
S.babu	532/98.7	99/433	0.2:4	7/1.2	0/7	-	76:1
S. baghdadis	282/91.55	24/258	0.09:10	26/8.44	0/26	-	11:1
S. dentata	283/81.55	283/0	-	64/18.44	64/0	-	4:1
S. bailyi	4/7.84	2/2	1:1	47/92.15	32/15	2.1:1	0.08:1
Total	**6535**	**5002/1533**	**3:1**	**621**	**535/86**	**6:1**	**11:1**

Among the total of 7156 individuals, 6535 were collected from indoor while only 621 were captured from outdoor habitats. A large proportion of sand flies were collected from indoor habitats than that of outdoor particularly in *P. salengensis* and *P. sergenti*. However, *S. bailyi* was predominantly present in outdoor habitats (Table 3.4 and Fig. 3.8). In most of the species males were collected far more in number than females but in cases of *S. babu* and *S. baghdadis* the ratio was in favour of females (Table 3.4). The overall male to female ratio was 3:1 for indoor and 6:1 outdoor sand flies collection. The difference in collection from these habitats was statistically not significant ($p>0.05$).

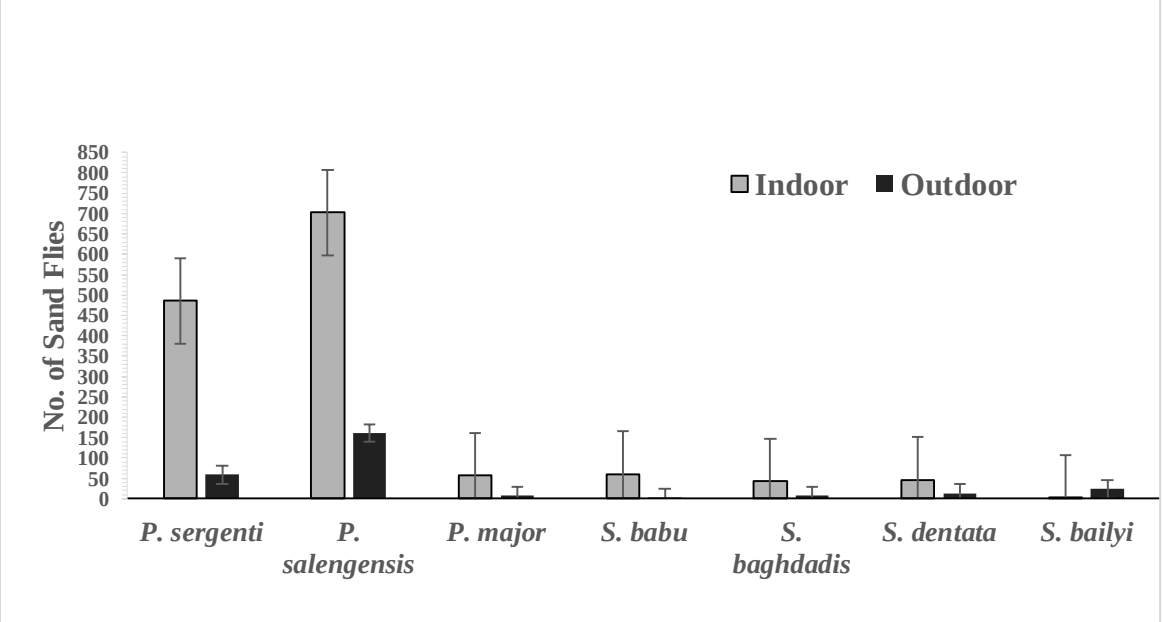

Fig. 3.8: Mean number (±SE) of sand flies collected from indoor and outdoor habitats

Table 3.5: Seasonal variation of sand flies found in night time's collection from indoor habitats in Dir districts, 2014

Months	**Species**						**Total/%**	**Average Temp**	**Average RH (%)**
	P. sergenti	***P. salengensis***	***P. major***	***S. babu***	***S. baghdadis***	***S. dentata***			
May	120	120	4	15	4	10	273/4.9	22.7	42.9
June	256	322	10	20	7	10	625/11.4	32.6	48.2
July	823	760	24	29	12	17	1665/	34.2	64.6
August	947	1085	55	29	14	5	2135/38.9	34.3	67.7
September	165	328	11	13	5	9	530/9.6	26.8	59.5
October	66	162	8	8	1	6	251/4.5	25.3	63.8
Total	**2377**	2777	112	114	43	57	5480	-	-

Table (3.5) and fig. (3.9) show monthly variation in abundance of sand fly species found in night time collection from indoor habitats. Sand flies showed their appearance in the warmer months of the year while total absence was observed in the colder parts of the year (Nov-Apr). After making their appearance in the month of May the population goes

up reaching to peak in the month of August (38.9%), starts declining in the following two months with complete disappearance in November. Population abundance seems to be associated with environmental factors, temperature and relative humidity, particularly it goes up with rise in humidity.

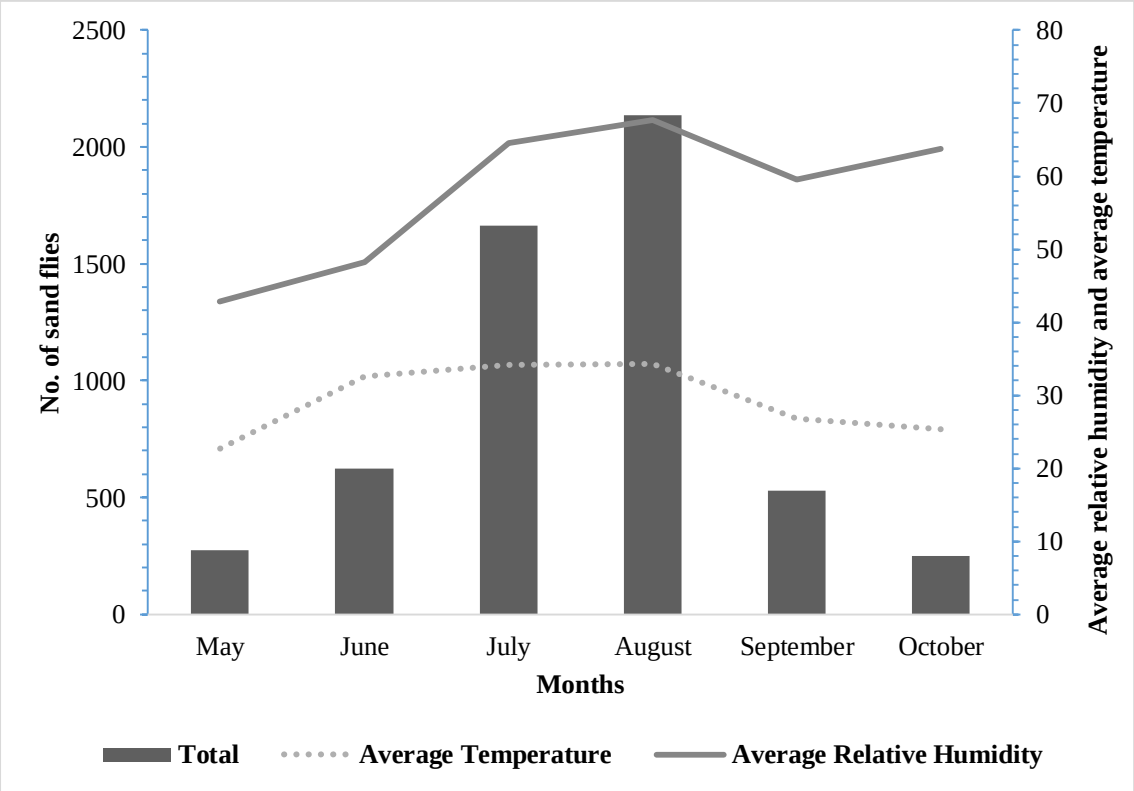

Fig. 3.9: Seasonal variation of sand flies found in night time's collection from indoor habitats in Dir districts

Table 3.6: Seasonal density (flies/trap/night) of sand flies collected from indoor habitats in Dir districts

Months	**May**	**June**	**July**	**August**	**September**	**October**
Density	**0.21**	**0.48**	**1.28**	**1.64**	**0.40**	**0.19**
Mean temperature	**22.7**	**32.6**	**34.2**	**34.3**	**26.8**	**25.3**
Mean relative humidity	**42.9**	**48.2**	**64.6**	**67.7**	**59.5**	**63.8**

Density of sand flies (specimens/ trap/ night) on monthly basis shown in table (6) confirms the same pattern shown in table 5 and fig 3 with peak level in the month of August and lowest in the months of May and October. Density of sand flies with different values of temperature and relative humidity in various months show the peak values of sand flies density coincide with the peak values of temperature and humidity. Low levels of density appeared with decreased values of the environmental factors (Table 3.6) and (Fig. 3.10).

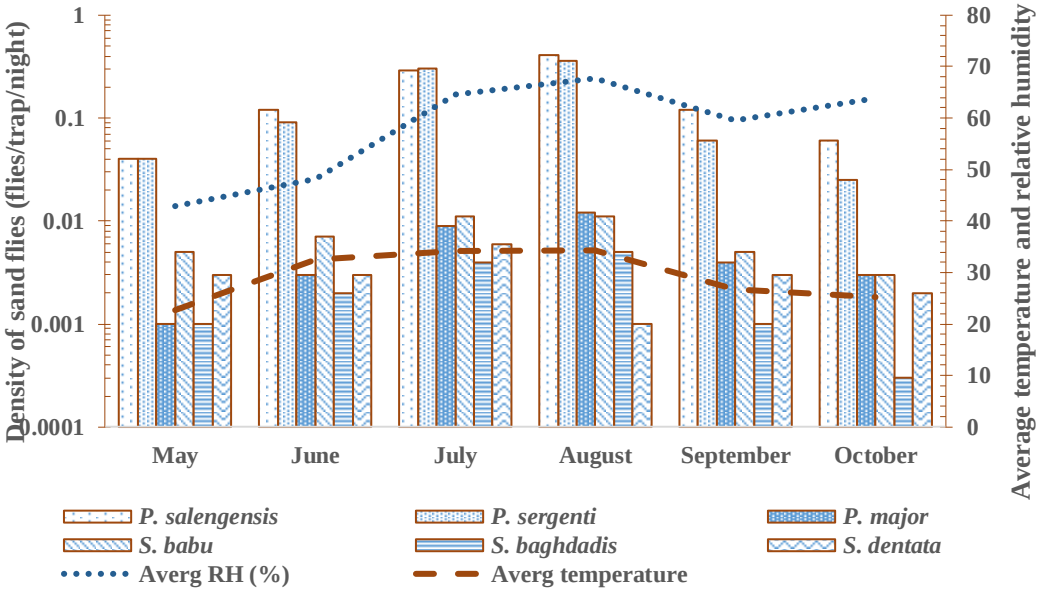

Fig. 3.10: Sand flies seasonal density of night times collection in Dir Districts

Table 3.7: Seasonal variation of sand flies collected from day time's collection of indoor habitats in Dir districts, 2014

Months	**May**	**June**	**July**	**August**	**September**	**October**	**Total**
P. sergenti	10	16	50	67	17	9	169
P. salengensis	1	1	3	1	-	-	6
P. major	-	-	-	1	-	-	1
S. babu	28	35	120	159	59	18	419
S. baghdadis	14	25	71	91	27	11	239
S. dentata	10	13	45	75	19	10	172
Total/%	**28/6.7**	**42/10.0**	**116/27.8**	**172/41.3**	**38/9.1**	**20/4.8**	**416**
Average temperature	**22.8**	**32.7**	**34.5**	**35.7**	**27.9**	**26.1**	
Average RH (%)	**43.8**	**48.9**	**65.6**	**76.0**	**61.4**	**66.5**	

Table (3.7) and fig. (3.11) show monthly abundance of sand flies collection from indoor habitats. Day time's collection of sand flies was done by insecticide spray and aspiration. In general the same activity pattern was observed as shown by sand flies collected through sticky traps with peak in August (41.3%) and minimum in May (6.7%) and October (4.8%) but by using this method contrary to the previous one, the number of female flies found was more than males in all the months of their availability. Positive association of sand flies with temperature and relative humidity is obvious (Table 3.7) and (Fig. 3.11).

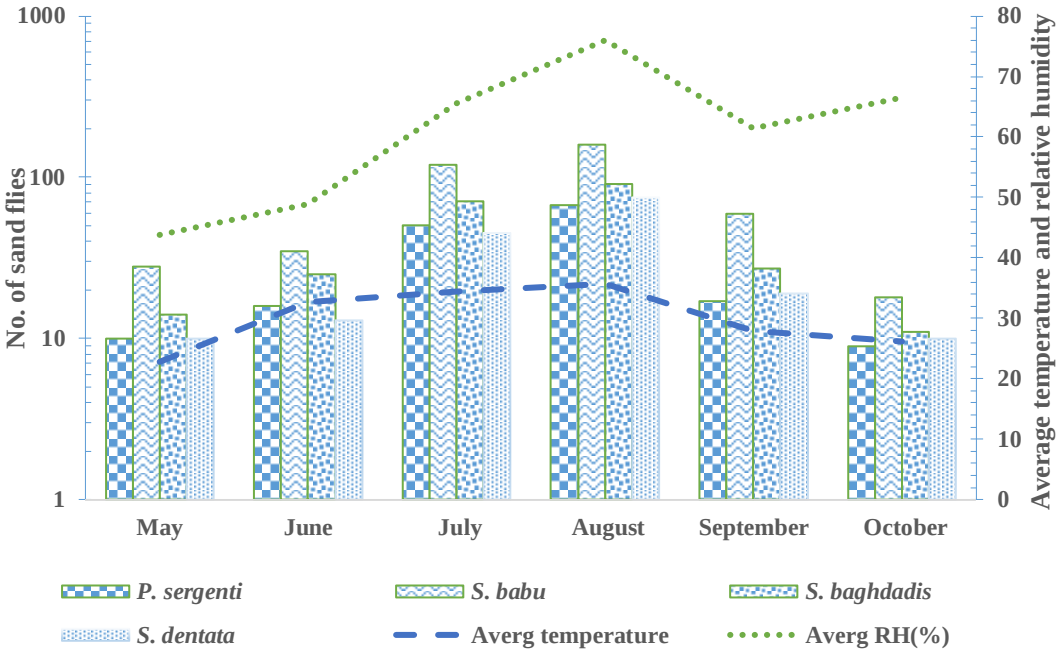

Fig. 3.11: Sand flies seasonal variation of day times collection in Dir Districts

3.4 DISCUSSION

The current study is the first detailed research in terms of sand flies species composition, seasonal variations and their density as well as, diurnal and nocturnal activities using different methods. Twenty species including nine species of genus *Phlebotomus* and 11 species of *Sergentomyia* were collected in both districts of Dir. All these species were reported for the first time from this region (districts), except *P. sergenti* which was recorded by (Rowland, 1999) while working on CL in Afghan Refugee Camps settled at Timergara (Lower Dir).

A total of 21 (Table 1.1) species are so for reported from KPK (Aslamkhan *et al.*, 1998). The present study recorded 12 species from Dir districts reported so far from KP, while eight species are new record to this province (Table 3.8). Among the eight species, (*P. ansari*. *P. salengensis, P. kazeroni, P. hindustanicus, P. bergeroti, P. andrejevi, S. grekovi* and *S. turkastanica*) *P. ansari*-is the new addition to the list of

sand fly species recorded in Pakistan. Four species, *P. andrejevi, P. bergeroti, S. grekovi* and *S. turkistanica* were reported by Kakar (2004) from Balochistan province while the rest of the three species were found in Azad Kashmir (Munir, 1994). The current study shared six species with those from North Waziristan Agency (NWA) (Ali *et al*., 2015).

Table 3.8: Current and previous list of sand flies species from Khyber Pakhtunkhwa, Pakistan, 2014

S.N	Species	Locality (Districts)
1	***P. salengensis***[b]	Districts Dir
2	***P. hindustanicus***[b]	Districts Dir
3	***P. ansari***[a]	Districts Dir
4	***P. kazeroni***[b]	Dir districts
5	***P. andrejevi***[b]	Districts Dir
6	***P. bergeroti***[b]	Districts Dir
7	***P. kandelakii burneyi***	Kalam (Swat)
8	***P. papatasi***	Bannu, Dera Ismail Khan, Tank, Kohat, Nowshera, Peshawar, districts Dir
9	***P. major***	Abbottabad, districts Dir
10	***P. nuri***	Peshawar
11	***P. sergenti***	Dera Ismail Khan, Chitral, Peshawar, Cherat, Lower Dir
12	***S. babu***	Landi Kotal, Districts Dir [a]
13	***S. baghdadis***	Bannu, Dera Ismail Khan, Peshawar, Landi Kotal, Districts Dir
14	***S. bailyi***	Dera Ismail Khan, Peshawar, districts Dir

15	***S. christophersi***	Peshawar
16	***S. clydei***	Peshawar
17	***S. dentata***	Peshawar, districts Dir
18	***S. grekovi***	Districts Dir [a]
19	***S. hodgsoni***	Peshawar, Cherat, Landi Kotal, districts Dir
20	***S. hospitii***	Abbottabad, districts Dir
21	***S. indica***	Peshawar,, Dera Ismail Khan, Cherat
22	***S. montana***	Chitral, Bahrain (Swat), districts Dir
23	***S. palestinensis***	Peshawar
24	***S. punjabensis***	Kohat, Dera Ismail Khan, Peshawar
25	***S. shorttii***	Peshawar
26	***S. theodori***	Dera Ismail Khan, Landi Kotal, districts Dir
27	***S.tiberidis pakistatiica***	Ahmad Khael, Landi Kotal, Peshawar, districts Dir
28	***S. turkistanica*** [b]	Districts Dir

[a]Species recorded for the first time from Pakistan. [b]Species recorded for the first time from KP.

Survey was conducted throughout the year but sand flies were observed only from May to October, where peak activity (highest abundance of sand flies) was seen in the month of August. The peak activity time coincided with the highest temperature and humidity indicating the two factors as environmental dynamics of ecosystem in which the flies survive (Oliveira *et al*., 2013). The positive correlation of humidity and temperature with population abundance of sand flies is confirmed by other workers like (Pérez *et al*., 2014; Gebresilassie *et al*., 2015). However, some studies reported negative association of humidity with sand fly population from Turkey (Kasap *et al*., 2009), Italy (Tarallo *et al*., 2010), Morocco (Lahouiti *et al*., 2013) and Portugal

(Branco *et al*., 2013). Moreover, higher temperature (30-35C^0) is considered ideal for sand fly activity (Lahouiti *et al*., 2013).

During day times females presented peak activities in August while at night males were in maximum number. There overall number decreased in September and October with lowering of climatological parameters. In general there were more male flies than that of female (3:1). Same observations were reported by some other workers (Morrison *et al.,* 1995; Killick-Kendrick, 1999). Different explanations are put forward for such findings. For example, Chaniatis *et al.,* (1974) suggested that these differences may be associated with sex mortality because females die soon after completing some gonotrophic cycles. It is also known that male sand flies arrive on their specific host first to form aggregation and wait for female to mate (Killick-Kendrick, 1999). Another study conducted in Israel confirmed that nocturnal activities of sand flies peaked soon after sunset. Females form minor peak aggregations before dawn and then subside gradually suggesting that they were crepuscular while males were active throughout the night (Kravchenko *et al*., 2004).

Three collection methods; sticky traps, spray and mouth aspiration were used for the sampling of sand flies in four tehsils. The current results confirmed that these methods differ in performance, sticky raps were found to be more effective than other collection methods. Certain advantages of sticky traps over other methods are being non-phototropic, inexpensive and can be distributed in short times, particularly useful for random collection of species (Kasap *et al*., 2009; Singorini *et al*., 2013). Tapping method was found more efficient for estimation of both sand flies species composition and diversity in three tehsils however; in Warae tehsil this method was not useful may be due to concreted houses with limited resting/breeding sites.

Sticky traps were more effective where domesticated animals were kept inside and sometimes around houses during night times. These traps were found ineffective in wind and rain. Ants (Order Hymenoptera) were also observed to feed on sticky material of the papers there by affecting their performance especially in Khall and Balambat. More males were captured, when collected by sticky traps as compared to collection made by insecticide spray. Kasap *et al*., (2009) and Gebresilassie *et al*., (2015) found that this might be due to the fact that the sticky traps were installed near emergence places (breeding sites) where males are generally abundant.

Insecticide spray and mouth aspiration were generally used in indoor sites (WHO, 1984; Samir, 2000; Senghor *et al*., 2011). Females of the genus *Sergentomyia* were sampled in high number than males in all tehsils using spray method. Similarly, Female's ratio most particularly of the genus *Sergentomyia* was high using insecticide spray during day time. Generally it has been observed that during day times sand flies stop their activities and take rest in dark and humid places inside houses.

Indoor and outdoor collection of flies were made from different sites where six species showing more than 1% abundance (*P. salengensis, P. sergenti, P. major, S. babu, S. baghdadis, S. dentata* and *S. bailyi)* exhibited both (exophilic and endophilic existence). Such places may be treated as breeding and suitable resting sites in the districts. Total ratio of the flies collected from indoor and outdoor was 10.5. Male to female ratio was 3:1 and 6:1 for indoor and outdoor flies respectively. More individuals of *P. sergenti, S. babu, S. dentata and S. baghdadis* were collected inside houses as compared to outdoor. Ali *et al*., (2015) and Sania (2008) also collected more number of these species from indoor habitats through insecticide spray. Identification of exophilic and endophilic behaviours of sand fly species is very

important in the control and prevention of the disease and in determining the suspected hot spots of transmission (Gebresilassie *et al.*, 2015).

P. salengensis in the current study was the most abundant (3101) sand fly, which is morphologically middle–sized, pigmented moderately thermophilic and hydrophilic (Lewis, 1967). This species was found in mountains areas of Afghanistan (Lewis, 1967) and Kashmir (Munir, 1994). Only six male sand flies were collected during day times while all the remaining were recorded at night times suggesting this species being more active at night times in the study sites. Its medical importance as a vector need to be investigated as suggested also by Lewis, (1967) and Munir, (1994), however, its abundance shows it being the big source of biting nuisance in the area.

P. sergenti (36%) was the second most abundant species in the total collection. This species is considered as the suspected vector of CL in the area (Rowland, 1999). *P. ansari,* so for recorded only from Iran is reported to be a vector of zoonotic leishmaniasis (Yaghoobi-Ershadi, 2012) was represented by two female sand flies from tehsil Timergara. Further detail studies are needed to explore the exact nature of these species which appear uncommon and possibly made its entry from the neighbouring country.

The seasonality of indoor sand flies in four tehsils were examined. Activity of sand flies was found from May to October during the dry season. The highest activity patterns and aggregate populations were observed in July and August, several studies support our conclusion (Kasap *et al.*, 2009; Doha and Samy, 2010). Day time's seasonality was investigated through insecticide spray in Balambat, Timergara, Warae and Khall respectively. This type of collection was mostly made of concrete houses. Such sites may provide suitable shelter for the flies. Secondly most of the collected

flies from these tehsils were *Sergentomyia* species indicating preference for such habitats.

Nocturnal seasonal variation was determined through sticky traps Greater number of flies were captured through this method in Timergara, followed by Khall and Balambat. Kasap *et al.,* (2009), investigated that sticky traps are used for determining species composition of an area for random sampling of flies in the area.

The life cycle of sand flies observed to be associated with environmental dynamics of the ecosystem in which the flies survive. This correlation analysis was investigated between sand flies and abiotic factors (temperature and humidity) in Dir districts. The present results showed that sand flies population have a significant correlation with temperature and humidity. These finding corroborate those reported by Oliveria *et al.,* (2013).

The study reports the species composition, seasonal variation of sand flies in Dir Districts. The efficacy of well-known collection methods was also determined during day and night time collection of sand flies from different habitats. The present study also provides help in determining the activity period and understanding the relationship of their density with meteorological factors in the region. The findings can contribute to the planning of preventive intervention periods to fight *Leishmania* vectors.

CHAPTER - 04
COMPARATIVE ACCOUNT OF SPECIES COMPOSITION AND ACTIVITY PATTERN OF PHLEBOTOMINE SAND FLIES IN FOUR TEHSILS OF DIR DISTRICTS

4.1 INTRODUCTION

Sand flies are frequently present in tropics and temperate regions of the globe (Alexander, 2000). They have a diverse habitats and each species have their own breeding sites ranging from semi desert to rain forest foci (Killick-Kendrick, 1999). Among approximately 700 species, medically important sand flies species belong to two genera *Lutzomyia* in New World and *Phlebotomus* in the Old World (Lewis, 1982; Lane 1993, Sharma and Singh, 2008). Some 30 species of sand flies are responsible for the transmission of *Leishmania* parasites in humans (WHO, 1990; Desjeux, 2001). Distribution of the sand fly vectors play a major role in the determining the spread of the disease in an area.

Eco-epidemiological studies of leishmaniasis require information about abundance and diversity of sand flies as well as their role as vectors in the concerned area. Frequent cases of leishmaniasis are reported from various parts of Dir districts particularly after the influx of Afghan refugees in the area. The area has also faced the major outbreak of CL in a refugee camp at Timergera (Rowland, 1999). *P. sergenti* was reported to be the suspected vector of CL in Dir. A comprehensive comparison of species diversity and activity pattern is presented in this chapter.

4.2 MATERIAL AND METHODS

4.2.1 Sand flies Collection

Sand flies were collected on monthly basis from May to October, 2104 in four Tehsils, Timergara, Balambat, Khall (from Lower Dir district) and Warae (from

Upper Dir district). Collection was made from indoor and outdoor habitats. A total of 499 houses; Balambat (195), Khall (154), Timergara (100) and Warae (59) were surveyed in four Tehsils. Indoor collection was made using both flit (day times) in 23 villages (Saddo, Sikaolae, Dhiry, Palusoo, Haji Abad, Koto, Shzadi, Munji, Baroon, Khall Kalone, Toor Mung Kalone, Akhagram, Warae Bala, Jugha Banj, Dab, Kotkae, Tangae, Shagae, Nawagae, Rani, Siar, Danvah and sticky traps (night times) in 17 villages (Yarkhanbanda, Afghan refugees camps, Khima, Watange, Mira, Sacha, Mandish, Malae, Manzari Tangae, Khall Banda, Luqmanbanda, Upper Lalo, Lower Lalo, Dwaba, Sarmu Obu, Shalfalm and Barkale). Outdoor sampling was done using sticky traps in seven villages, Yarkhanbanda, Khima, Watange, Sacha, Mandish, Shalfalm and Barkale and an Afghan refugees camp from 72 sites of Timergara (48), Khall (18) and Balambat (6).

4.2.2 Data Analysis

Sand flies data were transferred to Microsoft excel and were analysed in IBM SPSS Statistics 20. Diversity indexes of collected sand flies in different tehsils were estimated by Shannon-weaver ($\mathbf{H=\sum P_i \log_e P_i}$) and species richness ($\mathbf{D = \frac{S}{\sqrt{N}}}$). Sand flies abundance and seasonal activity parameters in each tehsil were calculated on monthly basis.

4.2.3 Determination of climatic variations

In order to determine local variations in temperature and humidity that might affect sand flies abundance in the study area, Kestrel 4500NV Pocket Weather Tracker, USA was used throughout the study times. They were programmed to record the climatological parameters (temperature and relative humidity) during day and night times from May up to October, 2014.

4.3 RESULTS

Table (4.1) shows sand fly species and their abundance in four Tehsils of Dir districts. Ratio of male sand fly individuals to that of females in each tehsils is also shown.

Table 4.1: Distribution, abundance of male to female ratio of sand fly species in four tehsils of Dir districts, 2014

Species	Timergara		Balambat		Khall		Warae		Grand Total
	T/%	M:F	T/%	M:F	T/%	M:F	T/%	M:F	
P. salengensis	1945/62.7	2.13	405/13.0	12.9	751/2.2	19.8	-	-	**3101**
P. sergenti	1320/49.5	19.6	581/21.6	11.9	756/28.2	11.8	23/0.8	**0.8**	**2680**
S.babu	193/35.8	0.4	181/33.5	0.1	94/17.4	0.2	71/13.1	0.07	539
S. dentata	67/19.3	-	112/32.2	-	153/44.0	-	15/4.3	-	347
S. baghdadis	65/21.1	0.03	150/48.7	0.1	42/13.6	0.07	51/16.5	-	308
P. major	129/99.2	7.06	-	-	1/0.7	-	-	-	**130**
S. bailyi	51/100	2	-	-	-	-	-	-	51
P. andrejevi	-	-	-	-	37/100	-	-	-	37
S. hospittii	11/39.2	-	9/32.1	0.5	7/25	-	1/3.5	-	28
S. montana	5/20.8	-	3/12.5	-	-	-	12/50	-	24
P. kazeroni	12/60		2/10	-	4/20	-	2/10	-	***20***
P. papatasi	4/28.5	-	6/42.8	1.7	2/14.2	-	-	-	***14***
P. bergeroti	-	-	8/100	-	-	-	-	-	8
P. hindustanicus	4/100	-	-	-	-	-	-	-	**4**
P. ansari	2/100	-	-	-	-	-	-	-	**2**
S. grekovi	1/100	-	-	-	-	-	-	-	1
S. hodgsoni	-	-	1/100	-	-	-	-	-	1
S. turkistnica	1/100	-	-	-	-	-	-	-	1
S. tiberidis	-	-	1/100	-	-	-	-	-	1
S. theodori	-	-	1/100	-	-	-	-	-	1
Total	**3810**	**52.24**	**1460**	**20.0**	**1847**	**25.3**	**175**	**2.3**	**7292**
Species/site	**15**		**13**		**10**		**7**		

Maximum number of species (15) was collected from Timergara, followed by Balambat (13) and Khall (10). Least number of species (7) was recorded in Warae. Highest number of sandflies (3810) was found in Timergara and least number in Warae (175). Among the six abundant species (>1%) Timergara, tehsil was most inhabited by *P. salengensis*, *P. sergenti*, *S. babu* and *P. major* while *S. dentata* and *S. baghdadis* were in maximum number in tehsils Khall and Balambat respectively.

The captured sand flies were ranked according to the presence in the studied tehsils of two districts, as common, occasional and rare. *P. sergenti*, *S. babu*, *S. dentata*, *S. baghdadis*, *S. hospittii* and *P. kazeroni* were common species present in all tehsils. *P. salengensis* and *S. montana* were occasional, present in three Tehsils. Other species were rare and were collected either from one or two Tehsils. *P. hindustanicus*, *P. andrejevi*, *S. dentata* and *S. grekovi* were represented by male individual/s while *P. kazeroni*, *P. ansari*, *P. bergeroti* and *S. montana* were shown by female only (Table 4.1, appendix).

Table 4.2: The Shannon-weaver, species richness and Jaccard's coefficient for the sand flies in four Tehsils of Dir districts, 2014

Biodiversity of sand flies in four tehsils of Dir districts				
Region	**Shannon-weaver index** $H=\sum P_i \text{ Log}_e P_i$	**Species Richness** $D=\frac{S}{\sqrt{N}}$		
Timergara	1.22	0.24		
Balambat	0.78	0.34		
Khall	1.28	0.23		
Warae	1.26	0.27		
Jaccard's coefficient (J= A/ a+b-A)				
	Timergara	**Balambat**	**Khall**	**Warae**
Timergara	*	*	*	*
Balambat	0.47	*	*	*
Khall	0.56	0.53	*	*
Warae	0.46	0.53	0.42	*

H = Shannon-weaver diversity index, Pi =the proportion of the species, Loge =Natural log.

D= Species richness, s= number of species in a community, N= total number of species in a community.

J= Jaccard's coefficient, A= common species in both habitats, a= representing species present in habitat a, b= representing species present in habitat b.

The higher the value of Shannon-weaver index indicating greater diversity (Belen and Alten, 2011). There were differences in diversity of sand flies in tehsils as indicated by H and S values in table (8) (Table 4.2, appendix). Species richness was high in Tehsil Balambat (0.34). The value of Jaccard's coefficient ranging from 0 to 1. If the value is one it estimates 100% similarity. Similarity was high between Timergara and Khall (0.56) and between Balambat and Warae (0.53) while it is low between Timergara and Warae (0.46) and Khall and Warae (0.42).

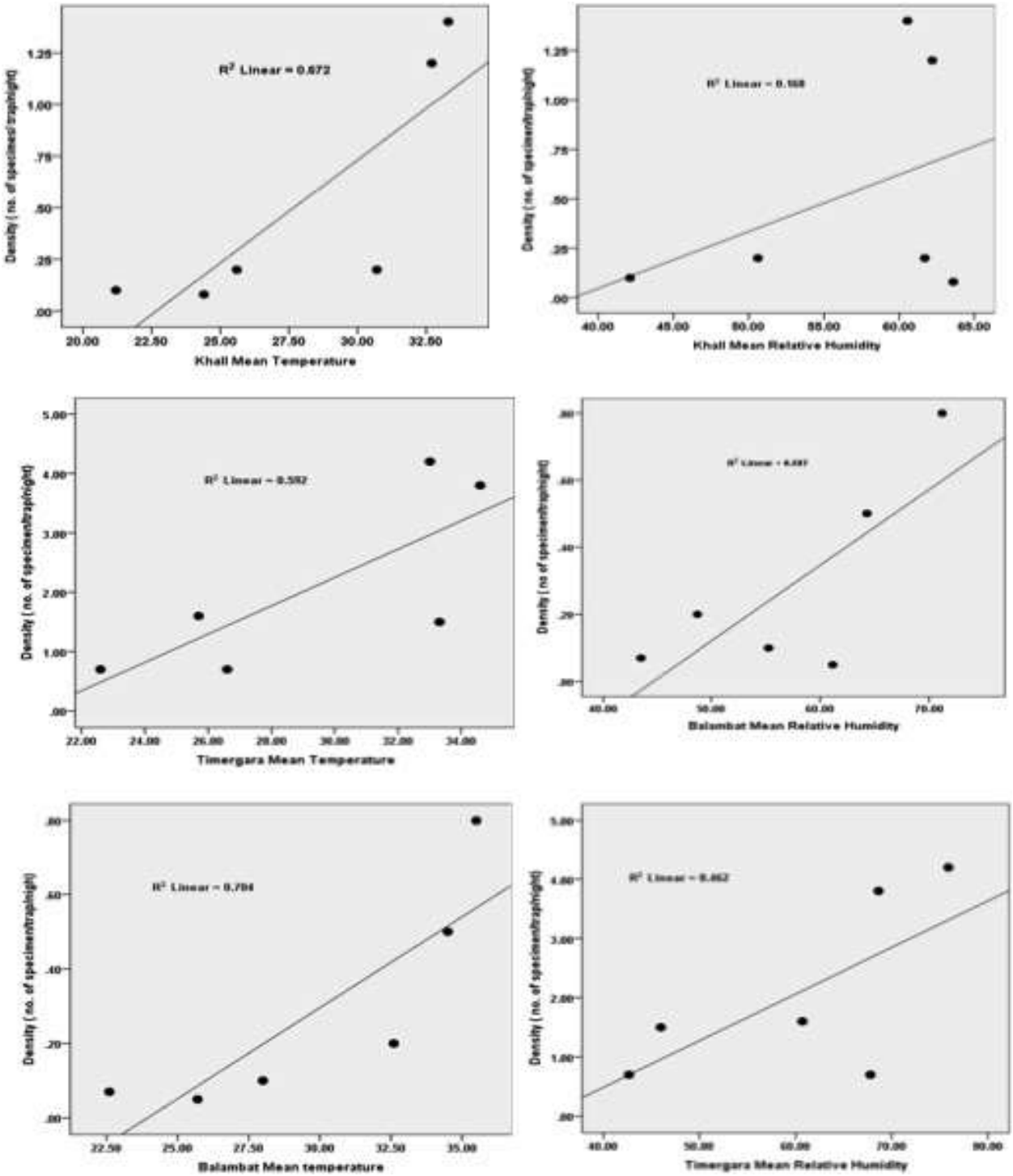

Fig. 4.1: Correlation between sand flies species density, temperature and relative humidity of three Tehsils Dir districts, 2014.

Density (specimens/trap/nigh) of five species of sand flies were investigated in three tehsils (Timergara, Balambat and Khall) in the active season. Warae Tehsil was not included as no collection at night time could be made in this Tehsil. Fig. (4.1),

temperature and relative humidity were positively correlated with density of sand flies in all Tehsils of Timergara, Balambat and Khall (A-F).

Table. 4.3: Seasonal variation of sand flies species in night time collection from Timergara, Balambat in Khall, 2014.

Species	Tehsils	May	June	July	August	September	October	Total
P. salengensis	**Timergara**	63	196	328	662	241	132	1622
	Balambat	12	21	210	105	37	16	401
	Khall	44	104	222	318	44	14	746
P. sergenti	**Timergara**	89	131	473	388	100	37	1218
	Balambat	11	31	133	305	20	10	510
	Khall	19	104	217	307	45	21	713
P. major	**Timergara**	4	10	24	55	11	8	112
	Balambat	-	-	-	-	-	-	-
	Khall	-	-	-	1	-	-	1
S.babu	**Timergara**	6	11	18	14	5	3	57
	Balambat	2	3	6	7	1	-	19
	Khall	7	6	5	7	12	5	42
S. dentata	**Timergara**	1	1	1	2	-	-	5
	Balambat	-	1	1	2	2	-	6
	Khall	8	14	13	48	8	6	97
S. baghdadis	**Timergara**	-	-	1	1	1	-	3
	Balambat	2	3	6	7	1	-	19
	Khall	-	-	1	1	1	-	3
Total/%		**268/4.8**	**636/11.4**	**1659/29.7**	**2230/40.00**	**529/9.49**	**252/4.5**	**5574**

Table 4.4: Average temperature and relative humidity in four tehsils during collection.

Tehsils	Temperature/ Humidity	May	June	July	August	September	October
Timergara	M T	21.8	32.3	33.7	29.5	24.7	26.8
	ET	23.4	34.3	35.5	36.5	26.7	24.4
	Average	22.6	34.1	34.6	33	24.7	25.6
	MRH	42.2	43	67.2	74.3	59.3	66.4
	ERH	43.3	49	70.1	77.5	62.1	69.2
	Average	42.7	46	68.6	75.9	60.7	67.8
Balambat	M T	20.0	31.0	33.4	34.0	26.6	25.1
	ET	23.3	34.2	35.7	37.0	29.4	26.3
	Average	21.6	32.6	34.5	35.5	28	25.5
	MRH	43.0	47.3	66.2	74.5	54.2	61.5
	ERH	44.1	50.1	62.4	68.0	56.3	60.8
	Average	43.5	54.8	62.3	71.5	55.2	61.1
Khall	M T	20.0	30.4	32.5	33.4	25.3	26.0
	ET	22.0	31.0	33.0	33.1	26..0	23.0
	Average	21	30.2	32.7	66.5	25.6	24.5
	MRH	42.0	49.1	64.3	65.9	60.3	61.2
	ERH	42.2	52.2	60.1	55.2	63.3	66.2
	Average	42.1	50.6	62.2	60.5	61.8	63.7

MT= morning Temperature, ET= Evening Temperature, MRH= Morning Relative Humidity, EMR= Evening Relative Humidity

Monthly abundance of *P. salengensis*, *P. sergenti*, *P. major*, *S. babu*, *S. dentata* and *S. baghdadis* were presented in table (4.3) and fig. (4.2 to 4.4). All six species population were lowest in May, some were totally missing. Aggregate population was observed in warmer months of July (29.7) and August (40.0%). Temperature and relative humidity were also in peak position (Table 4.4). Sand flies species number was decreased with lowering of climatological parameters. *P. major* seasonal variation was observed in Timergara and one individual in Khall in the month of August. *P. sergenti* showed peak activity in July while *P. salengensis* maximum

number was observed in August (Fig.4.2 & 4.3). In tehsil Khall both species population were in peak position in August (Fig.4.4, Table 4.4).

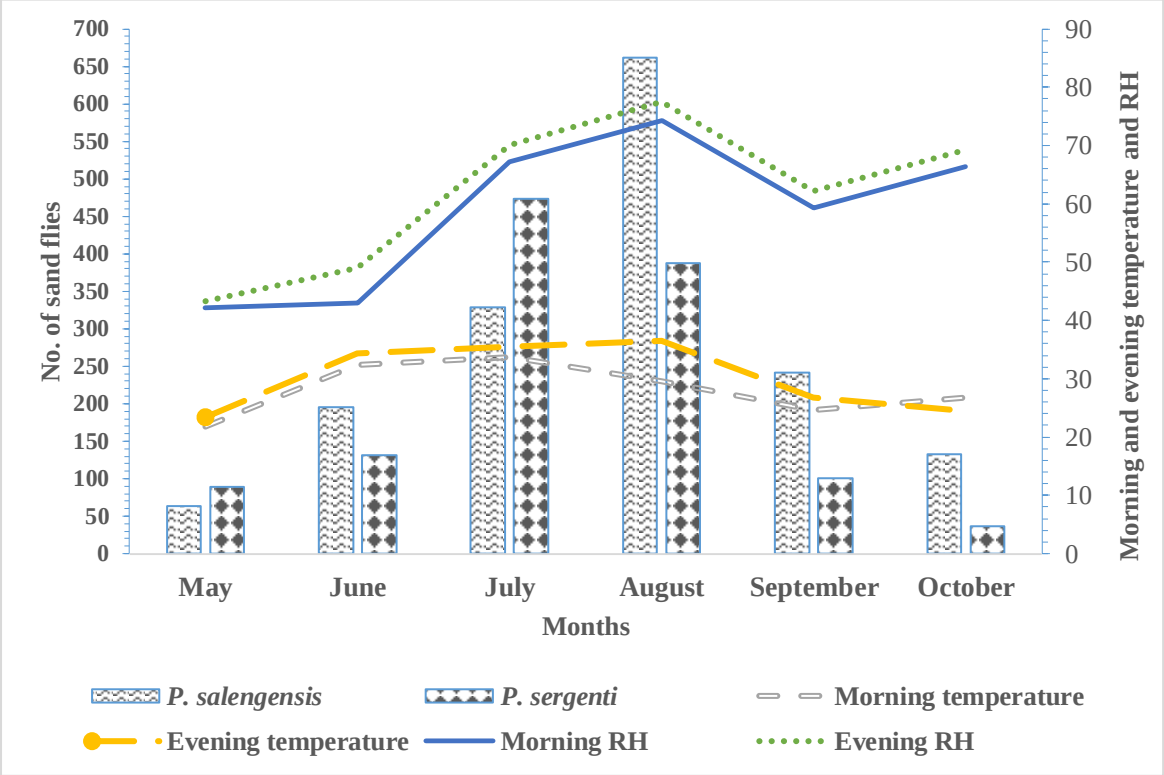

Fig. 4.2: Seasonal variation of sand flies species (night times) in three Tehsils of Dir districts

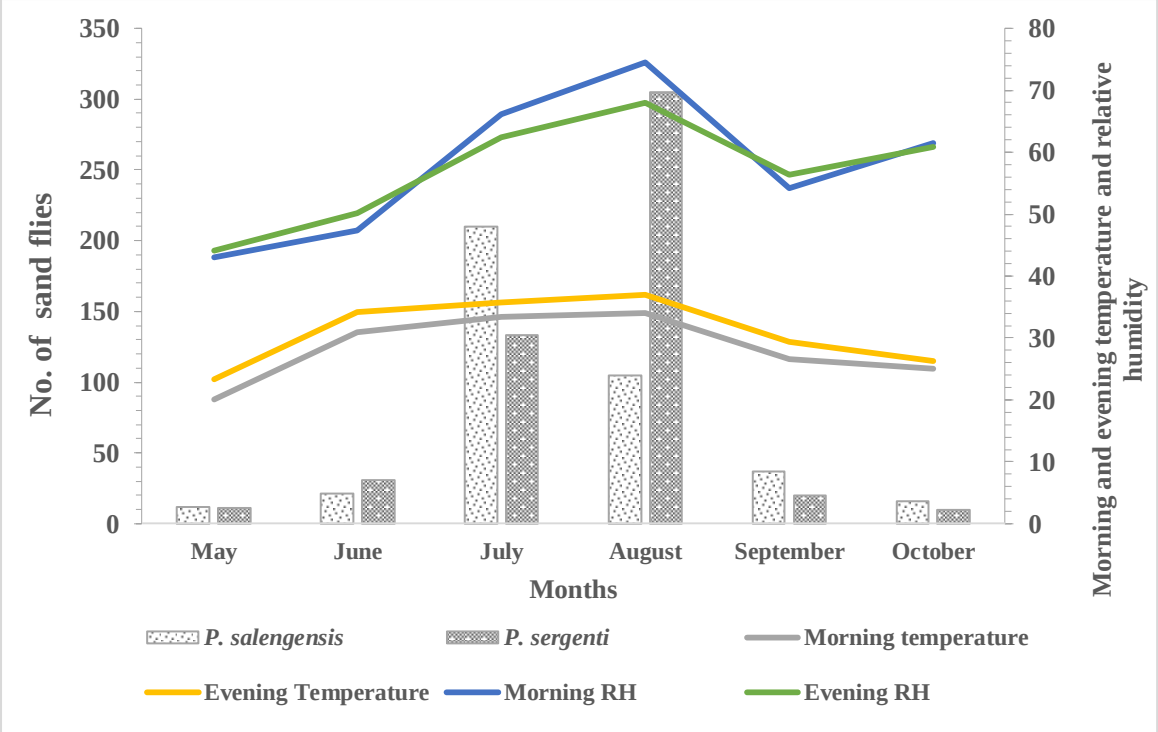

Fig. 4.3: Seasonal variation of *P. salengensis* and *P. sergenti* in night times collected from indoor habitats in Balambat

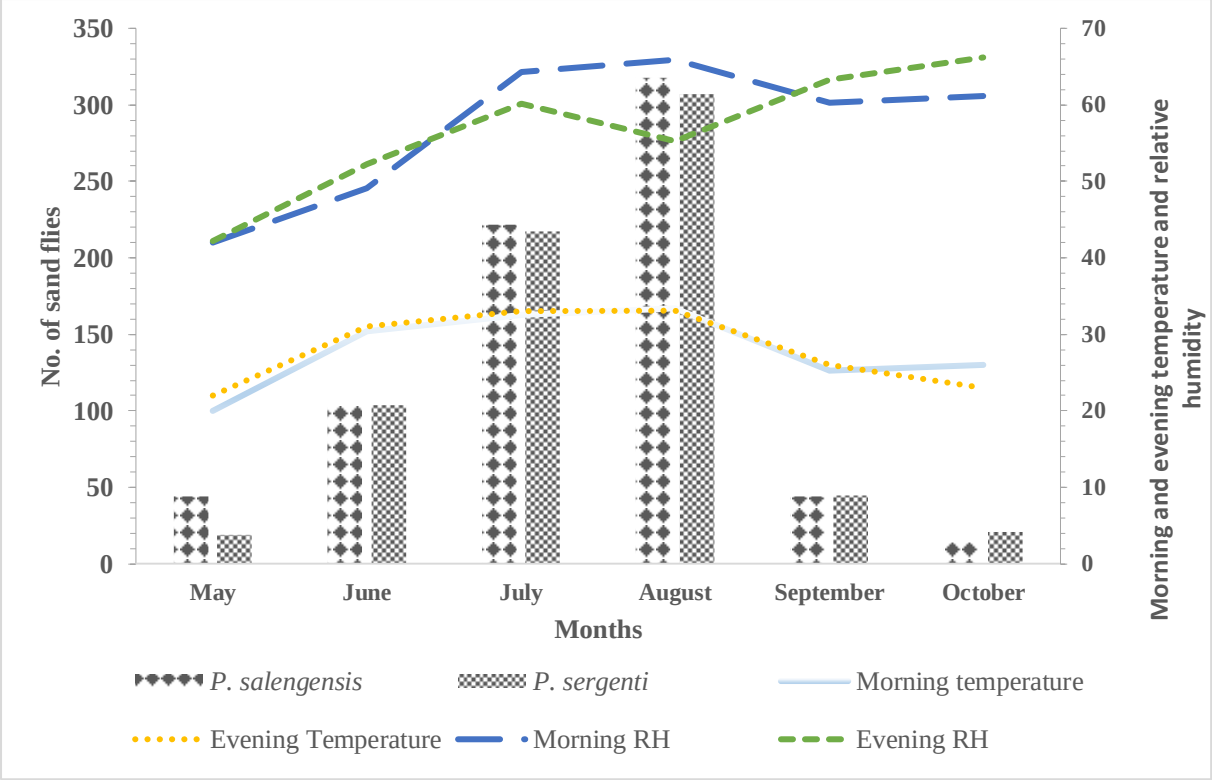

Fig. 4.4: Seasonal variation of *P. salengensis* and *P. sergenti* in night times collected from indoor habitats in Khall

Table 4.5: Monthly variation of sand flies species (day times) in four Tehsils of Dir districts, 2014

Species	Tehsils	May	June	July	August	September	October	Total
P. sergenti	**Timergara**	3	5	10	11	-	-	29
	Balambat	2	2	21	30	12	4	71
	Khall	5	6	14	12	4	4	45
	Warae	-	2	5	14	1	1	23
S. babu	**Timergara**	3	5	39	74	7	3	131
	Balambat	21	19	69	30	21	2	162
	Khall	2	5	5	27	6	5	50
	Warae	2	6	5	28	25	5	71

S. baghdadis	**Timergara**	1	3	19	24	4	5	56
	Balambat	13	14	33	36	26	-	122
	Khall	1	1	4	9	2	2	19
	Warae	1	6	15	22	5	2	51
S. dentata	**Timergara**	4	4	10	2	5	2	27
	Balambat	3	3	25	39	11	1	82
	Khall	2	5	6	6	2	2	23
	Warae	1	1	4	6	1	-	13
Total/%		**64/6.5**	**87/10.0**	**284/29.1**	**370/38.0**	**132/13.5**	**38/3.8**	**975**
Environmental parameters								
Timergara	**Temperature**	24.1	33.8	35.5	36.1	27.0	26.1	
	RH (%)	44.2	49.3	70.2	77.1	60.6	68.8	
Balambat	**Temperature**	24.5	33.9	35.3	36.8	29.1	26.2	
	RH (%)	44.5	51.2	64.5	68.3	57.5	61.6	
Khall	**Temperature**	24.1	33.8	35.5	36.1	27.0	26.1	
	RH (%)	44.2	49.3	70.2	77.1	60.6	68.8	
Warae	**Temperature**	20.7	30.8	32.5	35.4	29.8	26.7	
	RH (%)	43.3	48.5	57.9	78.8	69.2	62.4	

Table (4.4) and figure (4.5-4.8) shows sand flies species seasonal variation which was carried out through insecticide spray from May to October in four tehsils of Dir districts. *P. sergenti* showed lowest activities in May and June with minimum temperature and relative humidity. Their maximum peak was observed in July

(29.1%) and August (38.0%) in four tehsils. Other three species such as *S. babu*, *S. baghdadis* and *S. dentata* also presented lowest number with minimum temperature and relative humidity in colder month (May). A total number 6 of *P. salengensis* were recorded in day time's collection in different months and were found in Timergara and Khall only. *P. major* was totally missing in day time's collection. Overall sand flies species vanish with decreasing of environmental factors in September and October.

S. babu showed aggregate population in the month of August in Timergara, Khall and Warae while presented peak position in July in Tehsil Balambat fig. (4.5 to 4.8).

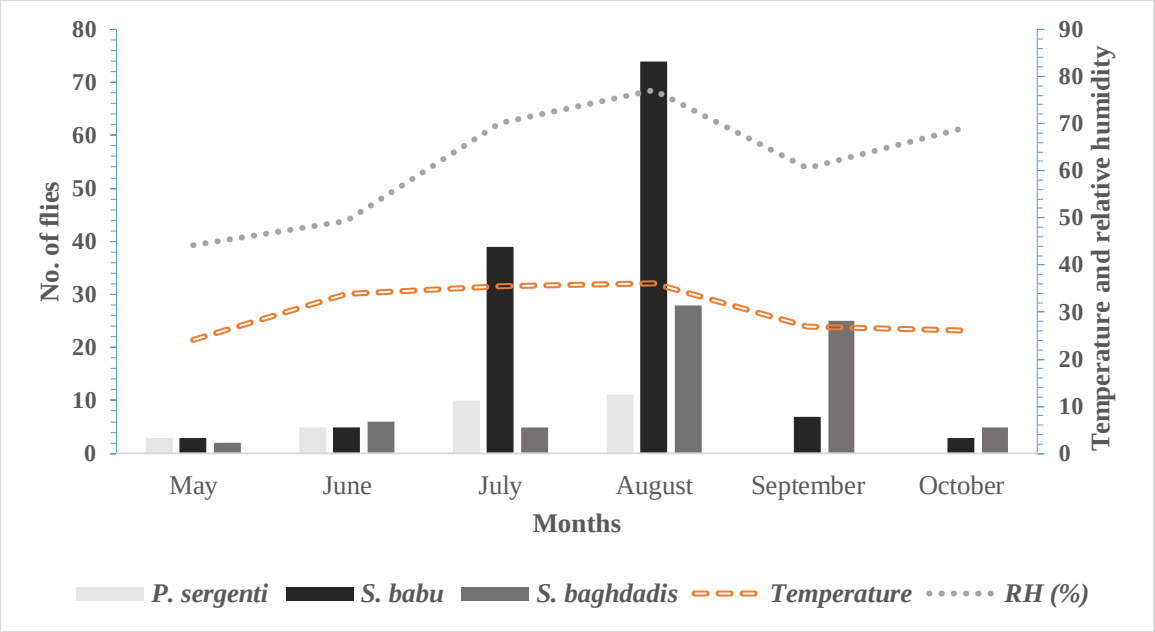

Fig. 4.5: Seasonal variation of *P. sergenti*, *S. babu* and *S. baghdadis* in day times collected from indoor habitats Timergara

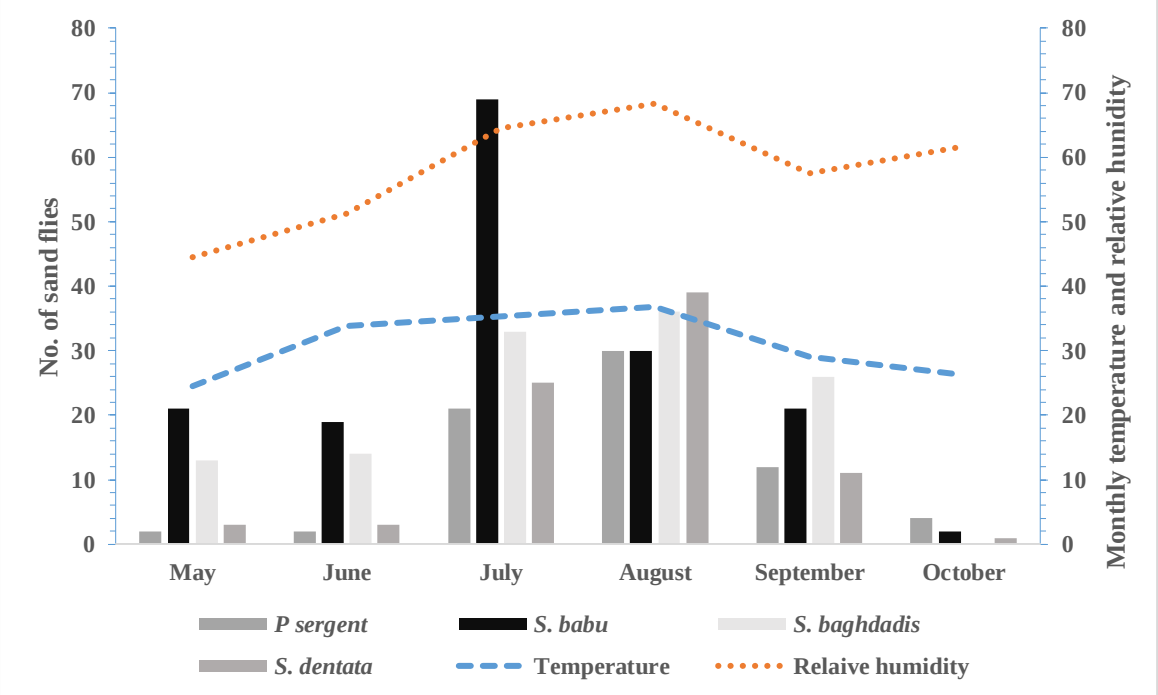

Fig. 4.6: Seasonal variation of *P. sergenti*, *S. babu*, *S. baghdadis* and *S. dentata* in day times collected from indoor habitats Balambat

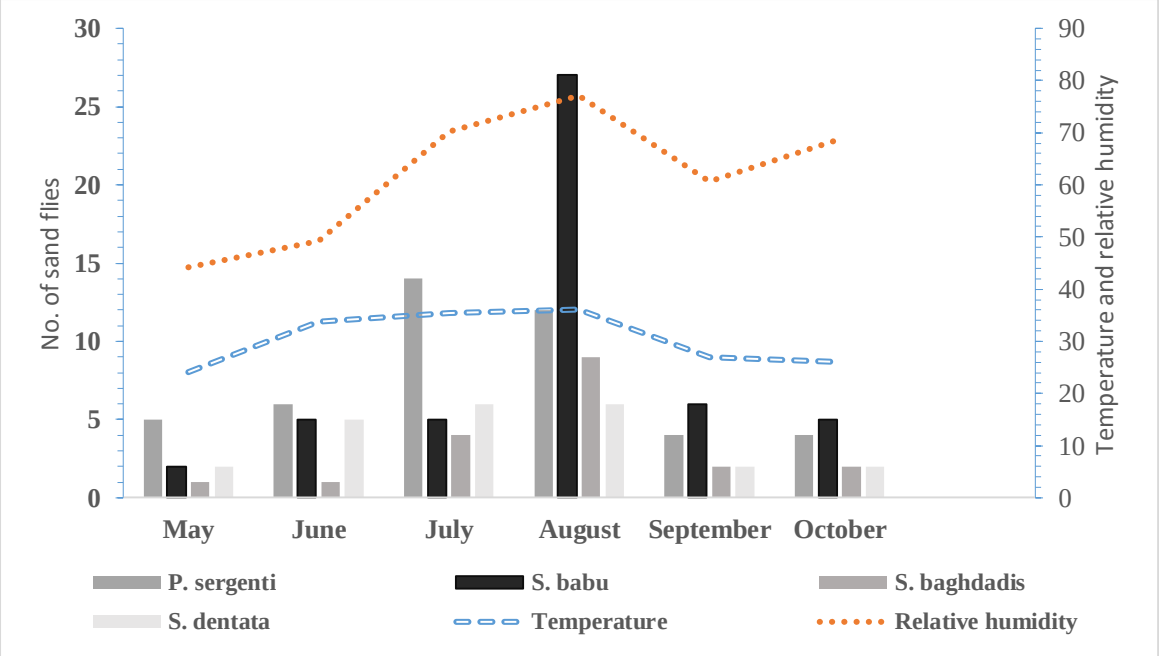

Fig. 4.7: Seasonal variation of *P. sergenti, S. babu, S. baghdadis and S. dentata* in day times collected from indoor habitats Khall

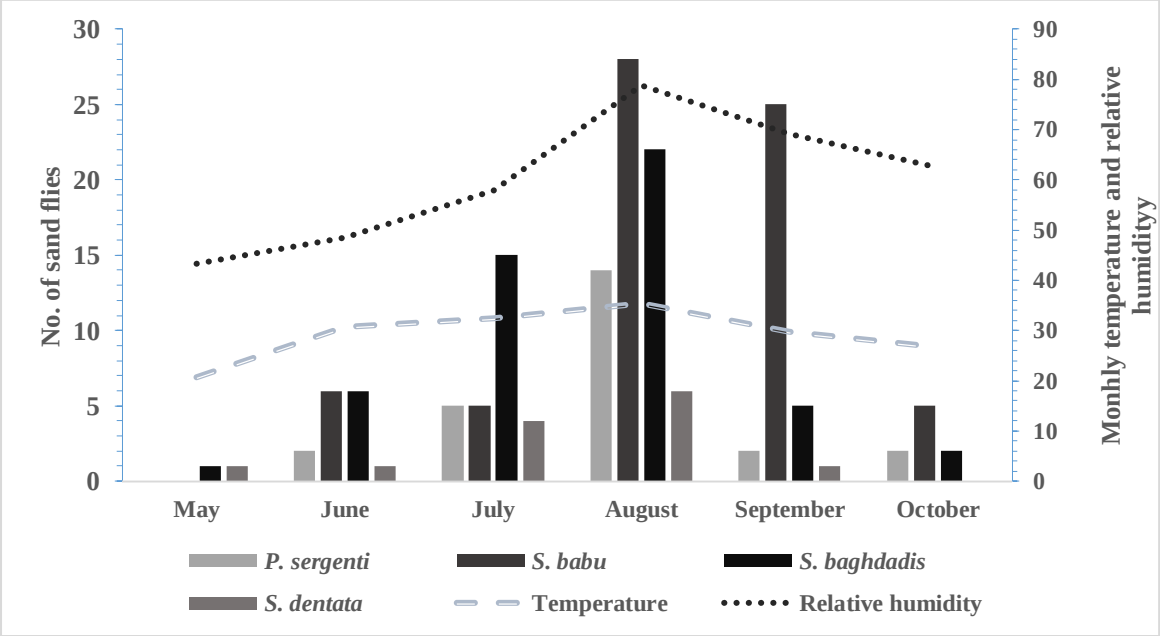

Fig. 4.8: Seasonal variation of *P. sergenti, S. babu, S. baghdadis and S. dentata* in day times collected from indoor habitats Warae

Table 4.6: Two way repeated measuring anova testing the effects of collected sand flies species, habitats and months on their activities in Tehsils of Dir districts, 2014

Source	Sum of square	df	Mean Square	F	Sig.
Tehsil	71945.212	3	23981.737	0.515	0.515
Method	80923.537	1	80923.537	0.308	0.308
Months	104562.371	5	20912.474	0.269	0.269
Species * Tehsil	108535.225	12	9044.602	0.486	0.486
Species * Method	178364.192	4	44591.048	0.028	**0.028**
Species * Months	96460.608	20	4823.030	0.676	0.676
Tehsil * Method	73465.246	3	24488.415	0.101	0.101
Tehsil * Months	38353.212	15	2556.881	0.432	0.432
Method * Months	56272.038	5	11254.408	0.185	0.185
Species * Tehsil * Method	106488.775	12	8874.065	0.000	**0.000**
Species * Tehsil * Months	91892.225	60	1531.537	0.523	0.523
Species * Method * Months	119133.858	20	5956.693	0.000	**0.000**
Tehsil * Method * Months	34808.479	15	2320.565	0.137	0.137
Species * Tehsil * Method * Months	93277.375	60	1554.623	-	-
Intercept	167851.704	1	167851.704	0.380	0.380
Species	132534.942	4	33133.735	0.605	0.605

Sand flies were collected in four Tehsils of Dir districts. They were collected through sticky traps, insecticide spray and aspiration. Sticky traps were used for night time while the other two methods were used for day time collection of sand flies. Results of Turkey's multiple comparison test revealed significant interactions between species and method ($p=0.028$). Similarly, significant interactions were found in species, habitats and methods ($p<0.05$) and species, methods and months ($p<0.05$).While species, months, Tehsil and methods presented a non-significant result (Table 4.5).

4.4 DISCUSSION

The species recovered from four tehsils differ in types of species, where high number (15) was found in Timergara and least was recovered from Warae (7). There is variation in bio geographical factors including environmental and socio economic parameters among these areas. Poche *et al.*, (2011), Akhoundi *et al.*, (2012), Oliveira *et al.*, (2013), Lahouiti *et al.*, (2013), Malaviya *et al.*, (2014) and Gebresilassie et *al.*, (2015), investigated the dependence of sand fly density on domestic and peridomestic factors like proximity to and amount of vegetation, number and types of domesticated animals and houses construction types etc.

P. salengensis was the most abundant species in three Tehsils except in Warae. *P. sergenti* (the second most abundant species in the total collection) was found in all Tehsils with greatest number sampled from Timergara followed by Khall, Balambat and Warae. This may be due to differences in climatic conditions, community structure and habitats variations. According to Seyedi-Rashti and Nadim, (1992) this fly is a 'mountainous fly commonly prevailing above 500m. Munir (1994) sampled this species in cool-humid climate of Kashmir, while Rowland *et al.*, (1999), isolated this species through light traps in Afghan Refugee Camps settled at Timergara. Ali *et*

al., (2015) also reported this species from Waziristan as the most abundant species. On contrary, Aslamkhan *et al*., (1998) treated this species as a common species in Pakistan that prefers dry and hot ecological condition in the country seems to be misconception.

P. major was collected from Timergara and Khall, mainly found in hilly areas of Pakistan with maximum rainfall in summer (Aslamkhan *et al*., 1998). Lewis (1967) reported this species from Abbottabad, Rawalpindi and Saidpur while Munir (1994) recorded this species from-Margella hills near Islamabad and Bagh district of AJ&K. Although no case of VL in the area is reported so far, however, *P. major* along with other five species of the same subgenus *Larrousius* are the suspected vector of VL in Pakistan and Afghanistan (Kakar, 2011; Artemiev, 1978) and confirmed vector in Southern Iran (Kassiri and Javadian, 2013). Keeping in view the medical importance the presence of *P. major* in the studied area should be considered important.

P. andrejevi were collected from mud houses in tehsil Khall. Kakar (2004) first time reported male individuals of this species from mud ruined walls of tomb at Dhadar, Balochistan and concluded that this is a rare, localized and Central Asian species and imported from Central Asia to Pakistan. Discovery of this species in the study area may be due to lack of previous exploratory surveys or its probable transport from Afghanistan through refugee's frequent travel. In Central Asia, the females of this species are suggested to be the vector of *L. tropica major* (Dubrovsky, 1976; Sergiev, 1979).

Only female individuals of *P. kazeroni* were reported from this study. Ali *et al*., (2015) also collected their female individuals from NWA. Kateo *et al*., (2010) found the females of this species naturally infected with amphibian *Trypanosoma* species in

Sindh, Pakistan. Sania (2008) also reported only females of this species from Peshawar. In all the above studies the number of sand flies were low, however, in Iran a large sized sample of this species were which collected still comprise of female individuals only in mountainous areas of the country (Kassiri and Javadian, 2012). Most probably special collecting techniques are required for capturing male of this species.

P. papatasi female individuals were recovered from Balambat, Timergara and Khall while their male individuals were collected in Balambat only. Ali *et al*., (2015) and Sania (2008) reported this species from Peshawar and NWA, Pakistan. Doha and Samy (2010) suggested their preference for indoor habitats and are truly endophilic nature. This species is considered to be the vector of *L. major* in Pakistan and Iran (Rowland *et al.,* 1999). The other two rare species belonging to genus *Phlebotomus* are *P. bergeroti* and, *P. hindustanicus*, represented by two and four individuals respectively in Tehsil Timergara, are previously recorded from Baluchistan and Kashmir (Kakar 2003; Munir, 1994).

P. bergeroti is reported as a suspected vector of VL in Ethiopia (WHO, 1990). Munir, (1994) suggested *P. hindustanicus* to be the probable vector of visceral leishmaniasis in AJK areas. *P. ansari*, was represented by two female individuals from Tehsil Timergara is reported for the first time from Pakistan. Its vectorial status is determined in Iran, playing role in the transmission of zoonotic leishmaniasis (Yaghoobi-Ershadi, 2012).

Among the 11 species of genus *Sergentomyia,* four species *S. babu*, *S. baghdadis*, *S. dentata* and *S. hospittii* were collected from all studied Tehsils, which, also reported from NWA (Ali *et al*., 2015). *S. babu* and *S. baghdadis* were-claimed to be prevalent

in several parts of the country, while *S. dentata* was considered as an uncommon fly with discontinuous distribution and found in hilly areas of the country having arid climate (Aslamkhan *et al*., 1998). The genus *Sergentomyia* species i.e. *S. babu* is considered medically important due to identification of *Leishmania* DNA in these flies (Mukherjee *et al.* 1997; Parvizi and Amirkhani, 2008; Berdjane *et al.,* 2012; Kanjanopas *et al.,* 2013).

S. bailyi, collected from Timergara, is an uncommon species with discontinuous distribution commonly found in hilly and mountainous areas of Pakistan and India (Aslamkhan *et al.,* 1998; Sinton, 1932). *S. hospittii* abundantly recorded from Timergara, first time recorded from Kashmir (Sinton, 1924) and from Abbottabad by Lewis (1967). Aslamkhan *et al*., (1998) described this species as a rare mountainous species, mostly found in cool sub-humid areas of Pakistan. *S. theodori* and *S. turkistanica* present in the current study are also reported by Ali *et al.,* (2105) from NWA. Other species such as *S. grekovi, S. hodgsoni* and *S. montana* collected in low number was also reported from Gilgit, Peshawar and Chitral (Sinton, 1932).

CHAPTER - 05
ISOLATION OF IMMATURE MALE AND VARIOUS REPRODUCTIVE STAGES OF FEMALE SAND FLIES BELONGING TO VARIOUS SPECIES, IN DIR DISTRICTS

5.1 INTRODUCTION

Scarce information is available on the ecology of the developmental stages and natural breeding sites of sand flies (Casanova, 2001) as searching for sand flies developmental stages is an extremely difficult and tedious job (Killick-Kendrick, 1986; 1999; Feliciangeli, 2004). The control efforts are therefore mainly confined to adult sand flies and personal protection (Alexander and Maroli, 2003). Grassi (1907) was the first to report an immature stage of phlebotomine sand fly in nature from Rome and was described as a new species, *Phlebotomus mascittii*.

Sand flies immature stages have been found in a wide range of ecotopes, and many species of sand flies employ rodent burrows, on buttress roots of trees in Panama, in termite hills in Kenya, in caves and rocks in East Africa and in the earthen floor of human habitations (Feliciangeli, 2004). Ecotopes occupied by immature phlebotomine are usually organically rich moist soils or contaminated soil of animal shelters.

Isolation of immature stages from a place will help to identify and describe types of breeding sites used by sand flies. Information on sand fly breeding sites can be used to facilitate their control by source reduction and to catch newly emerged adult sand flies for studies. The present study reports the first finding of immature male sand flies in natural habitats at district Dir. Reproductive stages in sand flies after insemination and before oviposition in natural conditions are also described.

5.2 MATERIAL AND METHODS

Permanent slides of the collected sand flies were made using Berlise media. Juvenile stages in male sand flies were identified by the incomplete rotation of the genitalia. Taxonomic identification of immature male flies was made using keys of Lewis (1967) and Artemiev (1978). Female sand flies were dissected for the determination of whether they are blood fed/half fed/unfed and gravid/semi gravid however, species identification could not be made in these females.

5.3 RESULTS

5.3.1 Age and reproductive grading of wild-caught sand flies

Like other Dipterans, male sand flies rotate their external genitalia on the longitudinal body axis through 180^0 at first 16-26 hours of adult hood to become fully mature (rotated) (Fig. 2.1). Juvenile males, about to mature with unrotated external genitalia (Fig. 5.2) were collected from cow sheds, belonging to three species, *P. sergenti*, *P. salengensis* and *P. andrejevi* (Table 5.1).

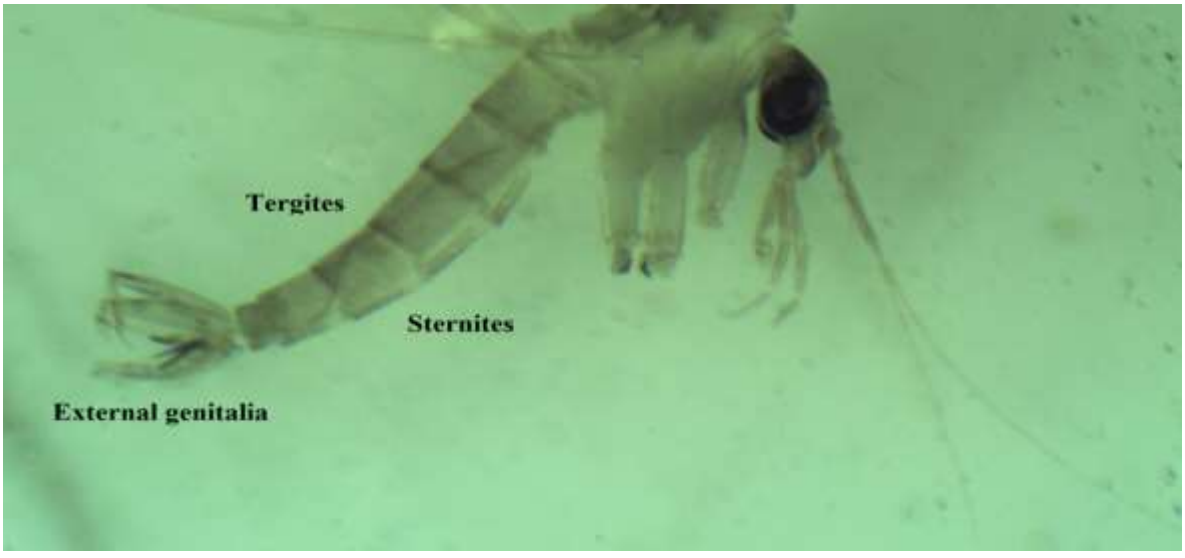

Fig. 5.1: A mature male of *P. salengensis* with the fully rotated genital apparatus, note the dorsal orientation of both the style and the tergites

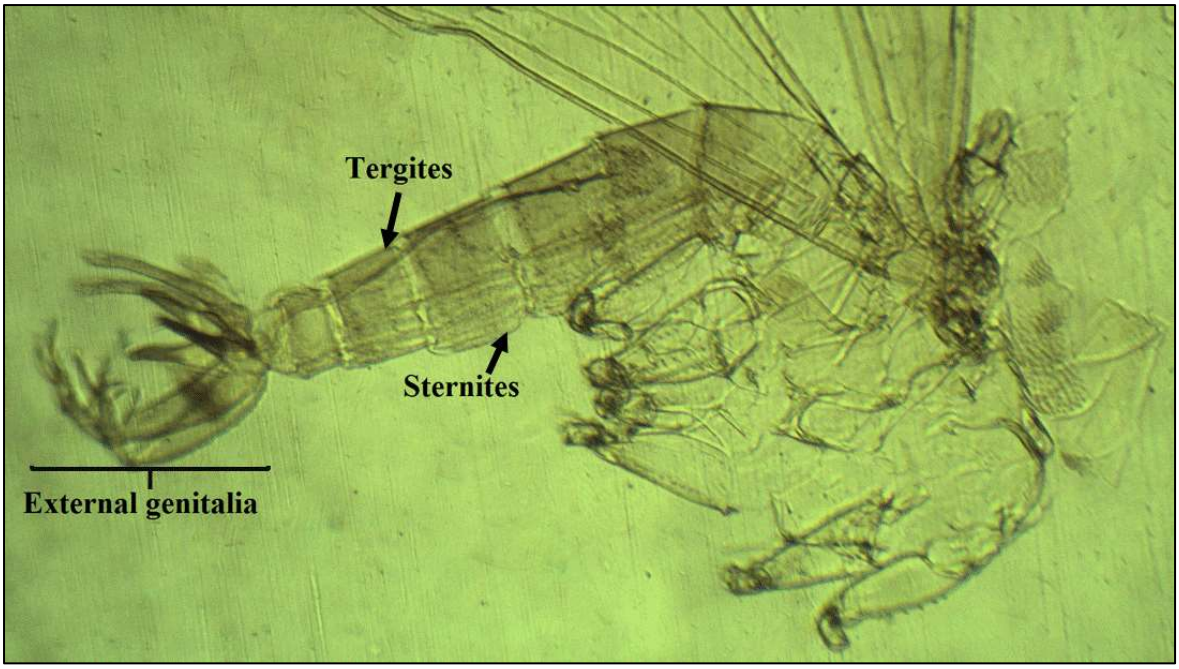

Fig. 5.2: Immature male of *Phlebotomus salengensis* with an un-rotated genital apparatus, note the ventral orientation of the style in relation to the dorsal tergites

Fig. 5.3: Collection of immature males from cattle corral

Table 5.1: Immature males of sand flies belonging to various *Phlebotomus* species collected from cattle sheds

Species	**Balambat**			**Timergara**			**Khall**			**Total**
	Cowshed	Sheep Shed	Goat shed	Cowshed	Sheep shed	Goat shed	Cowshed	Sheep Shed	Goat shed	
P. sergenti	2	-	-	20	-	-	4	-	-	26
P. salengensis	13	-	-	-	-	-	2	-		18
P. andrejevi	-	-	-	-	-	-	-	-	4	4
Total	15	-	-	20	-	-	6	-	7	48

Male immature stages were rare as compared to mature stages (48/4375) recognized by the complete rotation of male genatilia (Fig. 5.2). *P. sergenti* juvenile were observed from July to October, while those of *P. salengensis* were captured in August and September. Male immature individuals of *P. andrejevi* were sampled in August only. These three species were found in the captures from three studied tehsils except Warae (Table 5.2).

Table 5.2: Seasonal distribution of mature and immature (in parenthesis) males of three sand fly species

Species	**May**	**June**	**July**	**August**	**September**	**October**	**Grand Total**
P. sergenti	118	245	795 (3)	888(16)	134(6)	54(1)	**2234(26)**
P. salengensis	104	265	693	812(17)	171(1)	59	**2104(18)**
P. andrejevi	3	5	12	17(4)	-	-	**37(4)**
Total	**225**	**515**	**1500(3)**	**1717(37)**	**305(7)**	**113(1)**	**4375(48)**

Table 5.3: Status of wild caught female sand flies regarding blood feeding and maturation of eggs

Location	Site of collection	**Total Examined Females**	**Total dissected Females**	**Blood-fed females (%)**	**Half-feed (%)**	**Unfed (%)**	**Graved/semigraved (%)**
Timergara	Indoor collection	902	892	18(1.056)	45(2.64)	832(48.76)	7(0.40)
	Outdoor collection	60	58	3(0.176)	5(0.29)	50(2.94)	2(0.11)
Balambat	Indoor collection	379	371	06(0.35)	04(0.23)	361(21.23)	8(0.46)
	Outdoor collection	19	19	-	-	19(1.11)	-
Khaal	Indoor	214	213	02(0.117)	05(0.29)	206(12.11)	1(0.058)

	collection						
	Outdoor collection	8	7	-	-	7(0.41)	1(0.058)
Warae	Indoor collection	140	140	01(0.058)	-	139(8.176)	-
	Outdoor collection	-	-	-	-	-	-

Female flies require blood (post insemination) from the vertebrate host including humans for the development of eggs Female sand flies were separated into different categories; blood-fed, half-fed, un-fed and gravid/semi gravid on the basis of their anatomical examination. Unfed and half-fed sand flies were high as compared to blood-fed (Fig. 5.3), gravid/semi graved (Fig. 5.4). Gravid flies, blood–fed and half-fed flies collected from indoor sites were high as compared to collection from outdoor sites (Table 5.3).

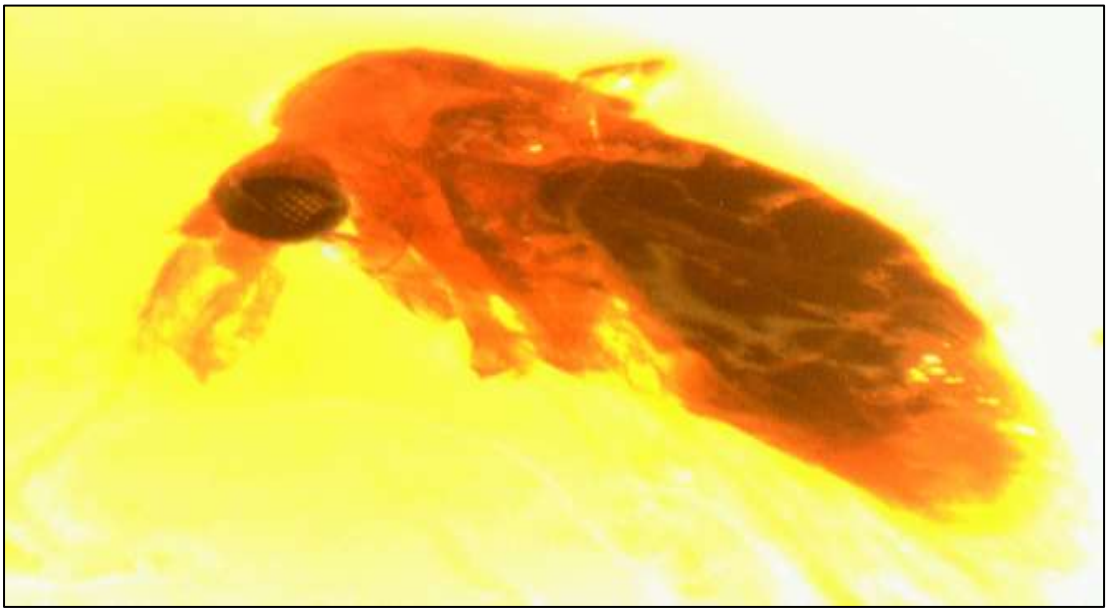

Fig. 5.4: Blood fed female sand fly

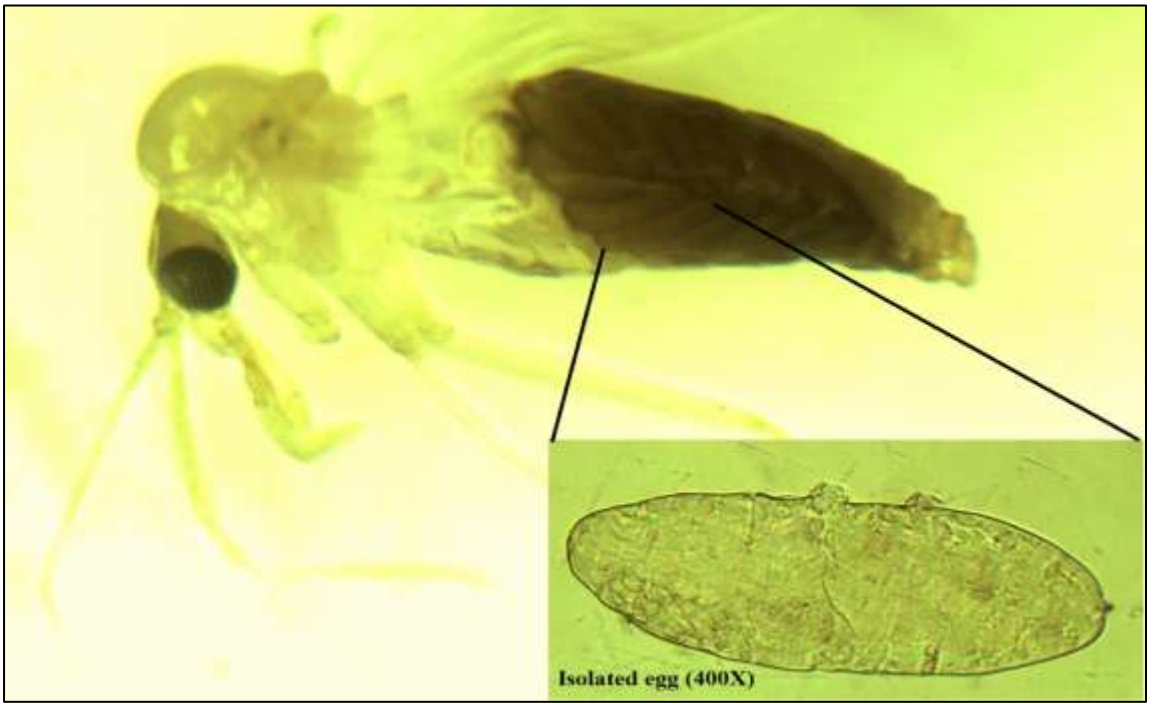

Fig. 5.5: Semigraved sand fly and the isolated egg

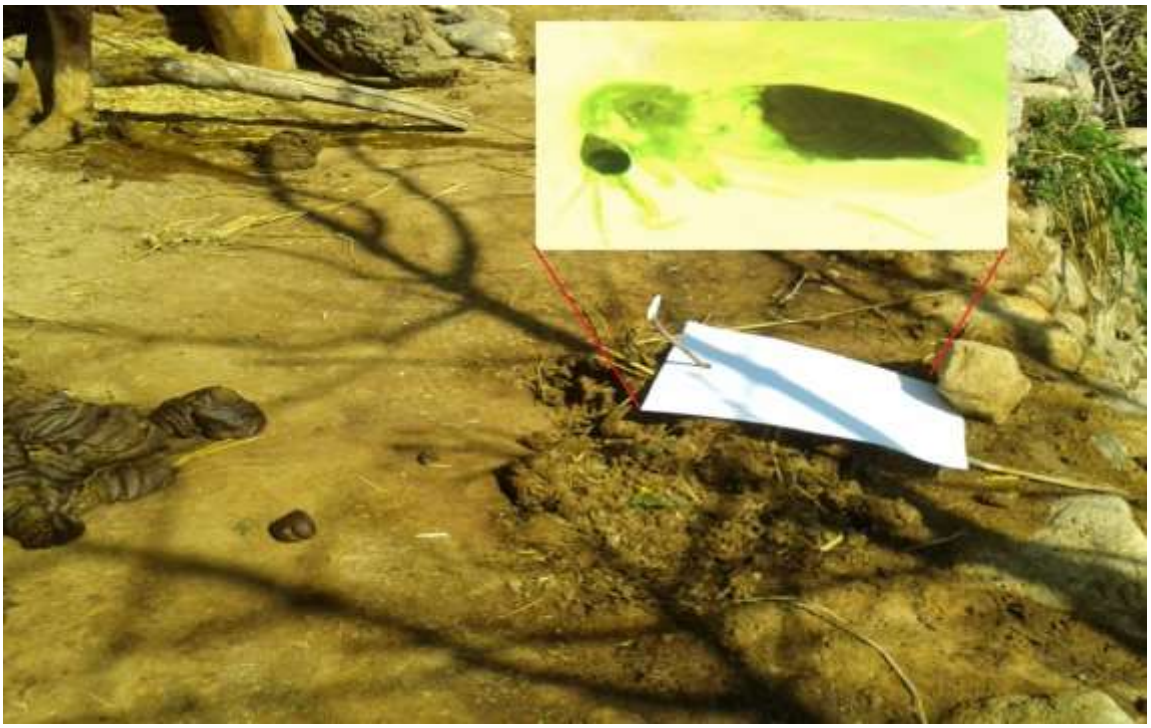

Fig. 5.6: Collection of semigraved female sand fly from cattle shed

5.4 DISCUSSION

No detailed and authentic information on biology of Pakistani sand flies is available, although several attempts were made to describe sand fly fauna from its different areas (Qutubuddin, 1951; Nasir, 1958; Burney and Lari, 1986; Barnett and Suyemoto, 1961; Lewis, 1967; Burney *et al.*, 1979; Kakar, 2004; Aslamkhan, 1996, 1998).

Unlike mosquitoes, sand flies do not breed in water; the environmental requirements on land for these flies are stable temperature, high humidity and decaying organic matters however very little is known about their natural breeding (Moncaz *et al.*, 2012-13). In adult sand flies collection some individuals were recognized as immature male, identified by the incomplete rotation of genatilia (most probably in the first night of adulthood). As the rotation process is the last stage of male maturation, these males were therefore still very close to the oviposition sites (Moncaz *et al.*, 2012).

Majority of the surveyed houses had domestic animals (mostly bovine species) the probable hosts for female sand flies. Both sexes of *P. salengensis* and *P. sergenti* (with blood fed, gravid/ semi graved females) were collected from cattle droppings and junks accumulated in open areas around human dwellings. Such sites probably serve as favourable breeding habitats for the early developmental stages of sand flies (Killick-Kendrick, 1999). *P. sergenti* collected from cow dung is the suspected vector of CL in the area (Rowland *et al*., 1999), also reported to be breeding in sheep pens by other worker (Branco *et al.*, 2013).

Alencer *et al.*, (2011) reported greater number of immature forms in soil with litter (which country), between roots and under fallen trunks. Resting or breeding sites are expected in the nearby places, where adult flies are captured as their flight capacity is limited and they tend to remain localized (Chaniotis *et al.*, 1974). Scanty literature

about sand fly larvae is available from diverse habitats including caves, crevices, animal burrows, termite mounds, and cracks in the soil, domestic animal shelters, cracked walls, tree-holes, birds' nests and leaf litter (Sharma and Singh, 2008).

Immature male sand flies herein are the first report not only from Pakistan but also its bordering countries, Afghanistan, Iran and India. This study identify the most probable breeding sites sand flies, which could be used as a base line for further investigation.

CHAPTER - 06
CHARACTERISTICS OF RESTING AND PROBABLE BREEDING SITES OF ADULT SAND FLIES (DIPTERA: PSYCHODIDAE) IN THE DIR DISTRICTS

6.1 INTRODUCTION

The complex biology of sand flies covering all aspects of reproduction, resting, feeding, dispersal and other activities of vector species is among the essential tools for disease control and prevention of outbreaks. Sand flies have wide distribution both in tropical and temperate regions, generally with species specific resting/ breeding sites. Natural breeding sites of sand flies, so far are very poorly investigated. Searching for developmental stages of sand flies is an extremely difficult and tedious job (Killick-Kendrick, 1986, 1999). The control efforts are therefore mainly confined to adult sand flies and personal protection (Alexander and Maroli, 2003).

According to the observations made by Newstead (1912) *P. papatasi* sand flies flourished in those micro habitats where specific conditions including the presence of organic matter, humidity and low levels of light are met. Alencer *et al*., (2011) reported greater number of immature forms in soil with litter, between roots and under fallen trunks. Resting or breeding sites are expected in the nearby places, where adult flies are captured as their flight capacity is limited and they tend to remain localized (Chaniotis *et al*., 1974). Scanty information about sand fly larvae have been reported from diverse habitats including caves, crevices, animal burrows, termite mounds, and cracks in the soil, domestic animal shelters, cracked walls, tree-holes, birds' nests and leaf litter (Singh, 2008).

Leishmaniasis (group of diseases) transmitted by various species of sand flies are wide spread mostly in tropical and sub-tropical regions. A spread in the distribution of

CL cases was observed in Pakistan (Rowland *et al*., 1999; Reithinger *et al*., 2003; Brooker *et al*., 2004) and neighbouring countries, Afghanistan (Ashford, 1992; Faulde *et al*., 2009) and Iran (Yaghoobi-Ershadi *et al*., 2004; Razmjou *et al*., 2009). In Pakistan and Afghanistan the leishmaniasis distribution is generally limited to the poverty and neglected areas with inadequate access to health care (Kassi *et al*., 2008). CL cases in district Dir was initially reported in the early 1990s (Rowland *et al*., 1999). The actual sand fly species responsible for the transmission of leishmaniasis is not known. However, *P. sergenti* and *P. papatasi* are considered as the suspected vectors of CL in the country (Rowland *et al*., 1999). The current study aimed to characterize bio ecological aspects of various sand fly species in and around human dwelling in four tehsils of Dir districts, could be possibly used in devising vector control strategy.

6.2 MATERIALS AND METHODS

6.2.1 Study design and flies sampling procedure

Overall, 40 villages in four Tehsils; Timergara (8), Warae (4), Khall (13) and Balambat (16) were randomly surveyed. Sand flies were sampled indoor and outdoor during the day and night times. Domestic collection was made from three kinds of houses such as mud made, concrete and stone made houses. Night time collection was more effective as the flies were active and out of their resting places. Peridomestic collection was made from different sites such as cattle corrals, dry and fresh cattle dungs, drainage holes in walls, plants, tree holes, caves, firewood stalks and grave yards.

Day times sampling was not very fruitful due to the flies mostly in inactive or resting condition. Diurnal collection from indoor sites was made in 24 villages of four

Tehsils, Timergara, (4) Balambat (13), Khall (1) and Warae (4) through spray and mouth aspirator. While nocturnal sampling was conducted in 18 villages of three Tehsils; Timergara (3), Balambat (4) and Khall (11) using sticky traps. Three to four traps per house were placed 30 cm above the ground and were fixed with wooden dowels in mud walls inside house as well as with nail in concrete houses between 7:00 PM in evening and 7:00 AM in morning.

Peridomestic sampling in different sites were made through sticky traps. In plants (leaves, stem and flowers) sticky traps were either placed folded or hanged vertically with the plant parts. Sand flies were monitored from chicken sheds through sticky traps placed vertical at the entrance and were fixed with wooden dowels. In cattle corrals traps were spread in various positions (vertical or horizontally placed). In drainage wall holes traps were in folded position, similarly folded and flat traps were also placed in firewood stalks (Fig. 6.7-6.19).

Survey was also carried out in 11 randomly selected caves (denoted by C1-C11 codes), in the hilly area of village Yarkhan Banda, tehsil Timergara, District Dir Lower (Table 1 and Fig. 6.1-6.6). These caves were visited once in the month of July, 2014. A total of 4-5 sticky traps per cave were fixed in different positions (horizontal, folded and flatted) in various parts of the caves in the evening and were collected next morning.

Soil samples of each cave ± 2kg were collected in plastic bags from the ground surface soil for analysis of different parameters in the laboratory at National Centre of Excellence in Geology, University of Peshawar as described by Moncaz *et al*., (2012). Co-ordinates of each cave were taken with the help of GPS.

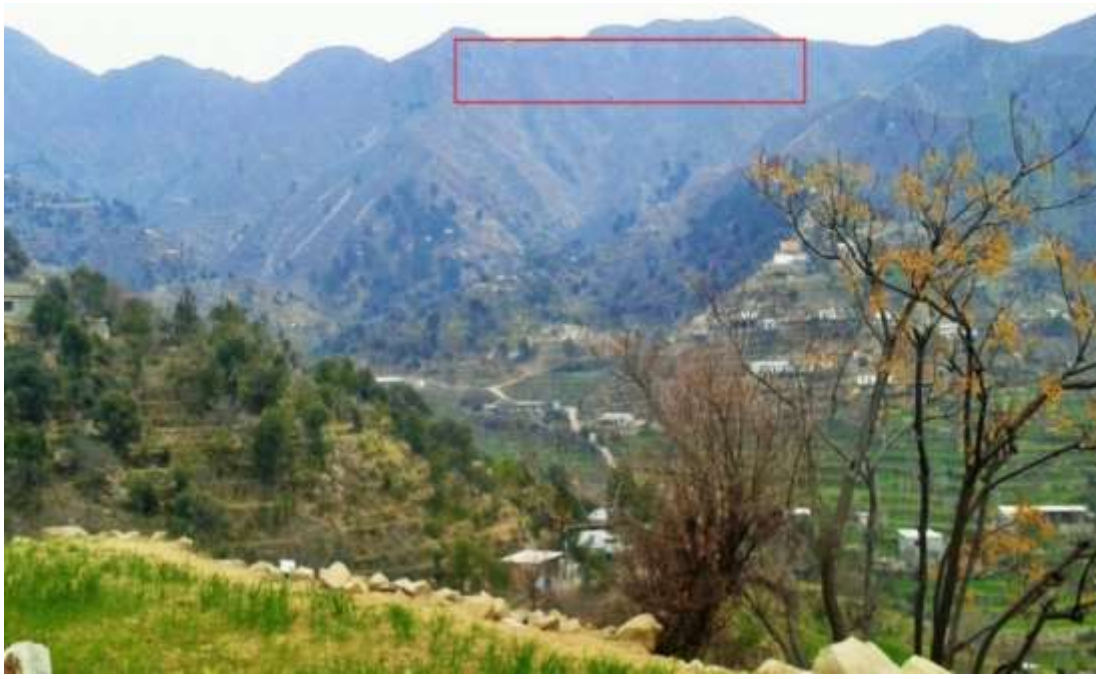

Fig. 6.1: Geographical localization of caves (rectangle) in village Yarkhan Banda, Tehsil Timergara.

Fig. 6.2: Overview of 11 caves represented by different IDs.

Fig. 6.3: Cows grazing in nearby caves.

Fig. 6.4: External as well as internal view of C2, vegetation are also evident in the surrounding area of the cave.

Fig. 6.5: The author is measuring the cave, cattle (goats or sheep) dry dung are shown by red arrows.

Fig. 6.6: Internal view of C10

Table 6.1: Locations and descriptions of the caves in Lower Dir.

Caves IDs	Latitude	Longitude	Altitude (m)	Climatic conditions of the caves
C1	34.78651	71.88386	2520	Humid, light penetration occurred, organic manures present
C2	34.78724	71.88294	2512	Damp, light penetration occurred, birds faeces present and were occupied by jackals
C3	34.78603	71.88405	2545	-do-
C4	34.78571	71.88397	2564	-do-
C5	34.78548	71.88493	2589	-do-
C6	34.78535	71.88451	2578	-do-
C7	34.78532	71.88464	2594	-do-
C8	34.78494	71.88515	2591	-do-
C9	34.78490	71.88508	2588	Humid, light penetration occurred, birds faeces present and were occupied by jackals
C10	34.78489	71.88473	2593	Humid, light penetration not accessible, occupied by porcupines
C11	34.78470	71.88585	2565	Dry, light penetration occurred, rats faeces present
C1	34.78651	71.88386	2520	Humid, light penetration occurred, organic manures present

6.3 Data Analysis of sand flies collected form caves

Relative abundance (RA) was calculated as no of specimens of species / total number of phlebotomine X 100. The degree of presence (D) was calculated using the no of cave containing species / total number of caves X 100 (Demir *et al*., 2010. Similarly, density was calculated as sand flies/ trap/night. Hierarchical Cluster Analysis (HCA) was analysed in SPSS version 20.0 using HCA. The result of the cluster analysis was displayed on dendrogram and was exported.

Fig. 6.7: Sticky trap kept in Rutaceae plant

Fig. 6.8: Sticky trap kept vertically in the nearby position of the Cucurbitaceae plant

Fig. 6.9: Sticky trap kept vertically in the nearby position of the Cucurbitaceae plant

Fig. 6.10: Internal and external views of C1 in the study site

Fig. 6.11: Sticky traps are evident inside the cave

Fig. 6.12: Sticky traps are placing in the tree holes

Fig. 6.13: Sticky trap are placed in the entrance of the chicken shed

Fig. 6.14: Vertically kept sticky trap in chicken shed

Fig. 6.15: Sticky trap placed in cattle corral

Fig. 6.16: Sticky trap kept in firewood stalk

Fig. 6.17: Seven sticky traps are evident in dry cattle dungs

Fig. 6.18: Sticky traps are placed in dry cattle dungs

Fig. 6.19: Sticky trap under firewood stalk

6.4 RESULTS

6.4.1 Nocturnal and diurnal collection of sand flies

Sand flies were collected during day and night in different habitats at four tehsils of Dir districts. For the day collection, spray catch and aspiration methods were used while sticky traps were set for night collection from different sites, table (6.2).

Table 6.2: Sand fly species composition and abundance at day and night time in Dir districts, 2014.

S. No.	Species	Night time collection		Day time collection		Grand Total
		M/F	Total/%	M/F	Total/%	
1	***P. salengensis***	2409/686	3095/99.8	6/0	6/0.19	3101
2	***P. sergenti***	2366/145	2511/93.6	139/30	169/5.18	2680
3	***S.babu***	28/93	121/22.4	71/347	418/77.5	539
4	***S. dentata***	173/0	173/49.8	174/0	174/50.14	347
5	***S. baghdadis***	3/65	68/22.07	21/219	240/77.9	308
6	***P. major***	112/16	128/98.4	2/0	2/1.53	130

7	***S. bailyi***	32/15	47/92.15	2/2	4/7.8	51
8	***P. andrejevi***	37/0	37/100	-	-	37
9	***S. hospittii***	0/12	12/42.5	3/13	16/57.14	28
10	***P. kazeroni***	0/11	11/45.8	0/13	13/54.16	24
11	***S. montana***	0/1	1/5	0/19	19/95	20
12	***P. papatasi***	5/9	14/100	-	-	14
13	***P. hindustanicus***	-	-	4/0	4/100	4
14	***P. ansari***	0/2	2/100	-	-	2
15	***P. bergeroti***	0/1	1/50	0/1	1/50	2
16	***S. grekovi***	1/0	1/100	-	-	1
17	***S. hodgsoni***	0/1	1/100	-	-	1
18	***S. turkistnica***	0/1	1/100	-	-	1
19	***S.tiberidis pakistanica***	0/1	1/100	-	-	1
20	***S. theodori***	-	-	1/0	1/100	1
Total		**5166/1059**	**6225/85.36**	**423/644**	**1067/14.6**	**7292**

Table (6.2) shows a total of 20 species, where 18 species were found at night time while 13 appeared in day time collections. *P. salengensis, P. sergenti, P. major, S. bailyi* and *P. andrejevi* were the most abundant species in night time collection but at day time *S. babu, S. dentata* and *S. baghdadis* were dominant. Individuals belonging to *P. papatasi* were found only at night time.

6.4.2 Indoor collected sand flies and the role of housing design on flies' distribution

A total of 499 houses were visited and sand flies were sampled from different sites of these houses. These houses were categorized into three types on the basis of the materials used in construction such as mud, concrete and stones.

Table 6.3: Sand flies species collected from different houses in Dir districts, 2014

Species	**Sand flies collected from different types of houses**			
	Mud made houses	**Concreted/painted houses**	**Stone/bricks made houses**	**Grand total**
P. salengensis	M/F	M/F	M/F	M/F
	2111/664	566/42	145/9	2387/160
P. sergenti	2387/160	187/18	453/24	2111/664
S.babu	31/130	17/20	17/20	98/434
S. dentata	126/-	145/0	12/0	83/0
S. baghdadis		17/176	2/15	24/252
P. major	100/15	-	0/1	100/15
S. bailyi	-	2/2	-	2/2
P. andrejevi	11/0	26/0	-	37/0
S. hospittii	0/8	3/10	0/6	3/34
P. kazeroni	0/13	0/8	0/2	0/23
S. montana	0/9	0/11	-	-/20
P. papatasi	2/7	3/1	0/1	4/9
P. hindustanicus	-	4/0	-	4/-
P. ansari	0/2	-	-	0/2

P. bergeroti	0/2	-	-	0/2
S. grekovi	-	-	-	-
S. hodgsoni	0/1	-	-	0/1
S. turkistnica	0/1	-	-	0/1
S.tiberidis pakistanica	0/1	-	-	0/1
S. theodori	-	1/0	-	1/0
Total	**5054/1606**	**1004/556**	**630/77**	**5054/1609**

As shown in the table (6.3) and fig. (6.20) greater number of flies was recovered from mud houses followed by concerted and brick houses respectively. *P. salengensis* and *P. sergenti* in greater number were recorded from mud houses. *P. major* was collected in mud made house while *S. bailyi* was recorded from concreted/painted houses. Multiple comparison test revealed no significant interaction ($p>0.05$) between species and houses design (Table 6.3).

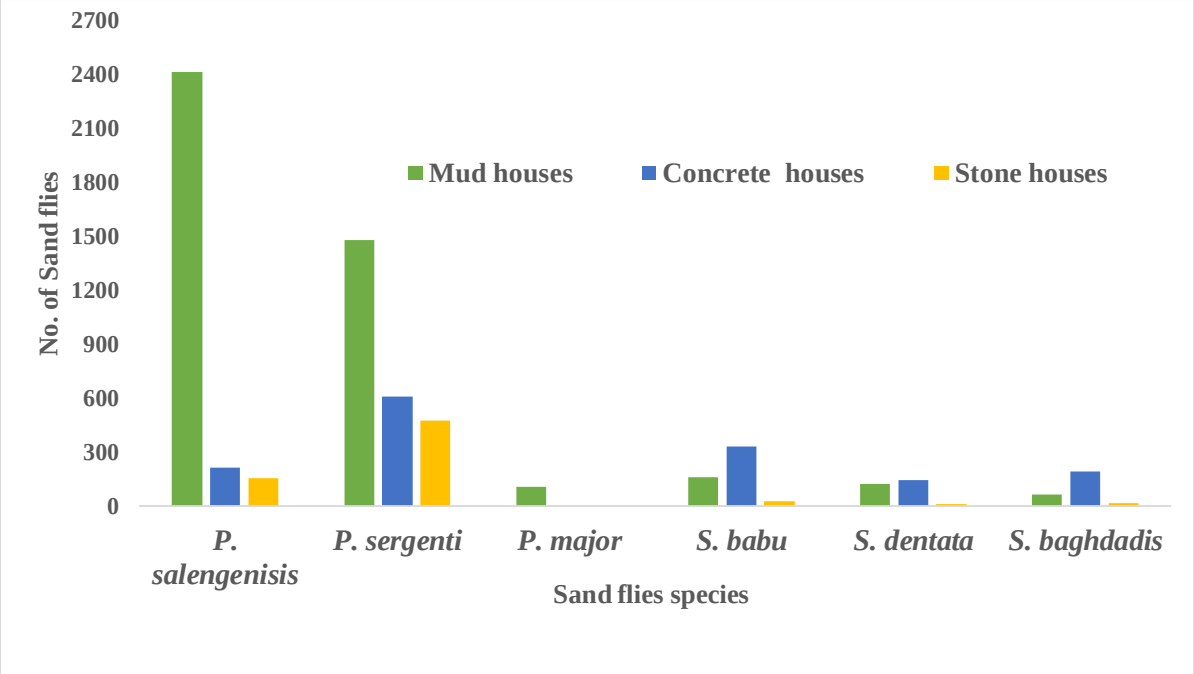

Fig. 6.20: Sand flies species collected from different houses in Dir Districts

Table 6.4: Multiple comparison (two way anova) testing the effects of houses designs on sand flies distribution in Dir districts, 2014

Source	Sum of Squares	Df	Mean Square	F	Sig.
Intercept	2036742.857	1	2036742.857	3.824	0.190
Species	2881680.476	6	480280.079	1.968	0.150
Houses	1065235.714	2	532617.857	2.182	0.155
Species * Houses	2928954.952	12	244079.579	-	-

Species	Sand flies collected from indoor sites					Total	Sand flies collected from outdoor sites										
	Combined dwellings	Bath rooms	Masques	Schools	Bedrooms		Cattles dung	Chicken sheds	Plants	Tree holes	Drainage holes	Corrals	caves	Grave Yards	Fresh cattle dung	Firewood stalks	Total	Grand Total
	♂/♀	♂/♀	♂/♀	♂/♀	♂/♀		♂/♀	♂/♀	♂/♀	♂/♀	♂/♀	♂/♀	♂/♀	♂/♀	♂/♀	♂/♀		
P. salengensis	2105/665	5/-	-	-	1/-	2111/665	158/2	60/10	12/2	17/	-	45/1	7/-	2/-	-/6	3/-	**304/19**	3101
P. sergenti	2245/133	93/20	4/-	-	45/7	2387/160	49/2	17/1	13/1	-/1	27/2	18/1	-	-/1	-	-	**124/9**	**2680**
S.babu	27/86	36/204	-	-/8	36/135	99/400	-/1	-/2	-/2	-	-/2	-	-	-	-	-	**0/7**	**539**
S. dentata	109/-	86/-	-	6/-	85/-	286/0	-	2/-	53/	3/-	-	-	-	3/-	-	-	**61/0**	**347**
S. baghdadis	3/39	7/127	-/4	1/-	9/92	20/262	-	-/2	-/18	-	-/2	-	-	-	-	-/4	**0/26**	**308**
P. major	98/15	-	2/-	-	-	100/15	10/-	-/1	1/-	-	-	2/1	1/0	-	-	-	**14/1**	**130**
S. bailyi	-	-	-	-	2/2	2/2	-	-	-	-	-	-	32/15	-	-	-	**32/15**	**51**
P. andrejevi	37/-	-	-	-	-	37/0	-	-	-	-	-	-		-	-	-	**-**	**37**
S. hospittii	-/11	3/6	-	-	-/7	3/24	-	-	-	-	-	-	-/1	-	-	-	**0/1**	**28**
P. kazeroni	-/10	-/5	-	-	-/8	0/23	-	-	-	-	-	-/1	-	-	-	-	**0/1**	**24**
S. montana	-/1	-/7	-	-	-/12	0/20	-	-	-	-	-	-	-	-	-	-	**-**	**20**
P. papatasi	5/9	-	-	-	-	5/9	-	-	-	-	-	-	-	-	-	-	**-**	**14**
P. hindustanicus	-	-4	-	-	-	4/0	-	-	-	-	-	-	-	-	-	-	**-**	**4**
P. ansari	-/2	-	-	-	-	0/2	-	-	-	-	-	-	-	-	-	-	**-**	**2**
P. bergeroti	-/1	-	-	-	-/1	0/2	-	-	-	-	-	-	-	-	-	-	**-**	**2**
S. grekovi	-	-	-	-	-	-	-	-	1/-	-	-	-	-	-	-	-	**1/0**	**1**
S. hodgsoni	-/1	-	-	-	-	0/1	-	-	-	-	-	-	-	-	-	-	**-**	**1**
S. turkistnica	-/1	-	-	-	-	0/1	-	-	-	-	-	-	-	-	-	-	**-**	**1**
S.tiberidis pakistanica	-/1	-	-	-	-	0/1	-	-	-	-	-	-	-	-	-	-	**-**	**1**
S. theodori	-	1/-	-	-	-	0/1	-	-	-	-	-	-	-	-	-	-	**-**	**1**
Total	**4628/975**	**277/351**	**6/4**	**7/8**	**178/236**	**5055/1620**	**221/5**	**80/16**	**82/20**	**20/1**	**27/6**	**65/4**	**40/16**	**5/1**	**-/6**	**3/4**	**535/80**	**7292**

Table 6.5: Sand flies collected from indoor and outdoor habitats in Dir districts, 2014

Sand flies were collected from 15 different habitats including five from indoor and ten from outdoor (Table 6.5). The number of sand flies collected from indoor habitats was far more than that of outdoor. Highest number of flies was collected from common dwelling (rooms used for both people and animals) followed by the flies collected from bath rooms and bed rooms. Among the outdoor habitats studied, highest number was collected from cattle dung (semi dry) followed by plants and chicken sheds. Least number of flies was collected from mosques and schools among outdoor habitats. In the studied outdoor habitats, grave yards, fresh cow dung and firewood stalks inhabited lowest number. *P. salengensis* and *P. sergenti* showed wide distribution as found in most of the indoor and outdoor habitats, but like rest of the species were collected in large number from indoor sites.

6.4.3 Sand flies prevalence association with different types of vegetation/plants

A small number of sand flies were collected from different trees, vegetables and ornamental plants (Table 6.6). A variable number of sticky traps were placed especially in leaves and young stems of plants in peridomestic habitats in the evening and were collected the following morning (Table 6.6).

Table 6.6: Sand fly species collected from different plants in District Dir 2014

Family	**Collected sand flies species**														**Total**
	P. sergenti		***P. salengensis***		***P. major***		***S. babu***		***S. baghdadis***		***S. dentata***		***S. grekovi***		
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	
Cucurbitaceae	4	-	-	1	-	-	-	-	-	6	39	-	-	-	**50**
Eupherbiceae	-	-	-	-	-	-	-	-	-	1	10	-	-	-	**11**
Moraceae	1	-	7	-	-	-	-	-	-	-	-	-	-	-	**8**
Asclepiadaceae	-	-	-	-	-	-	-	-	-	8	-	-	-	-	**8**
Rutaceae	-	-	-	-	-	-	-	-	-	2	4	-	-	-	**6**
Polygonacae	1	-	2	1	1	-	-	-	-	1	-	-	-	-	**6**
Sepotacae	1	2	1	-	-	-	-	-	-	-	-	-	1	-	**5**

Acanthacaeae	2	-	-	-	-	-	-	2	-	-	-	-	-	-	**4**
Nyctiginaceae	1	1	-	-	-	-	-	-	-	-	-	-	-	-	**2**
Rosaceae	-	-	1	-	-	-	-	-	-	-	-	-	-	-	**1**
Canvolvulaceae	1	-	-	-	-	-	-	-	-	-	-	-	-	-	**1**
Mimosaceae	1	-	-	-	-	-	-	-	-	-	-	-	-	-	**1**
Olaeceae	-	-	1	-	-	-	-	-	-	-	-	-	-	-	**1**
Verbenaceae	1	-	-	-	-	-	-	-	-	-	-	-	-	-	**1**
Total	**13**	**3**	**12**	**2**	**1**	**-**	**-**	**2**	**-**	**18**	**53**	**-**	**1**	**-**	**105**

A total of seven species of sand flies were collected from twelve plant families. Overall, male sand flies were observed in greater number as compared to females. In general, high number of flies was sampled from Cucurbitaceae family followed by Moraceae and Eupherbiceae respectively (Table 6.6). Among the collected species *S. dentata* was collected in highest number followed by *S. baghdadis, P. sergenti* and *P. salengensis*.

6.4.4 Sand flies existence in Caves

The area where the caves are located is deforested during the last few decades, therefore tall trees (*Pinus roxburghii*) once a dominant species in the area were rare and replaced by dense vegetation comprising *Indgofera heterantha, Rumex hestatus, Cannabis sativa, Monotheca buxifolia, Myrsine africana, Isodon rugusus, Themeda anathera, Pennesetum orintale* and *Dodonea viscosa* (Fig. 6.1). Grazing animals (sheep, goats and cows), predators (jackals, foxes wild dogs, leopard) and partridges (*Alectoris chukar* and *Francolinus francolinus)* were observed nearby caves. Organic manure, animal faecal matters was also noted in these caves (Fig. 6.5). Locals of the village routinely shifted their herds to the area and they also collect dry fire wood and fresh grasses for domestic animals in the houses (Fig. 6.3).

Sand fly species collected from caves were identified as belonging to two genera, *Phlebotomus* and *Sergentomyia*. Only male individuals of two species (*P. salengensis*

and *P. major*) were collected while *S. hospittii* was presented by one female individual (Table 6.7). *S. bailyi* (62.33%) was the abundant sand fly followed by *P. salengensis* (15.58%). High number of sand fly individuals were found in C1 (28.57%) and C7 (23.37%). Regarding species maximum number (4) was recorded from C1 followed by three from C3. Rest of the caves inhabited two species each except C8 where only one species was found. Two species *S. bailyi* and *P. major* were collected from nine caves and *P. salengensis* was found in five caves. Only one individual of *S. hospittii* was collected from C11 (Table 6.7). A total of seven male specimens of *P. salengensis* were collected in four caves (C2, C3, C9 and C11). A single immature male individual (identified on the basis of its unrotated external genitalia) was also recorded in C11 as shown in table 6.7.

Table 6.7: Sand flies collected from different category caves in tehsil Timergara, 2014

Cave IDs	*S. bailyi*		*P. major*		*P. salengensis*		*S. hospittii*		Grand Total	Relative abundance	Degree of presence (%)
	♂	♀	♂	♀	♂	♀	♂	♀			
C1	-	1	4	-	5	-		1	22	28.57	36.37
C2	-	-	2	-	1	-	-	-	3	3.89	18.18
C3	5	3	2	-	2	-	-	-	12	15.58	27.28
C4	2	-	1	-	-	-	-	-	3	3.89	18.18
C5	3	2	3	-	-	-	-	-	8	10.38	18.19
C6	3	-	1	-	-	-	-	-	4	5.19	18.19
C7	12	5	1	-	-	-	-	-	18	23.37	18.19
C8	2	2	-	-	-	-	-	-	4	5.19	9.09
C9	2	1	-	-	2	-		-	4	5.19	18.19
C10	3	2	2	-	-	-	-	-	7	9.09	18.19
C11	-	-	1	-	1	-	-	-	2	2.59	18.19
Total	32	16	17	-	11	-	-	1	77	100	-

Table 6.8: Summary of soil parameters and size of the caves located in Tehsil Timergara, Lower Dir.

Caves ID	length	Height	Width	Electric conductivity (µS/cm)	Salinity (g/L)	pH	Organic matter	Hygroscopic Water Content (%)
C1	18	7.7	10	303	0.1	7.01	6.05	3.2
C2	8	3	4	178.7	0.1	6.95	4.83	2.63
C3	5.4	2.2	3.7	47.3	0	7.09	5.25	1.83
C4	4.1	2.4	3.7	371	0.2	7.23	4.97	3.12
C5	8	4.5	4	957	0.5	7.29	4.07	2.48
C6	5.9	2.8	4.5	79.1	0	6.76	4.08	2.1
C7	5.9	2.7	7.4	223	0.1	7.8	5.3	2.06
C8	13.2	4.6	8.2	271	0.1	6.26	3.7	40.56
C9	7.2	4.5	4.1	163.4	0.1	7.6	4.6	3.8
C10	11.6	8.5	11	113.4	0.1	6.35	3.9	36.42
C11	1.5	1.3	3	100.7	0.1	7.94	5	4.08
Average	**8.072**	**4.018**	**5.781**	**255.236**	**0.127**	**7.11**	**4.704**	**9.29818**

Soil samples of eleven caves were analysed for the presence of different parameters (Table 6.8). Overall, pH of all caves ranged between 6.2 and 7.9. Highest values were obtained for organic content, hygroscopic water (%) content, electrical conductivity and salinity in C1 (6.05), C8 (40.56), C5 (957µS/cm) and C5 (0.5 g/L) respectively.

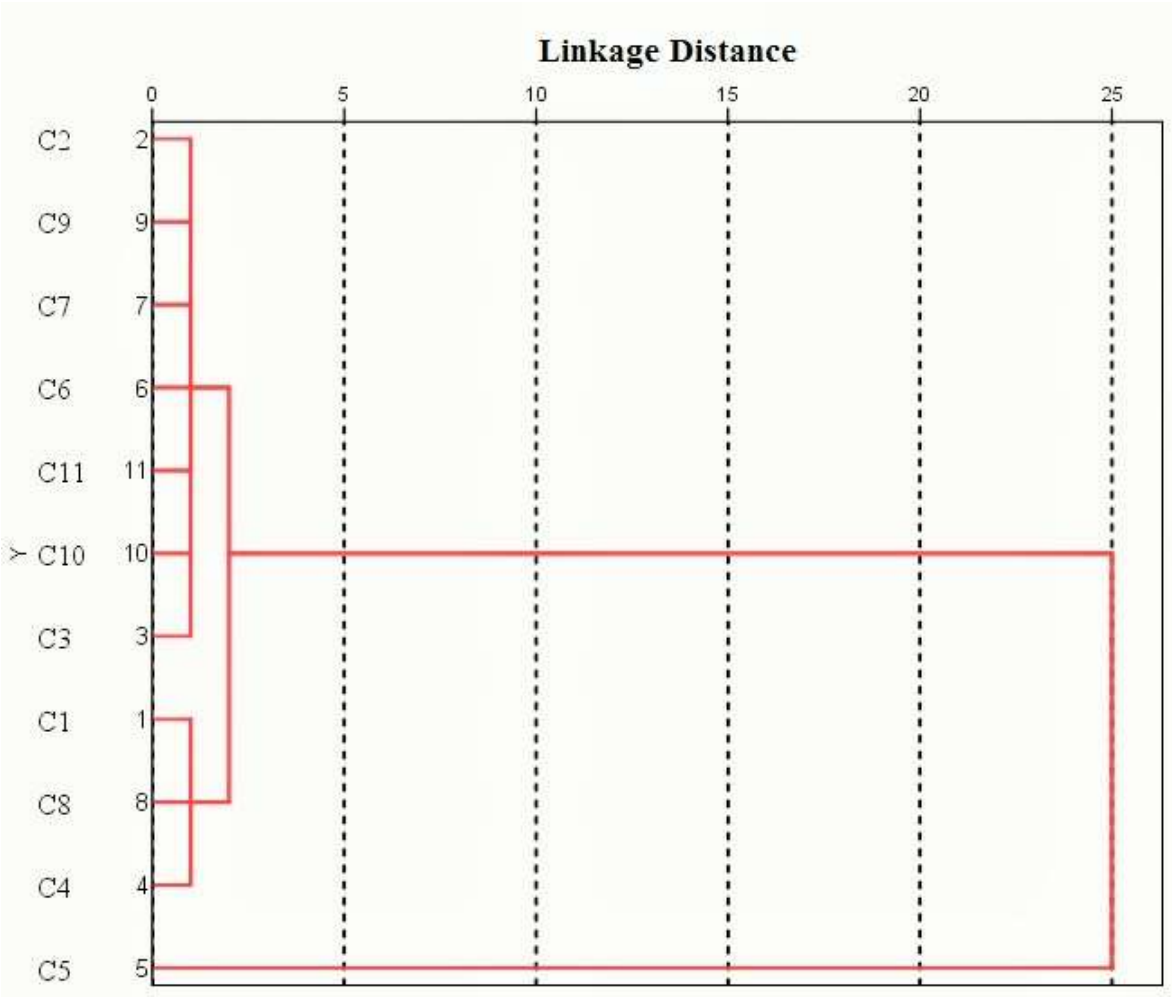

Fig. 6.21: Dendrogram showing similarities in resting or probable breeding sites of four sand fly species.

Cluster analysis regarding sand flies resting sites and probable breeding habitats form clusters (Fig. 6.21). Cluster includes C1,C2,C3,C4,C5,C6,C7,C8,C9,C10 and C11. Linkage distance was 0 to 25. Seven caves presented closed linkage while three caves 8, 1 and 4 showed closed affinity. Similarly C5 was very different from the other caves in this analysis

6.5 DISCUSSION

The present study was aimed to identify sand flies activity sites (resting/breeding), the essential prerequisites in devising appropriate control methods. Specific ecological

niches are required by various species of sand flies prevailing around human and domestic animal dwelling (Lawyer and Perkins, 2004). Such information is essential step in devising up vector control strategies. Favourable micro habitat is required for sand flies survival in houses, latrines, cellars, stables; caves, fissures in walls, rocks or soil; dense vegetation tree holes and buttresses; burrows of rodents and other mammals or bird's nests (Grassi, 1907; Killick-Kendrick, 1999). Till now no detailed study is available about the sand flies biology in Pakistan.

Sand flies collected from plants using sticky traps shows the presence of mostly *Sergentomyia* species. It was observed that among different plants those belonging to Cucurbitaceae family, (creeper plants) had more flies indicated having appropriate environment for resting/ breeding of sand flies. These plants are cultivated as vegetables in the summer season in the surrounding areas of houses. Early developmental stages (eggs and larvae) are reported from tree buttresses, root leaf litters and epiphytes (Alencar *et al.,* 2011). This study provides the preliminary information regarding this aspect which could be used as base line for further investigation.

An important aspect that deserves attention is the housing conditions where sand fly collection is being made. It was observed that mud made houses have high number of genus *Phlebotomus* species. Such type of construction provides more cracks and crevices, microhabitats required for resting and other activities of sand flies sites (Feliciangeli, 2004; Kumar *et al.,* 2009; Malaviya *et al.,* 2014). In contrast very low number of sand flies was collected from concrete houses. Poche *et al.,* (2011) reported the correlation of sand flies populations in houses with the type of construction. They recorded just a few cases of VL from cemented houses in India.

P. sergenti and *P. papatasi* are considered suspected vectors of cutaneous leishmaniasis in Pakistan (Rowland *et al.,* 1999; Rahim *et al.,* 2003). Both the species were observed in the present study but the former was in much more number than the latter, probably playing more active role as was also recorded in the houses where active cases of human cutaneous leishmaniasis were recorded. *P. salengensis* was observed to be the most dominant species in this study was collected in the night collection particularly from cattle sheds. The medical importance of this species is not confirmed however, its role in zoonotic leishmaniasis needs to be explored.

Caves have been postulated to provide suitable breeding environment for sand fly species (Feliciangeli, 2004). Four species; *P. salengensis, P. major*, *S. bailyi* and *S. hospittii* were found in caves. Such places could be considered extra ordinary habitat for these flies as their ecology are completely different from the external habitats (Carvalho *et al.,* 2013). Two genera, *Phlebotomus* and *Sergentomyia* exhibited nearly close association with each other in the caves. This co-occurrence of the genera indicated that they share the same habitats (Moncaz *et al*., 2014).

S. bailyi was the dominant species in the surveyed caves. In Pakistan, this species was recorded from indoor sites in (Dera Ismail Khan, Jandola, Lahore, Larkana, Pano Aqil, Tando Mohammad Khan, Peshawar, Shikarpur, Tank, Mir Muhammad, Rawalpindi and Taxila) regions of Pakistan. This species were also presented their distribution in caves in Thailand (Suankratay, 2014).

Previous studies also emphasized that caves have unique characteristics (high humidity, stable temperatures and the risk of starvation due to scarce food resources). Therefore, these factors most likely effect the distribution of sand flies and other

organisms in the cave environment (Auler and Zogbi 2005; White and Culver 2005; Barata and Apolinário, 2012).

Physiochemical properties of the soil (pH, conductivity, water retention capacity, organic matters) are very important for sand flies larval development. Alder and Theodor (1957) confirmed that sand flies require moist soil rich in organic and nitrogenous matter to breed. In the present study, the average pH of the caves soil was (7.11). Polseela *et al*., (2011) found that average pH of the soil was alkaline 7.6 in Saraburi Province, Thailand and concluded that soil pH plays an important role in sand fly breeding. Vivero *et al*., (2015) analysed that alkaline soil is the characteristics of a stable environment, which effects larvae development by conditioning growth of soil microorganisms as possible larval food. Physical structure and pH of the soil can be used to control sand flies breeding sites.

The sites identified in the present study could be considered as resting/or breeding places, the information could be used for source reduction of sand flies including vector species for controlling cutaneous leishmaniasis.

CHAPTER - 07
GEOSPATIAL DISTRIBUTION OF SAND FLIES (DIPTERA: PSYCHODIDAE): ENVIRONMENTAL AND BEHAVIORAL RISK FACTORS ASSOCIATION WITH CUTANEOUS LEISHMANIASIS IN DIR DISTRICTS

7.1 INTRODUCTION

Sand fly species are distributed in tropics and temperate regions of the globe with specific ecological requirements extending from semi desert to forest environment. Sand fly distribution pattern is influenced by climatic factors, altitude and soil types and other biotic factors like availability of host and sugary plants (Hassan *et al*., 1999; Ferreira *et al*., 2001; Rispail *et al*., 2002). Kirton (1998) observed that spatial distribution of leishmaniasis was limited to the geographical range of sand fly specific vectors. Correlation of sand flies species spatial distribution with environmental and climatological layers may provide a tool for planning disease control and modelling of transmission potential as well as distribution of sand flies especially exotic vectors (Karimi, 2014).

Remote Sensing (RS) and Geographic Information System (GIS) were used to digitalised maps of sand fly vector species and leishmaniasis in Middle East for highlighting endemic and risk areas in the region (Cross *et al*., 1999). GIS has also been used for ecological analyses, disease mapping, predicting parasite occurrence/seasonality and surveillance (Cringoli *et al*., 2005; Rinaldi *et al*., 2006). Many studies has associated the disease with sex, age , domestic animals and clustering of disease cases at the household level (Reithinger *et al*., 2003-10; Brooker *et al*., 2004). Other factors such as poverty, low educational level and dog's ownership were associated with increased risk of VL (Bashaye *et al*., 2009).

Similarly, frequent movement of population between endemic and non-endemic sites is also suggested to be the main cause of the spread of leishmaniasis (WHO, 1990).

Risk factors data is very important to design and project public health response towards epidemics (Bashaye *et al*., 2009) and such factors can be modified to control and prevent future outbreaks. In Pakistan and Afghanistan, leishmaniasis distribution is generally limited to the poverty and neglected areas with inadequate access to health care (Kassi *et al.,* 2008). A spread in the distribution of CL cases was observed in Pakistan and neighbouring countries, Afghanistan and in Iran (Rowland *et al*., 1999; Faulde *et al*., 2008; Razmjou *et al*., 2009). The current study was designed to examine incidence of *Leishmania* infection and risk factors associated with the disease in Dir districts, keeping in view the identification of potential threats regarding this disease.

Leishmaniasis particularly CL is being reported in various parts of Pakistan but the ecology and distribution of sand fly species have not been systematically documented in the country. The disease is endemic in different parts of KPK, particularly in the northern belt. The present work is an attempt to overlay the spatial distribution of sand flies species with environmental factors which could be helpful to predict the presence or absence of sand flies species and disease risk in the study sites.

7.2 MATERIALS AND METHODS

7.2.1 Study sites and design

The present study was carried out in 40 villages of four tehsils of Dir districts. The area is hilly and surrounded by high mountains of Hindu Kush Range. Coordinates of each surveyed houses were taken with the help of GPS and clinically confirmed cases of cutaneous leishmaniasis of each household were recorded at the time of survey.

7.2.2 Risk factors data

The risk factors questionnaire data covering household information (Appendix) were collected at the time of collection. The data from each houses were taken either from the head of the household or representative (health workers, school teachers and students) of the house.

7.2.3 Statistical Analysis

A database was created in MS Access and was imported to ArcGIS version 10.2.2 where Dir districts boundary shape file was created. Digital Elevation Model (DEM) of both districts was extracted from ASTER GDEM. Digitalized distribution maps of two genera and most abundant sand flies species; *P. salengensis* and *P. sergenti* of genus *Phlebotomus* were produced. To investigate correlation of elevation of the studied area with the presences or absence of sand flies, the location of each species were overlaid on the elevation layers.

7.2.4 Statistical Analysis of risk factors data

The questionnaire data was first entered into a MS Access database. In addition, explanatory variables collected during the household surveys, a number of explanatory variables were derived. All the analysis of univariate and bivariate were done with the help of Stata software, version 12.

7.3 RESULTS

Overall, 7292 sand flies (5589 males and 1703 females) specimens were collected in four tehsils, comprising two genera; *Phlebotomus* and *Sergentomyia*, the former genus was represented by nine species and later one was showed by eleven species. GIS based maps of both genera and abundant sand fly species; *P. salengensis* and *P. sergenti* were further analysed (Table 7.1).

Table 7.1: Sand flies and CL cases found in study villages in Dir districts, 2014

Study Sites		Altitude (m)	*Phlebotomus*		*Sergentomyia*		*P. salengensis*		*P. sergenti*		CL cases
			♀	**Total**	♀	**Total**	♀	**Total**	♀	**Total**	**Total**
Tehsil Timergara	**Yarkhan Banda**	1289	662	1619	57	67	597	1022	41	493	-
	ARC2	803	8	558	13	35	0	1	8	549	1
	ARC1	795	11	104	18	26	0	0	7	93	37
	Danvah	836	2	10	39	49	0	1	0	7	4
	Saddo	761	1	1	24	42	0	0	0	0	1
	Siar	996	0	0	36	76	0	0	0	0	2
	Sikaolae	762	0	0	20	26	0	0	0	0	2
	Total		**684**	**2292**	**207**	**321**	**597**	**1024**	**56**	**1142**	**47**
Tehsil Khall	**Watange**	749	11	504	7	9	3	341	8	152	10
	Shalfalm	964	17	166	0	0	6	25	10	124	12
	Dwaba	969	16	132	3	3	9	53	6	63	10
	Upper Lalo	923	0	113	4	4	0	87	0	26	1
	Lower Lalo	941	2	54	3	5	2	40	0	12	-
	Mira	739	3	45	22	31	0	5	2	37	3
	Sacha	731	3	45	22	31	0	5	2	37	-
	Sermubu	1018	3	45	22	31	0	5	2	37	-
	Barkale	1189	5	42	14	17	0	10	4	27	18
	Khall Banda	895	1	40	20	24	1	26	0	11	-
	Luqmanbanda	918	0	13	0	0	0	7	0	6	-
	Kalone	914	3	3	38	59	0	0	3	0	26
	Total		**64**	**1202**	**155**	**214**	**21**	**604**	**37**	**532**	**80**
	Mandish	831	27	549	14	14	24	307	2	125	-

Tehsil Balambat	**Khima**	1100	38	310	8	10	0	17	36	255	-
	Manzaetange	1093	4	78	9	13	2	8	2	66	-
	Malae	883	2	64	4	7	2	43	0	19	-
	Baroon	887	1	25	26	43	0	0	1	24	32
	Shzadi	820	2	7	8	40	0	0	0	5	1
	Rani	847	0	0	30	32	0	0	0	0	9
	Dab	906	0	13	20	36	0	0	0	13	7
	Haji Abad	952	1	12	48	57	0	0	0	11	6
	Kotkae	914	1	8	7	18	0	0	0	7	4
	Nawagae	839	1	5	37	55	0	0	0	4	4
	Munji	1062	2	3	12	35	0	0	2	1	7
	Koto	847	1	1	1	1	0	0	1	0	-
	Tangae	893	0	2	2	3	0	0	0	2	-
	Palusoo	757	0	0	35	50	0	0	0	0	-
	Dairy	890	0	0	7	9	0	0	0	0	-
	Shagae	912	0	0	2	3	0	0	0	0	3
	Total		**80**	**1077**	**270**	**426**	**28**	**375**	**44**	**532**	**73**
Tehsil Warae	**Akhagram**	1094	3	14	51	53	-	-	2	1	2
	Turmung	1130	2	4	35	38	-	-	2	2	-
	Jugha Banj	1098	3	4	36	40	-	-	3	2	1
	Warae Bala	1058	1	1	13	19	-	-	-	1	0
	Total		**9**	**23**	**135**	**150**	**-**	**-**	**7**	**16**	**3**
Grand Total			**217**	**3504**	**715**	**1004**	**70**	**1583**	**125**	**1612**	**203**

Sand flies were collected in 40 villages (sites) comprising four tehsils (Table 7.1). Overall, greater number of flies were recorded from Yarkhan Banda (Tehsil

Timergara) followed by Mandish (Tehsil Balambat) and ARC2 (Afghan Refugees Camp 2) in Tehsil Timergara. Regarding *P. sergenti*, high abundance was found in ARC 2, Yarkhan Banda and Khima while *P. salengensis* maximum number was recorded in Yarkhan Banda, Mandish and Watange. Sergentomyian sand flies were most abundant in Siar, Yarkhanbanda and Nawagae. High number of CL cases were reported in ARC1 (37), Baroon (32) and Kalone (26).

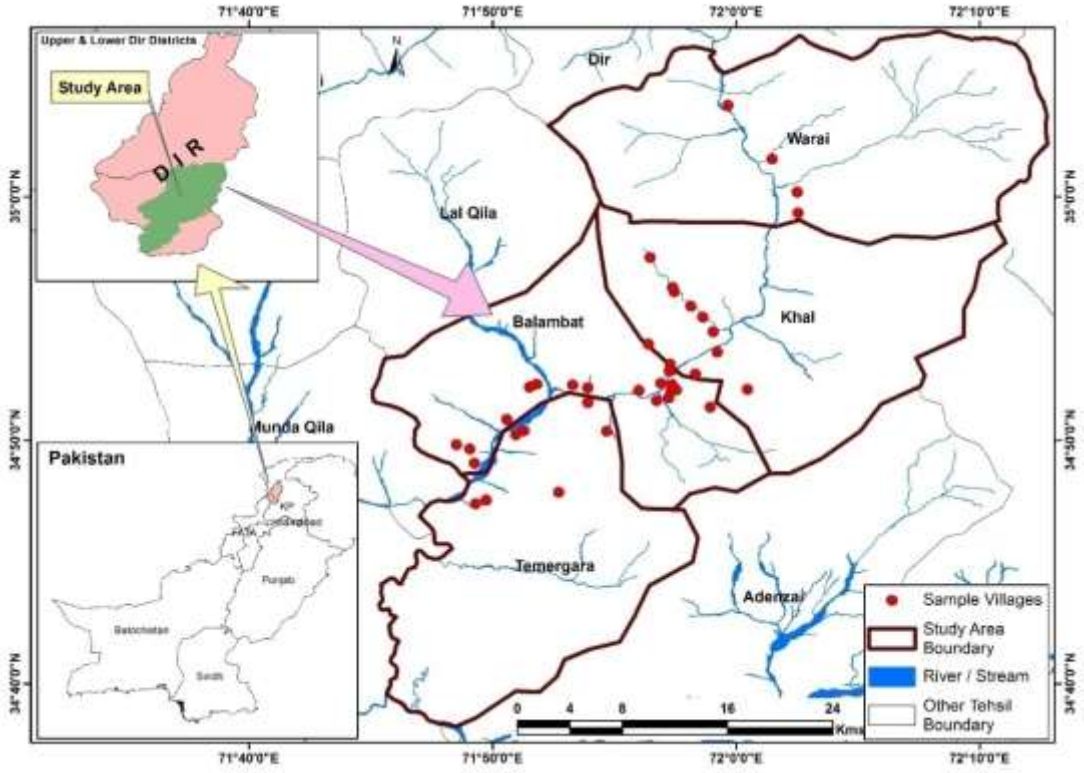

Fig. 7.1: Sand flies sampling location sites in Dir Districts

Sand flies collection was carried out in 40 villages of four tehsils in two Dir districts on both sides of the river Panjkora (Fig.7.1). Greater number flies were recorded in Timergara, followed by Khall, Balambat and Warae.

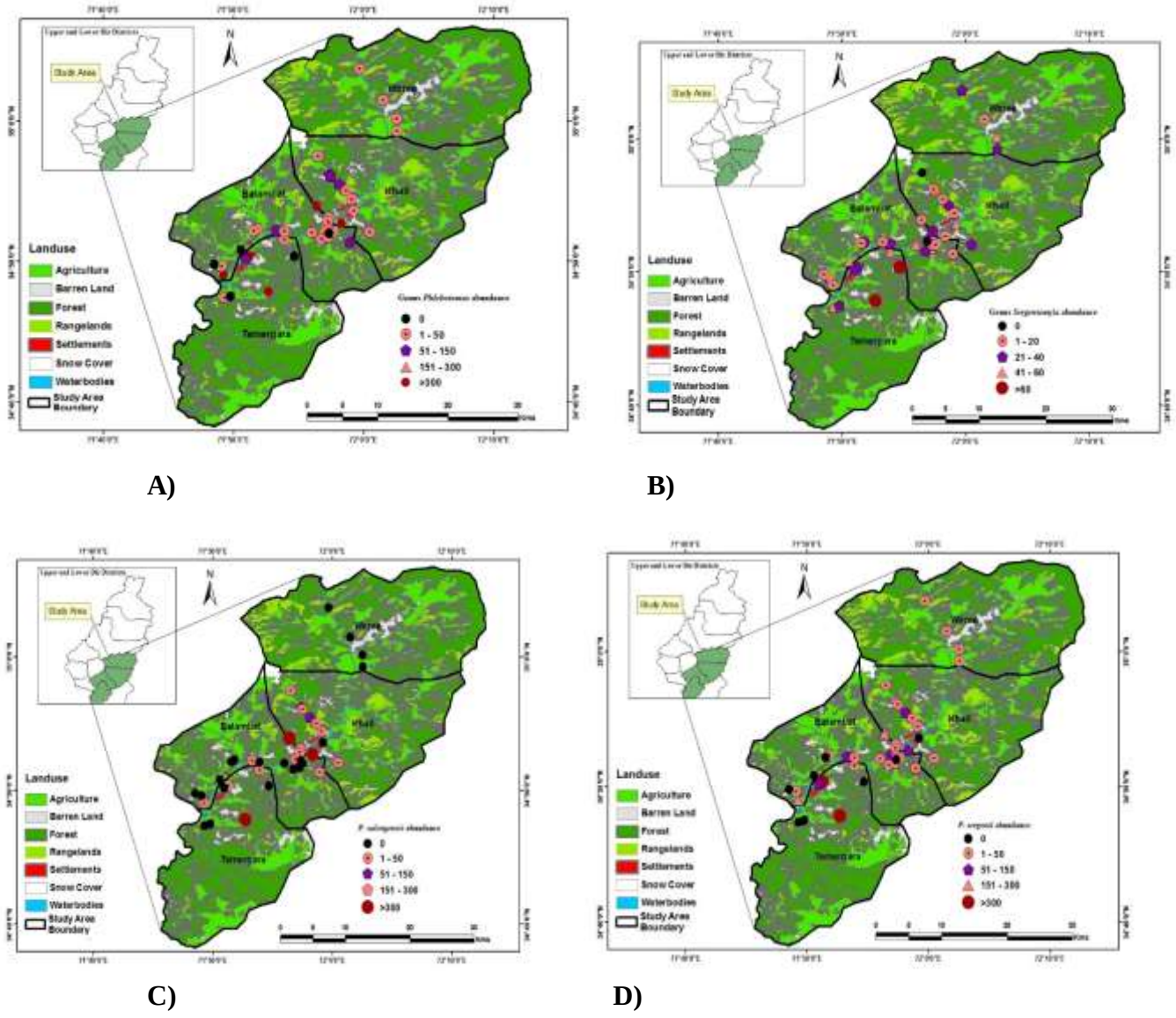

Fig. 7.2: Abundance of sand flies projected on land use maps in four Tehsils of Dir Districts (A) *Phlebotomus* (B) *Sergentomyia* (D) *P. salengensis* (E) *P. sergenti*

Human settlements are evident in Timergara, Balambat, Khall and Warae on the sides of the river. Majority of the area of four tehsils are forested. Sampling villages are more near to human population (Fig.7.2 A-D).

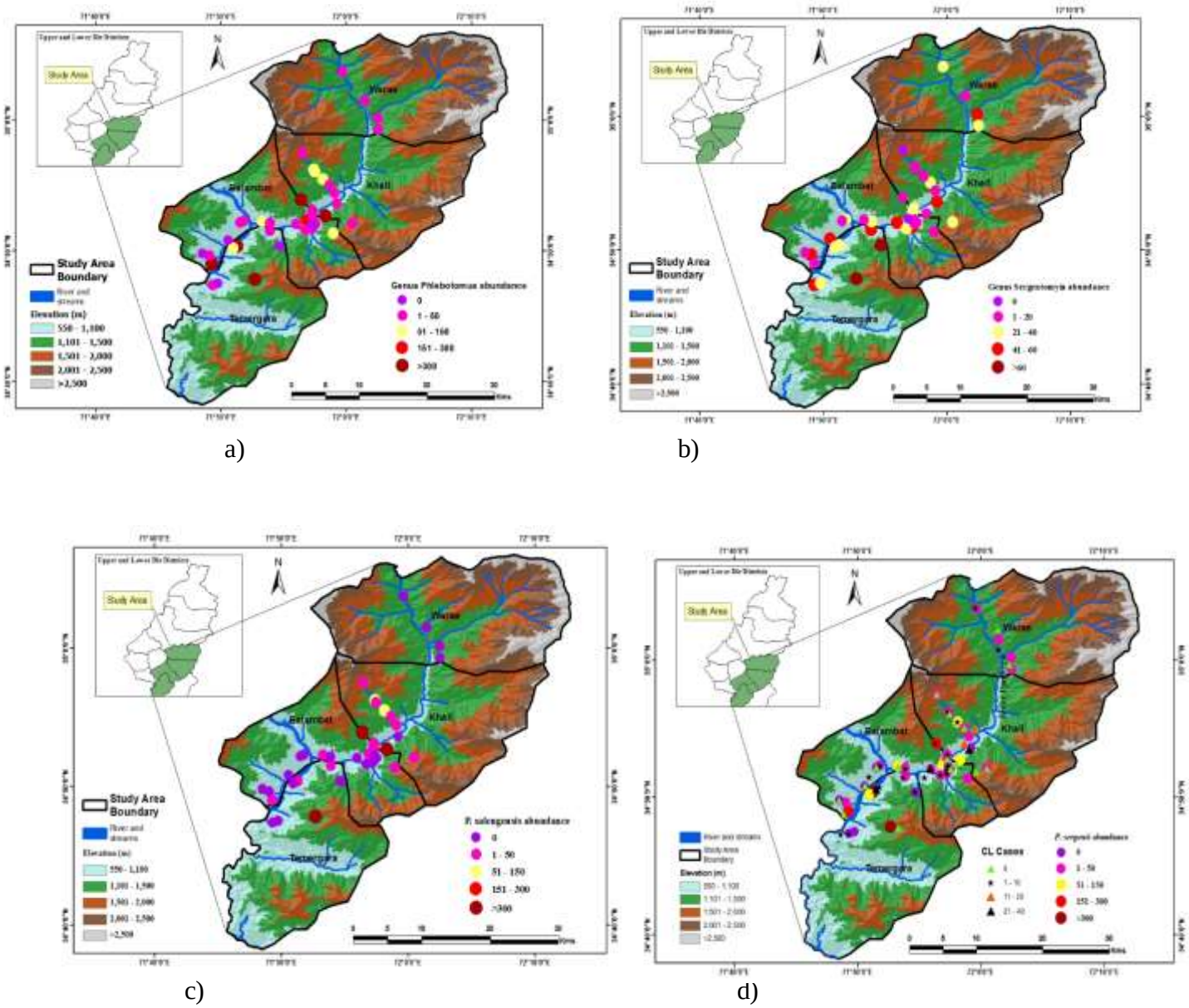

Fig. 7.3: Abundance of sand flies projected on elevation maps in four Tehsils of Dir Districts (a) *Phlebotomus* (b) *Sergentomyia* (c) *P. salengensis* (d) *P. sergenti (*associated with incidence of CL cases).

Abundance of *Phlebotomus* sand flies are shown in fig. (7.3 A) and table (7.1), indicated by different coloured spots, showing number of sand flies in different categories ranging from <50 to >300. The studied villages are along both sides of the river Panjkora shown as a blue line passing through all the four tehsils having

elevation of about 550 m (shown as blue). Cluster of abundance sports are evident in Khall, Balambat, Timergara and Warae Tehsils.

Abundance of flies at high altitude (indicated by light brown) was with the elevation ranging between 1,500 to 2,000m. Only four abundance spots (comprising the first two categories) are indicated in the map for Tehsil Warae, both at the 1,101 to 1,500m elevation. Similarly, in Balambat no abundance spot is there at high altitude and all the ten spots comprising all the number categories are there at lower 5,00 to 1,100m altitude. High abundance spots are indicated only in two Tehsils, Khall (7) and Timergara (2) comprising different categories. Abundance of *Sergentomyia* sand flies are shown in fig (7.3 B) and table (7.1). Similar to fig. (7.3 A) cluster of abundance sports are shown in Khall, Balambat and Timergara. Abundance spots of flies at high altitude are absent in Balambat, however are there in Khall (7), Warae (4) and Timergara (2).

Abundance of *P. salengensis* sand flies are shown in fig. (7.3 C) and table (7.1). Like fig. (7.3 A) cluster of abundance sports are shown in Khall, Balambat and Timergara. This species presented high abundance spots at high elevation in Timergara (1) and Khall (8), while missing in Balambat and Warae however are there in Khall (7) and Timergara (1).

Clinically confirmed cases of CL in each study sites are coincided with the *P. sergenti* population (Table 7.1 and Fig. 7.3 D). The disease showed close association with the sand fly species in some sites in Timergara, Balambat and Khall. This association was no observed in Warae Tehsil. The close overlapping relationship between disease and species are recorded at low elevation spots at 550m in three tehsils (Timergara, Balambat and Khall) as compared to Warae.

7.4 RISK FACTORS ANALYSIS

Table 7.2: Household risk factors, univariate analysis in Dir districts, 2014

Variables	Odds Ratio	Std. Err.	Z values	p values	[95% Conf. Interval]
District	0.2728674	0.6090417	-2.13	0.033	-2.492469 -0.1050695
Village	0.8540152	0.031872	-4.95	0.000	-0.2202743 -0.0953382
Rooms	1.070656	0.0253474	2.69	0.007	0.0185917 0.1179515
Family size	1.038503	0.0115293	3.28	0.001	0.0151831 0.0603772
Level of education	1.498291	0.1239647	3.26	0.001	0.1613587 0.6472914
Animals in the house	0.9113155	0.250207	-0.37	0.711	-0.5832628 0.3975305
Dogs in the houses	1.778803	0.5014365	1.15	0.251	-0.406857 1.558738
Houses construction materials	0.931333	0.1180854	-0.60	0.547	-0.3025815 0.1603047
Toilet	-0.3027188	0.1823047	-1.66	0.097	-0.6600294 0.0545918
Material of the roof	0.7078556	0.1688961	-2.05	0.041	-0.6765455 -0.0144849
Material of the floor	1.078362	0.209089	0.36	0.718	-0.3343633 0.4852504
Windows glassed	0.9375002	0.3117252	-0.21	0.836	-0.6755085 0.5464319
Eaves	0.4930072	0.2377745	-2.97	0.003	-1.173261 -0.2412021
Guestroom	1.664272	0.2569546	1.98	0.047	0.005766 1.013009
Kitchen in/out	1.313397	0.2298048	1.19	0.236	-0.1777921 0.7230261
Ceiling/ pedestal fan	2.036146	0.2276581	3.12	0.002	0.2648573 1.157261
Water facility	0.329932	1.046955	-1.06	0.290	-3.160863 0.9431253
Water storage	2.600954	1.072748	0.89	0.373	-1.146669 3.058425
family members residing outside	0.5399941	0.23548	-2.62	0.009	-1.07773 -0.1546647

country					
Out province	1.189158	0.1789542	0.97	0.333	-0.177498 0.5239896
Vegetation	2.187472	0.3596837	2.18	0.030	.0777793 1.487713
Types vegetation	1.169858	0.0841709	1.86	0.062	-.0080896 0.3218541
Perfume usage	0.4924616	1.229925	-0.58	0.565	-3.118948 1.70227
Light source	2.225337	0.9632289	0.83	0.406	-1.087986 2.687802
Humid places inside houses	0.1218603	1.229967	-1.71	0.087	-4.51557 0.3058102
Humid places nearby hoses	0.7894057	0.5832198	-0.41	0.685	-1.379565 0.9066149
Dunes	1.229789	0.2218624	0.93	0.351	-.2279994 0.6416853
Sleeping in/out	1.466418	0.2120593	1.81	0.071	-.0328056 0.7984516
Bath rooms	0.7340611	0.1921499	-1.61	0.108	-.6857699 0.0674439
Clothes for covering of children	0.8113094	0.2237492	-0.93	0.350	-.6476461 0.2294345
Mosquitos bed net	5.772894	0.5524434	3.17	0.002	.6704042 2.835943
Seen sand fly	0.9457539	0.2258525	-0.25	0.805	-.4984356 0.3868899
Biting time	0.9677155	0.1255024	-0.26	0.794	-.2787972 0.213163
Attack of sand fly	1.053606	0.1303361	0.40	0.689	-.2032357 0.3076726
Net hanging	1.010204	1.123712	0.01	0.993	-2.192283 2.212588
Spray against other flies	2.363915	0.6371246	1.35	0.177	-.3884219 2.109061
Monthly spray	2.794331	0.655624	1.57	0.117	-.2574067 2.312592
Whose prayed	0.696128	0.2927925	-1.24	0.216	-.9360844 0.2116409

In the current study, a total of 499 houses were surveyed in 40 villages. Study population consisted of 6939 individuals and 203 (2.9%) have clinically confirmed cases of CL. Number of rooms per households range from 1–40 and average family

size was 13.91 person per house. Association of leishmaniasis cases with level of education, family size, household members residing outside country and number of rooms are statistically significant $p<0.05$ (Table 7.2).

The analysis showed that factors such as eves, ceiling /pedestal fans and mosquito bed net are positively associated $p<0.05$ with the disease. The remaining factors were not significantly ($p>0.05$) associated with infection in the present study. OR (Odder Ration) was observed low for the use of mosquito bed nets, ceiling/pedestal fans and spray against other flies and regular monthly spray while in case of presence of water storage inside houses, vegetation and illumination sources were observed with high risk of disease (Table 7.2). The significant factors were used to identify positive association in multivariate analysis.

Table 7.3: House hold risk factors, multivariate regression analysis in Dir districts, 20155

Variables	Odds Ratio	Std. Err.	Z values	p values	[95% Conf. Interval]
Villages	0.8662265	0.0285954	-4.35	0.000	0.8119551-0.9241253
Level of education	1.508458	0.1951881	3.18	0.001	1.170554-1.943905
Eaves	0.5435773	0.1406288	-2.36	0.018	0.3273762-0.9025588
Family members residing outside country	0.5700183	0.1434285	-2.23	0.025	0.3481037-0.9334023

Multivariate analyses shows that villages (not clear), low level of education, eaves and family members residing outside the country increased the risk for leishmaniasis (Table 7.3).

7.5 DISCUSSION

Sporadic epidemics of CL have been seen in different regions of Pakistan including Baluchistan, Multan (Punjab) and some areas of KPK (Kassi *et al*., 2010). Some outbreaks of the disease have also been reported in refugee settlements along the Pak-Afghan border most particularly in northwest Pakistan during the mass migration in the Afghanistan crisis (Rowland *at al*., 1999). The disease (CL) is now a key public health problem with significant stigma (disfiguring of the infected site) associated with it (Reithinger *et al.*, 2003; Chaves and Pascual, 2006; Kassi *et al*., 2008).

Comprehensive knowledge of specific environmental requirements of vector species is one of the essential tools to understand vectorial capacities of disease causing

insects. In the current survey, remote sensed and GIS are used to map and model the distribution of abundant sand fly species including suspected vector of CL in Dir districts. This study was established on the basis of biological and ecological data of sand flies requirements for specific distribution. These maps will provide a not clear for sand flies collection, particularly potential vector species in the study area. Moreover, these maps will help to provide distribution information of sand fly species to medical entomologists, epidemiologists and health planners for vector control in the study region.

In the current study, risk maps of sand flies of the genera *Phlebotomus* and *Sergentomyia*, as well as of the two abundant species *P. salengensis* and *P. sergenti* (found in the present study), were designed using remote sensing and GIS techniques. These techniques highlighted an unequal distribution of the suitable areas for these species while they clustered in some localities. These sites were characterized by specific environmental topographies (hilly areas, river body, elevations, temperature, relative humidity and vegetation) with probable unchanging biological and ecological requirements for the species. The present GIS-assisted spatial analysis results showed that the species of genus *Phlebotomus* such as *P. salengensis* and *P. sergenti* were found widely distributed (adopt to varied ecological areas).

Guernauoi *et al*., (2006) showed that sand flies density was related to elevation and was one of the important factors playing role in the dispersion pattern of sand fly species. Sand flies species in four Tehsils of Dir districts, showed clear association with altitudinal ranges. The most contributable power to the current GIS and remote sensing models were elevation because DEM (Digital Elevation Model) closely related to topography of the study area (Signorini *et al*., 2014). Sand flies direct

relation to elevation was also found in Egypt (Kassem *et al.,* 2012), however, sometimes this variable fails to work in some regions (Signorini *et al*., 2014). The common sand fly species are generally considered mountainous species found at high altitude by other workers. *P. salengensis* the most abundant species found in the present survey was collected mostly at higher altitude of the study area. Munir (1994) sampled this species from Azad Jammu and Kashmir (AJ&K) at 1500-2600 meters above the sea level. In Afghanistan this may be found in mountainous areas (height not known) of the country (Lewis, 1967).

The second abundant species, *P. sergenti* was found at different altitude on both sides of the river Panjkora in the study area. Aslamkhan, (1998) argued that this species may live at the height of 2,438 meter in Pakistan and 2832m in Kashmir. In Saudi Arabia *P. sergenti* was found at al altitudes, with maximum abundance at low altitudinal ranges between 200-1200 meters (Doha and Samy, 2010) but Buttiker and Lewis, 1983 found it most common at elevation 550–700 metres, while Seyedi-Rashti and Nadim (1992) treated this species as a mountainous one in Iran living at high elevations. Kasap *et al*., (2009) collected this species with low abundance (0.1%) at lower altitude of 180 metres in Turkey.

In the current study, GIS based map of *P. sergenti* distribution and clinically confirmed cases of the CL relationship at every altitudinal sites were made in four tehsils of Dir districts. The results showed that the abundance of the fly closely coincides and overlap well with the distribution of disease at low elevation in some sites of the area. Aslamkhan *et al*., (1998) proposed that this species closely associate with CL cases in Pakistan while Rowland *et al*., (1999) also collected this species from the site with high number of CL cases in Afghan Refugees camp at Timergara.

The present study revealed *P. sergenti* as the plausible vector of leishmaniasis in the surveyed region because approximately 95% study area was exposed to this species. Other *Phlebotomus* species have restricted distribution and were omitted from the association with CL in the study area.

Rowland *at al*., (1999), reported *L. tropica* to be responsible for leishmaniasis in Afghan Refugees settled at Timergara, Dir Lower. The epidemiology of leishmaniasis is composite and a number of important questions remain unanswered. Rowland *at al*., 1999 reported that older children appeared to be more at risk than any other age-groups because they are less self-controlled in covering during sand flies active season in summer. CL distribution is also associated with poverty and neglected areas of the country with limited access to health care and hygiene as well as low level of education (Rahim *et al*., 2011; Argaw *et al*., 2013).

According to Killick-Kendrick (1999), sand fly vector species have a short flight range and usually confined to a limited area and thus the disease can be cluster within a households. In the present study, family size (no. of household members) as well as house designs (number of rooms) were found to be positively (no of rooms is negatively negative association as you explained later) associated with this skin disease. (Reithinger *et al*., 2010) concluded that house design and construction materials are among the risk factors of ACL in Kabul (Afghanistan).

Majority of people in the study area were living in mud made houses with domesticated animals. *P. sergenti* was particularly abundant in such places, also observed to be an endopghagic species in Afghanistan (Killick-Kendrick *et al*., 1994). More number of people in bed rooms is expected to attract higher number of sand flies as compared to less number of people per room. In the total of 40 surveyed

villages 23 were found having healed or active cases of leishmaniasis. In univariate analysis, results suggested that they were significantly associated with this cutaneous disease.

A large number of people in the study area were found to be working in Middle East, mostly in Saudi Arabia, where leishmaniasis is endemic (Mustafa *et al.*, 1994; Khan and Zakai, 2014) which is possibly one of the spreading factor of the disease in locals. Vegetation was also positively linked with the leishmaniasis in the region. In the study area different kinds of plants ranging from small creeping shrub to tall trees were present around human houses in peridomestic sites, this vegetation could be attracting the sandflies as the source of sugary juice.

Use of ceiling and pedestal fans in houses were found to be negatively associated with CL. Such facilities were usually missing in the mud made houses. Similar association was found with the use of bed nets for protection against biting insects. Both types of provisions were scared in mud made houses. Bern *et al.*, (2000) found that bed net were more protective and reduced the VL transmission more than 70% in Nepal. Number of out lets (eaves) showed positively associated with the CL (Poche *et al.*, 2011) mostly observed in mud made houses and less frequently in concerted houses.

Other factors most particularly, domesticated animals, dogs, construction materials, sleeping inside/outside rooms, screened/unscreened windows although showed significant association with sand fly abundance but were found not significantly associated with the leishmaniasis in the study area, although reported positively associated with the disease in other countries especially in Afghanistan (Reithinger *et al.*, 2010), Ethiopia (Bashaye *et al.*, 2009) and Nepal (Bern *et al.*, 2000). In the current study, the largest odds ratios, however, were associated with mosquito bed

nets, monthly spray and water storage in the houses indicating the importance of general hygiene and use of insecticide spray in the control of disease.

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Chapter -08

Numerical taxonomic analysis of sand flies (Diptera; Psychodidae) from Dir districts, Khyber Pakhtunkhwa, Pakistan

8.1 INTRODUCTION

Sand flies are haemotophagus insects of great medial and veterinary significance, due to their ability to transmit protozoal, bacterial and viral diseases to humans and other animals (Rangel and Lainson, 2009). Currently three genera: *Phlebotomus*, *Sergentomyia* and *Lutzomyia*; present in tropics and temperate regions of the world are known for their medical importance as disease vectors (Lewis, 1971; Lane, 1993). Among them, *Lutzomyia* species presented their distribution in North and South Americas while the other genera are present in the Old world.

In Pakistan, a total of 37 (Kakar, 2004) sand flies species are reported which belong to two genera; *Phlebotomus* and *Sergentomyia*. More species may exist as many regions of the country are to date unexplored (Shakila *et al*., 2006). Pre-partition studies were carried out in the region now included in Pakistan by Sinton (1924-27). Up till now no evolutionary studies are present on this medically important group of insects in the whole country (Kakar, 2008).

The present study is a preliminary effort to describe the phylogeny of Phlebotomine of four Tehsils of Lower and Upper Dir using numerical taxonomy. It aims at resolving the dubious relationships among species; simultaneously demonstrating the taxonomic position of each taxon.

8. 2 MATERIALS AND METHODS

8. 2.1 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 (cattle corrals, dry cattle dung, caves, tree holes etc.) sites. For identification purposes taxonomic keys as described by Lewis (1967) and Artemiev, (1978) were used.

8.2.2 Numerical taxonomy

A preliminary list of 60 morphological characters was drawn for both sexes which was inclusive of antennae, palps, cibarium, setae (thorax and abdomen) and genital structures (male and female). Based on these characters a matrix was constructed (Table 8.1) as described by (Sun *et al*., 2009).

The matrix composed of taxa and characters was analysed software Statistical Package for the Social Sciences (SPSS) version 20.0 using HCA. The results of cluster analysis classification were exported and displayed on dendrogram.

8.3 RESULTS

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 and occurrence of body hairs (character 3). Phylogenetic investigation of the sand flies using Hierarchical Cluster Analysis (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 8.1).

For species diagnosis our analysis revealed the utility of several characters such as head structure and male and 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 8.1: 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	Mesanepisternum	Without postspiracular hairs(0)	Present(1)	-
11	Spermatheca morphology	Segmented or incompletely segments(0)	Smooth(1)	Spindle(2)
12	Termanila 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)
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)	-

Table 8.1: Sand flies taxonomic characters and their state value used for the study.

Table 1. continued	Characters	Character state and their values		
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 tubercule(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)	-

The HCA dendrogram is consistent with traditional taxonomy of Phlebotomine sand fly species (Fig. 8.1). The analysis group 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 without any dubious branching. 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, Paraotmyia, Sintonus, Rondanomyia and Grossomyia*. Our analysis therefore describes Phlebotomine flies as a distinct clad of the family Psychodidae.

Table 8.2: 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**
P. papatasi	0	0	0	1	0	0	0	0	0	0	2	0	2	1	2	2	2	1	2	0
P. bergeroti	0	0	0	1	0	0	0	0	0	0	2	0	2	1	2	2	2	1	2	0
P. sergenti	0	2	2	0	1	1	1	1	1	1	2	0	2	1	2	2	2	0	0	2
P. andrejevi	0	2	2	0	1	1	1	1	1	1	2	0	2	1	2	2	2	0	0	2
P. kazeroni	0	2	2	0	1	1	1	1	1	1	2	0	2	1	2	2	2	0	0	2
P. ansari	0	2	2	0	1	1	1	1	1	1	2	0	2	1	2	2	2	1	2	1
P. hindustanicus	0	2	2	0	1	1	1	1	1	1	2	1	2	2	0	2	2	2	2	2
P. salengensis	0	2	2	0	1	1	1	1	1	1	2	1	2	2	2	2	2	2	2	2
P. major	0	2	2	0	1	1	1	1	1	1	2	0	2	0	0	2	1	2	2	2
S. babu	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
S. baghdadis	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
S. bailyi	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
S. grekovi	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
S. montana	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
S. dentata	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
S. theodori	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
S. hospittii	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
S. tiberidis	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
S. hodgsoni	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
S. turkistanica	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

Table 8.2: The character states matrix for the 20 sand fly species from Dir districts

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

Table 8.2: The character states matrix for the 20 sand fly species from Dir districts

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

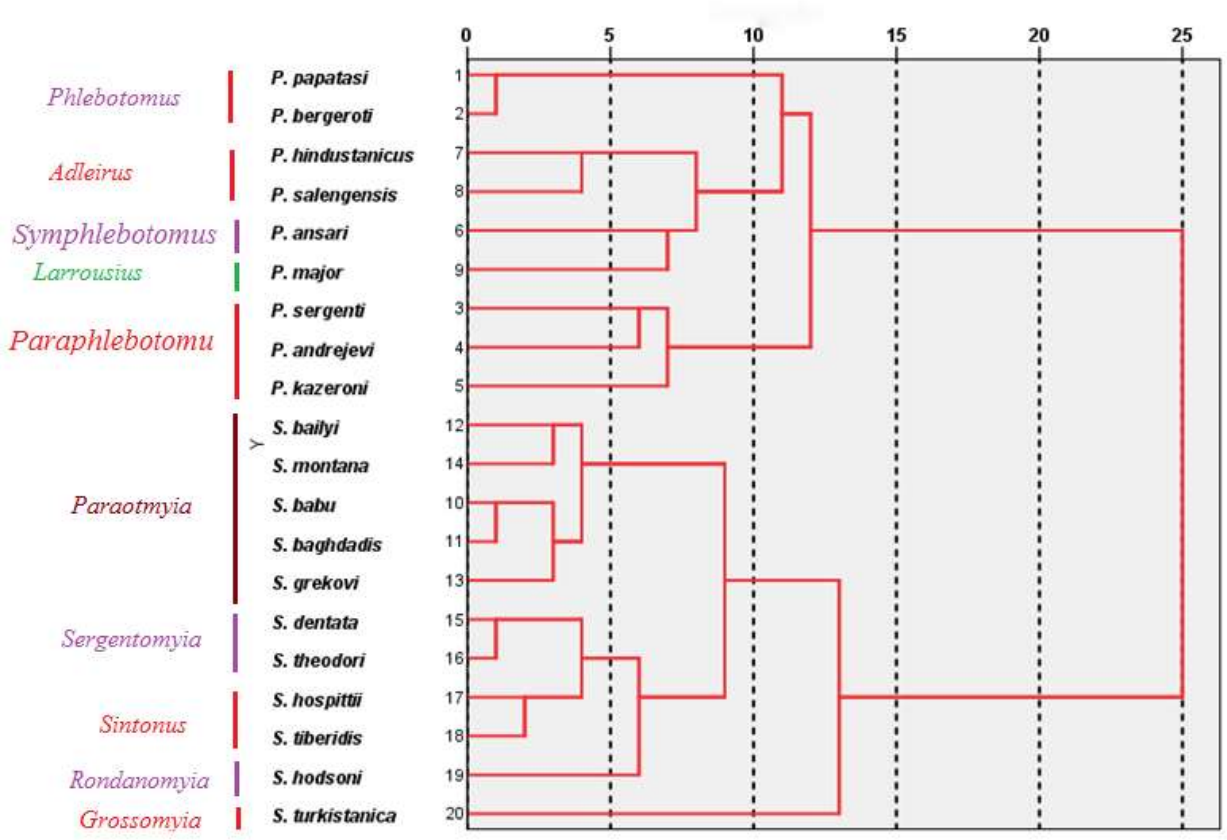

Fig. 8.1: HCA dendrogram of sand fly species constructed using linkage between groups.

8.4 DISCUSSION

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 (Horaiellinae, Sycoracinae, Trichomyiinae, Bruchomyiinae, Phlebotominae and Psychodinae) within the family Psychodidae (Stebner *et al*., 2015). Among these six sub families Phlebotomine are the sole or 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 were doubtful in the country. No authentic taxonomic keys are available to identify sand flies specimens up to species level. Considering the importance of both visceral and cutaneous leishmaniasis vectors, knowledge about their morphological and morphometric characteristics may indicate important diagnostic characters as tools for a future phylogenetic approach.

In this study, a total of 60 morphological characters of sand flies were included in phylogenetic analysis based on numerical taxonomy. Previous studies have shown that taxonomy of the genus *Phlebotomus*, relies on a small number of adult characters (Rispail 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 showed the significance of these characters especially the genitalia. Thus the structure of the male genitalia (França, 1919; França and Parrot, 1920-21) aided in the erection of subgenera. Similarly female characters such as the ciberial and pharyngeal armatures and Spermathacae as introduced by Adler and Theodor (1926), and developed by Sinton (1927-28) and Nitzulescu (1931) further helped in sand fly taxonomy (Rispail and Léger, 1998b).

Numerical polythetic taxonomy simultaneously takes into account numerous characters without according any discriminant value, using advancements in information technology. Phenetic analysis expresses the intergroup relationships by means of factorial graphs and dendrogram (Rispail 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 subgenus. The HCA dendrogram strictly adheres to the traditional taxonomy of the group (fig. 8.1).

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 improved by the use of 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 complete analysis of Phlebotomine (Rangel *et al*., 1996, Dujardin, 1999). Therefore we aim at carrying out a more elaborate study in future to utilize other characters as well in the identification of sand fly species

Our analysis showed that using integrative taxonomy can be vital in improving species identification and solving the problems in the misidentification of certain groups of species of sand flies. Sand flies identification based on morphological structure are widely accepted around the globe but such studies are limited and sporadic in Pakistan. Therefore, the present study will be helpful to other sand flies researchers in classifying this group in Pakistan particularly. Phylogenetic analysis of sand flies species will be better determined and understood when representatives of every species from all provinces of the country are included in the same phylogenetic tree.

CHAPTER - 09
GENERAL DISCUSSION AND CONCLUSION

The current study is the first detailed research, carried out in four Tehsils; Timergara, Balambat, Khall and Warae of Dir districts. The study mainly comprised sand flies species composition, seasonal variations, habitats and their density as well as diurnal and nocturnal activities using different methods. Risk factors for the presence of leishmaniasis were also assessed. In addition, Remote Sensing (RS) and Geographic Information System (GIS) were used to design maps for highlighting areas of sand flies abundance in the surveyed region of Dir districts.

CL caused by *Leishmania tropica* is considered to be an emerging disease known as Kaal Dana (a sore taking at least one year to heal) in local language (Pashtu) in north-east Afghanistan and north-west parts of Pakistan including Dir districts (Rowland *et. al*., 1999). Only the female Phlebotomine sand flies are responsible for natural transmission of leishmaniasis. Some species of sand flies are also vectors of sand fly fever and visceral leishmaniasis, one of the fatal forms of disease in the country (Ahmad *et al*., 1962; Barnett and Suyemoto, 1961; Nasir, 1964; Aslamkhan and Barnett, 1967; Lewis, 1967).

Some scattered information about the number of sand fly species reveals 37 species prevailing in different parts of Pakistan (Kakar, 2004). The present study reports nine phlebotomine and eleven Sergentomyian species including all known and suspected vectors of leishmaniasis in the country. Among the collected species *P. ansari* is the new addition to the known list of sand fly species in Pakistan. *P. ansari* is a hydrophilic and thermophilic species, mostly found in Central and Northern Iran and Western Afghanistan. This species finding in the area is significant as it is included among the suspected vectors of *L. tropica* by (Lewis, 1971) in Western Afghanistan.

Seven more species (*P. salengensis, P. hindustanicus, P. kazeroni, P. andrejevi, P. bergeroti, S. turkistanica* and *S. grekovi*) are those which are reported for the first time from the KPK province. *P. salengensis* was the most abundant in the current study was sampled from three tehsils. Lewis (1971) suggested that this species was mostly found in low rocky mountains in Afghanistan and is thermophilic and hydrophilic. In the present study its collection was made from domestic and peridomestic habitats. Munir (1994) recovered this species from Kashmir. The medical importance of this species is yet to be determined. However, its prevalence in close vicinities to humans may be reflecting their anthropophagic behaviour. *P. sergenti* was the second most abundant species in the current study. Based on circumstantial evidence *P. sergenti*, is considered as a suspected vector of CL in Pakistan (Rowland, 1990) but proven vector of *L. tropica* in Kabul, Afghanistan (Killick-Kendrick *et al*., 1995), Iran (Seyedi Rashti and Nadim, 1992) and Turkey (Kasap *et al*., 2009).

The studied localities differ in species composition with 15 (Timergara), 13 (Balambat), 10 (Khall) and 7 (Warae) species most probably reflecting changes in the environment within the larger scale area (Akhoundi *et al*., 2012). Four species (*P. sergenti, S. babu, S. baghdadis* and *S. dentate*) were found in all the studied tehsils while others were restricted to some sample localities indicating that these flies have a limited distribution in these sites. Species collected from one site only, were *P. hindustanicus, P. ansari, S. baily, S. grekovi, S. turkistanica* (Timergara). *P. andrejevi* (Khall) *S. theodori, S. hodgsoni* and *S. tiberidis pakistanica* (Balambat). Many factors including precipitation and temperature, physical factors like geographical barriers and habitat availability and abundance of vertebrate hosts are possibly responsible for

various sand fly species richness and diversity (Cross *et al*, 1996; Ghosh *et al*., 1999; Oliveria *et al*., 2013).

Sand flies presence was monitored throughout the year on monthly basis from January to December, 2014. In the study area mean temperature in winter season (using weather station) recorded below -2°C, while in summer the average temperature from May to October 2014 ranged between 20℃-33℃, when sand flies showed their presence. Optimum temperature for the completion of the flies' life cycle was available only in the summer months as reported by (Maroli, 1983; Maroli *et al*., 1987) based on his experimental evidences. WHO (1984) reported a worldwide distribution of sand flies in those areas where the average temperature is 20℃ for at least one month. The onset of warm weather trigger sand fly emergence in the month of May, reaching highest density in the month of August with gradual decrease in the next two months and complete disappearance from November–April indicating only one single peak activity and generation in the year.

Temperature and relative humidity seemed to be the determining factors for the maintenance of sand flies in nature in the studied regions (Fig. 3.5 and 3.5). Similar activity pattern was noted in Waziristan (mountainous area) (Ali *et al*., 2015), indicating these insects have a well-defined seasonal activity pattern. This study is in accordance with the previous surveys regarding high abundance of sand flies at high temperature and relative humidity (Kasap *et al*., 2009; Tarallo *et al*., 2010; Lahouiti *et al*., 2013). These finding can be helpful to determine disease transmission time which may lead to design effective control and prevention tools for the disease. Besides temperature, relative humidity also influences the activity pattern of sand flies in surveyed area.

Searching for developmental stages of sand flies in their ordinary biotopes is thought to be one of a tiresome job and has established to be generally unfruitful (Killick-Kendrick, 1999). A noteworthy finding of the current study was the sampling of immature males belonging to three species (*P. salengensis, P. sergenti* and *P. andrejevi*). Immature males were recognized by the incomplete rotation of male genatilia (characteristic of dipterous insects). Complete rotation (mature male formation) takes place within 24 hours after imago emergence (Killick-Kendrick, 1986). Finding of immature males of sand flies is being reported for the first time in Pakistan. These juveniles were collected in specific months (June, July and August) the active period of sand fly activities, by using sticky traps. The collection of immature males in the current study could be used as a base line work for further detailed study.

Regarding the types of habitats surveyed, domestic and peridomestic habitats were found to be favourable sites for finding various sand fly species. Some observations made in the current study could be used as evidences for the recognition of breeding sites of sand flies. The presence of domestic animals increases the chances of sand fly prevalence. Cattle sheds attracted highest number of flies indicating these places as their most suitable breeding sites. The presence of organic manure and moisture in such sites are attributed to be the provision of suitable conditions for sand flies (Moncaz *et al*., 2014). In addition the finding of immature males in the sheds, and the gravid/semi graved female sand flies confirm the mentioned conclusion. Moreover, sand flies are usually found near to their breeding sites as they have a limited power of distribution. Killick-Kendrick (1986) reported *P. ariasi* from in indoor sites having organic matter.

Collection was made from various indoor and outdoor sites in order to determine the endophilic and exophilic nature of sand flies, the knowledge important for the control point of view (Gebresilassie *et al*., 2015). *P. salengensis*, *P. sergenti*, *P. major*, *S. babu*, *S. baghdadis*, *S. dentata*, *S. bailyi*, *S. hospittii* and *S. grekovi* were recorded from both indoor and outdoor sites while the remaining species were recovered from indoor habitats only. Sand flies found indoor are the probable anthropophagic and outdoor are zoophagic in nature and still some are of mixed nature feeding on wide range of hosts including both animals and humans.

A small number of flies were collected from different plants belonging to twelve families, at night times. All these plants were found around the houses (peridomestic sites). Majority of species of sand fly were collected from creeping plants (Cucurbitaceae) because these were the dominant shrubs in summer season around human houses.

A total of four species (*P. salengensis, P. major*, *S. bailyi* and *S. hospittii)* were collected from eleven caves located in the hilly area of Tehsil Timergara, where *S. bailyi* was the dominant species. The presence of sand fly species in the caves may be associated with the occurrence of animals, most particularly bats, rodents, birds, reptiles and wild canine species probably acting as blood source for sand flies (Barata *et al*., 2012). The cave sand flies was observed to play essential role in the spreading of leishmaniasis (Carvalho1 *et al*., 2013), the animals inhabiting such places may also be acting as reservoir hosts of the disease. Moreover, the large amount of organic manure inside the caves may provide suitable breeding sites for sand flies (Alves *et al*., 2011).

Better understanding of the caves physical features and their sand flies fauna were important to study as it may be a great risk for the locals regarding spread of the disease (Carvalho *et al*., 2013). These caves are visited by the local people for various purposes especially for shelter. It is important for the control and prevention of the disease to aware locals and to consider care of the caves particularly during the months of high abundance of sand fly species. In addition, it is also important to explore the feeding behaviour of *S. bailyi* and other species prevailing in such sites.

Singorini *et al*., (2014) suggested *Phlebotomus* species distribution to be related with elevation and environmental factors such as temperature, humidity, slope, water bodies, vegetation and air pressure. In the current study, GIS and remote sensing tools were used to map and project the sand flies distribution in the study area, which is suggested to be one of the important tools in all vector borne infections control programs (Surendra, 2013). Sand flies species abundance in Dir districts were observed high with nine *Phlebotomus* and eleven species of *Sergentomyia* collected from about 571 sampled sites. The impartially heterogeneous landscape (elevation) seem to contribute to flies species distribution in four tehsils.

Therefore, GIS and RS parameters are used to recognize geographical areas that may be suitable for sand fly species and Leishmaniasis transmission. Although the recent study did not identify specific areas where disease spreading and transmission occurs because of the local nature of the species and the infection. There must be (reservoir hosts) for the sand fly species to feed and animal burrows, cracks where the microclimate is suitable for resting, breeding and survival of the vectors species other than the study sites.

In the current study influence of altitude was analysed on vectors density. Species abundance of females sand flies were found at various elevations suggesting that sand flies can survive in different environments. Females of abundant species (including the suspected vector) were taken into considerations because they stabilize the ecosystem of species in which they survive (Muller and Schliden, 2004). Males usually have a restricted distribution, live on the available food resources and were found near to the breeding sites while female individuals may go for the blood feedings and other plant juices (Killick-Kendrick, 1999).

Most of the studied villages in these tehsils were present on both sides of river Panjkora which provides ideal temperature and humidity for sand flies development. A study in Iran (Rassi and Hanafi-bojd, 2006) confirmed that river banks were the suitable breeding sites for sand flies because the river provides high humidity and temperature. Elnaiem (2011) also recorded the same observation on the costal side of East Africa. Moreover, rivers are usually present at lowest attitudes, where sand flies abundance is generally higher than that of high elevation (Galvez *et al.*, 2010).

Present results demonstrate that the prevalence and abundance of sand flies is dependent on several factors including building construction type, proximity and amount of vegetation and type of livestock kept so that flies don't need to fly long distances for blood meal or sugar. Poor construction material like mud walls and thatched roofs provided more cracks and crevices for sand fly resting and entry to indoor. Sand flies most particularly *P. sergenti* (suspected vector) were more abundant in mud made houses, may be providing better resting and breeding sites as compared to concreted and iron sheeted houses,

Among the studied Tehsils, high number of sand flies regarding both species and individuals were found in three Tehsils, Timergara, Khall and Balambat as compared to Warae. The former Tehsils were inhabited by economically poor population where houses were mostly mud made with poor hygienic conditions in the domestic and peri domestic environment and domesticated animals/cattle's were generally present. People drop the organic manure of the livestock in to open areas around their houses and agricultural fields in a specific sites known as 'Ghoshanu Diran'. Such sites are generally considered the best breeding places for sand flies (Poche *et al*., 2011). Warae Tehsil was found to be inhabited by economically better class of people where the houses were concreted and lest number of domesticated animals were there.

Sampling of flies was done by using various methods including sticky traps, spray catch and aspiration. The purpose was to catch maximum number of flies and to assess the efficacy of these methods regarding number and diversity of caught sand flies. Collection by aspiration could only be done occasionally for individual fly collection while the former two methods were used for collection on larger scale in relatively short time. Sticky traps provide much better results as compared to the insecticide method in agreement with some previous studies (Lahouiti *et al*., 2013; Kasap *et al.,* 2009). Proximity to animal's shelters improved the efficacy of sticky traps performance. This method was particularly useful in sites where insecticide spray or sucking methods could not be employed easily.

More males as compared to females were collected through sticky trap method, possibly indicating the sites of emergence of flies and therefore the breeding places of sand flies. Such sites may be treated as breeding sites and will be kept in consideration during sand flies control programs in the study area. Similar association

was revealed between the collection methods and number of flies collected as well as between the methods and the proportion of males and females by Hesam-Mohammadi *et al*., 2014 while studying these flies in Kashan district, Isfahan province of Iran.

Insecticide spray collection method was less efficient in general as compared to sticky traps but was more effective for the collection of *Sergentomyia* species at day times. Sand flies prefer to take rest in dark humid, cool and closed places during day times (Feliciangeli, 2004), suggesting spraying method more suitable for collection from such sites. This method, was more productive at 8:00 am to 10:30 am as at these times flies are usually not active and prefer to rest are therefore easily be collected by spray (Samir, 2000). Certain limitations were observed using insecticide method like some taxonomic parts of flies got damaged during collection and occasionally flown away as they were not fully killed by the insecticide. Its efficacy was also influenced by factors such as large sized indoor sites, mud and iron sheet houses and houses with large number of openings and eaves in the bedrooms. In addition hazardous effect of insecticide on food stuff and personal safety measures is also an issue to use this method of collection.

Information about insect vector behaviour in natural setting is required to understand the status of disease caused by them. Despite of its medical importance, studies on sand flies are neglected in Pakistan in general and Khyber Pakhtunkhwa in particular. A thorough account on biodiversity of sand flies in the country is yet to be explored, followed by the recognition of active and potential vector species in endemic regions of the country. Further studies are needed to determine the behaviour, infection rate, and the natural reservoir hosts of sand fly vectors in the region on larger scale.

Moreover in order to prevent the disease from spreading, public awareness of the disease also need to be considered.

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APPENDIX

Table. Abundance of sand flies found in Dir districts, 2014

Species	M/F	M:F	Total	%
P. sergenti	2505/175	14:1	2680	36.66
P. salengensis	2415/686	3:1	3101	42.55
P. major	114/16	7:1	130	1.89
P. kazeroni	0/24	0:24	24	0.329
P. papatasi	5/9	5:9	14	0.193
P. hindustanicus	4/0	4:0	4	0.054
P.ansari	0/2	0:2	2	0.027
P. bergeroti	0/2	0:2	2	0.027
P. andrejevi	37/0	37:0	37	0.507
S.babu	99/440	1:4	539	7.413
S. baghdadis	24/284	1:11	308	4.228
S. dentata	347/0	347:0	347	4.763
S. hospittii	3/25	1:8	28	0.384
S. montana	0/20	0:20	20	0.274
S. bailyi	34/17	2:1	51	0.700
S. grekovi	1/0	1:0	1	0.013
S. hodgsoni	0/1	0:1	1	0.013
S. turkistnica	0/1	0:1	1	0.013
S. tiberidis pakistanica	0/1	0:1	1	0.013
S. theodori	1/0	1:0	1	0.013
Total	**5589/1703**	**3:1**	**7292**	

Table. Diversity of sand flies in districts Dir, 2014

Species	Total	Proportion (P_i)	$Log_e p_i$	P_i Log_e P_i	Shannon-weaver Diversity index $H=\sum P_i \log_e P_i$	Species richness $D=\frac{S}{\sqrt{N}}$	Simpson Index(D) $\sum_i n_i(n_i-i)N(N-1)$
P. sergenti	2680	0.367	-1.002	-0.367	1.4044	0.23	0.32
P. salengensis	3101	0.425	-0.855	-0.363			
P. major	130	0.017	-4.07	-0.069			
P. kazeroni	24	0.003	-5.809	-0.017			
P. papatasi	14	0.0019	-6.265	-0.011			
P. hindustanicus	4	0.00054	-7.600	-0.004			
P.ansari	2	0.0002	-8.517	-0.0017			
P. bergeroti	2	0.0002	-8.517	-0.0017			
P. andrejevi	37	0.005	-5.298	-0.026			
S.babu	539	0.073	-2.617	-0.191			
S. baghdadis	308	0.042	-3.17	-0.133			
S. dentata	347	0.047	-3.05	-0.143			
S. hospittii	28	0.0038	-8.11	-0.030			
S. montana	20	0.002	-6.21	-0.012			
S. bailyi	51	0.006	-5.11	-0.030			
S. grekovi	1	0.00013	-8.94	-0.001			
S. hodgsoni	1	0.00013	-8.94	-0.001			
S. turkistnica	1	0.00013	-8.94	-0.001			
S.tiberidis pakistanica	1	0.00013	-8.94	-0.001			
S. theodori	1	0.00013	-8.94	-0.001			
Total	7292			1.4044			

Table. Sand flies species abundance in tehsil Timergara, Dir, 2014

Species	Total	%	M/F	M:F
P. sergenti	1320	34.64	1256/64	19:1
P. salengensis	1945	51.04	1324/621	2:1
P. major	129	3.38	113/16	7:1
P. kazeroni	12	0.31	0/12	0:12
P. papatasi	4	0.104	0/4	0:4
P. hindustanicus	4	0.104	4/0	4:0
P.ansari	2	0.052	0/2	0:2
S. babu	193	5.065	57/136	1:2
S. baghdadis	65	1.706	2/63	1:31
S. dentata	67	1.758	67/0	67:0
S. bailyi	51	1.338	34/17	2:1
S. hospittii	11	0.288	0/11	0:11
S. montana	5	0.131	0/5	0:5
S. grekovi	1	0.026	1/0	1:0
S. turkistanica	1	0.026	0/1	0:1
Total	**3810**		**2858/952**	**3:1**

Table. Abundance of sand fly species in tehsil Balambat, 2014

Species	Total	Percentage	M/F	M:F
P. sergenti	581	39.79	536/45	12:1
P. salengensis	405	27.73	376/29	12:1
P. kazeroni	6	0.410	0/6	0:6
P. papatasi	8	0.547	5/3	2:1
P. bergeroti	2	0.136	0/2	0:2
S. babu	181	12.397	17/164	1:9
S. baghdadis	150	10.273	19/131	1:6
S. dentata	112	7.671	112/0	112:0
S. hospittii	9	0.616	3/6	1:2
S. montana	3	0.205	0/3	0:3
S. hodgsoni	1	0.0684	0/1	0:1
S. tiberidis pakistanica	1	0.0684	0/1	0:1
S. theodori	1	0.0684	1/0	0:1
Total	**1460**			2:1

Table. Abundance of sand fly species found in tehsil Khall, 2014.

Species	Total	Percentage	M/F	M:F
P. sergenti	756	40.93	697/59	12:1
P. salengensis	751	40.66	715/36	19:1
P andrejevi	37	2.003	37/0	37:1
P. kazeroni	4	0.216	0/4	0:4
P. papatasi	2	0.220	0/2	0:2
P. major	1	0.005	0/1	0:1
S. babu	94	5.089	20/74	3:1
S. baghdadis	42	2.273	3/39	1:13
S. dentata	153	8.283	153/0	153:0
S. hospittii	7	0.378	0/7	0:7
Grand Total	**1847**			7:1

Table. Abundance of sand flies in tehsil Warae, 2014.

Species	Total	%	M/F	M:F
P. sergenti	**23**	**13.14**	**16/7**	**2:1**
P. kazeroni	**2**	**1.14**	**0/2**	**0:2**
S. babu	**71**	**40.57**	**5/66**	**1:13**
S. baghdadis	**51**	**29.14**	**0/51**	**0:51**
S. dentata	**15**	**8.57**	**15/0**	**15:0**
S. hospittii	**1**	**0.57**	**0/1**	**1:0**
S. montana	**12**	**6.85**	**0/12**	**0:12**
Total	**175**		**36/139**	**1:3**

Table. Name and locations of 40study villages in Dir districts 2014.

Serial No.	Site name	Latitude (◦N)	Longitude (◦E)	Altitude (m)
1	Barkale	34.4033	71.51115	3866
2	Kalone	34.89080	71.98214	2971
3	Shalfalm	34.54364	71.59325	3135
4	Banda	34.86815	72.00805	2908
5	Dwaba	34.92552	71.96894	3151
6	Lower Lalo	34.93556	71.95828	3061
7	Luqman	34.95844	71.94112	2986
8	Mira	34.88593	71.95610	2404
9	Sacha	34.88009	71.95289	2378
10	Sarmu Obu	34.91885	71.97785	3310
11	Upper Lalo	34.93998	71.95644	3000
12	Watange	34.90057	71.93876	2435
13	Shzadi	34.87001	71.86349	2666
14	Rani	34.51389	71.55483	2755
15	Baroon	34.86652	71.92554	2885
16	Tangae	34.86782	71.95902	2966
17	Shagae	34.86737	71.95348	2903
18	Dairy	34.83138	71.80939	2894
19	Dab	34.86081	71.94638	2945
20	Haji Abad	34.85786	71.85371	3097
21	Koto	34.86999	71.85847	2755
22	Kotkae	34.86984	71.95733	2971
23	Munji	34.87214	71.89503	3454
24	Nawagae	34.86403	71.95560	2729
25	Palusoo	34.84636	71.84129	2461
26	Khima	34.81283	71.81269	3578
27	Malae	34.86416	71.99257	2871
28	Mandish	34.87628	71.97637	2702
29	Manzaetangae	34.87190	71.88759	3553
30	ARC.1	34.84568	71.86503	2585

31	Danvah	34.85766	71.89512	2719
32	ARC. 2	34.84621	71.86298	2612
33	Siar	34.83917	71.91024	3239
34	Saddo	34.78906	71.82323	2475
35	Sikaolae	34.79165	71.82499	2477
36	Yarkhan Banda	34.79302	71.88764	4192
37	Toor Mung	34.81126	71.81643	3675
38	Akhagram	34.92823	72.03936	3556
39	Jugha Banj	35.06369	71.99706	3571
40	Warae Bala	35.00012	72.04098	3439

Table. Risk factors for the collection of sand flies based on household and individual based questionnaireDate: / /2013

1.1	Districts (1=Dir Lower 2= Dir Upper)
1.2	Tehsils 1=Adezai 2=Timrgara 3=Balambat 4=Summer Bagh, 5=Lal qala 6= Ware 7= Dir khas 8= Sharin gal……..
1.3	Name of union council:
1.3.1	Name of village:
1.4	No. of households in the compound
1.5	Household ID Tehsil Formula: (tehsil+village+ house no =) Village House number
1.6	Who is the respondent? 1=Household head 2=Representative
1.7	Does the respondent agree? 1= yes 2= no
1.8	Family size ( ) No. of people in each age group: 0-5( ) 6-20( ) > 20( )
1.9	What is the highest level of education completed by Household head? 1=None 2=Primary 3=Secondary 4=Higher
1.9.1.	Is there any one who owns? houshold asset ownership? And his/ her job
1.10	Transport links 1= yes 2= no
1.11	No. of rooms in the house
1.12	Fridge 0= no 1= yes
1.13	Radio/TV/ Computer 1= R 2= TV 3= C
1.14	Bicycle , motor car etc. 0= no 1= yes
1.15	Mobile phone / Telephone 1= yes 2=no
1.16	Is the house connected to electricity? 0= no 1= yes

1.17	The main type of fuel used by family for cooking? 1=Electricity/gas 2=charcoal 3=kerosene 4=dung 5=Wood 6=other
1.18	Do you have animals in this household like goat, sheep or cattle-how many? 0= no 1= yes no of cattles
1.19	Dogs in the house 0= no 1= yes
1.20	What type of wall was used for the construction of this house? 1=clay/mud 2=bricks/stones 3=cement/painted 4=other
1.21	What kind of toilet facilities does your household have? 1 = Flush toilet 2 = Pit toilet/latrine 3 = No facility/bush/field
1.22	What is the main material of the roof? 1= Iron sheets or tiles 2= Thatch/grass or leaves 3= Other (explain)
1.23	What is the main material of the floor?]1=eath/sand 2=cement 3=dung 4=carpeted 5=other
1.24	How many windows are glazed? 0= no 1= yes
1.25	Are the windows open or closed? 0= open 1=closed
1.26	Is guest room present? 0= no 1= yes
1.27	Kitchen inside/outside 0= inside 1= out side
1.28	Ceiling fan/ Pedestal fan 0= C fan 1= P fan
1.29	Water facilities 0= well 1= hand pump 3= lake 4= river 5=stream 6= rain water 7= lending on money
1.30	Water storage 0=yes 1=no
1.31	Family member residing outside country 0= no 1= yes
1.32	Family member residing outside province/ city 0= P 1= C

1.33	Vegetation in house 0= no 1= yes
1.34	Which type of vegetation
1.35	Perfume usage 0 = no 1=yes
1.36	Presence of mice/ rats 0= no 1= yes
1.37	Light source (bulb/ energy saver)
1.38	Organic manure dump near 0= no 1= yes
1.39	Humid and shady places inside house 0= no 1= yes
1.40	Humid and shady places near house 0= no 1= yes
1.41	Muddy dunes in the area 0= no 1= yes
1.42	Sleeping out/ inside in rooms in summer 1= inside 2= out side
1.43	Washrooms/ bathrooms inside/ outside rooms 0= inside 1= out side
1.44	Clothes used for children during sleeping(complete cover/ incomplete cover)
1.45	Leishmaniasis cases in the house 0= no 1= yes
1.46	No. of family members suffered/ suffering from leishmaniasis
Section 2: Sand flies Control/Prevention	
2.1	Do you have mosquito bed net at home? 0= no 1= yes
2.2	Have you ever seen sand flies 0= no 1= yes

2.3	Do you know about the biting time of sand flies (day time/ night time)? 0= day time 1= night time
2.4	Type of attach of sand flies: a= individual b= group
2.5	Use of any mosquitoes repellent 0= no 1= yes on exposure parts at night

2.6	Where these nets hanging last night? 0= no 1= yes
2.7	Is the net observed? 0= no 1= yes
2.8	Use of mosquito spray 0= no 1= yes
2.9	Name and composition of spray
2.10	Spray used against other flies 0= no 1= yes
2.11	In the past 1- 12 months, has any one sprayed the interior walls of the house 0= no 1= yes
2.12	Who sprayed the house?1=Govt.progm 2=NGO 3=house hold member 4= other(explain) 5=don't know
2.13	Have any of these been used in your house over the last week/month/ year? 1=Mosquito coils 2=Insecticide spray 3=Repellents 4=other(explain)
3.1	ID number
3.2	Sex 1= male 2= female
3.3	Age/date of birth
3.4	Have you lived here for at least last 6 months 0= no 1= yes
3.5	Did you stay here last night? 0= no 1= yes
3.6	Have you always lived in this district? 0= no 1= yes
3.7	If no which other district have did you live in most recently?
3.8	Have you travelled outside from this district in the last 3 months? 0= no 1= yes
3.9	When did you come back from your most recent trip? 1= <two weeks 2= <four weeks 3= >4 weeks
3.10	Have you been infected with leishmaniasisat any time in your life? 0= no 1= yes 2=not sure

3.11	Do you know that any afghanrefuges living in your village 0= no 1= yes
3.12	Do you know that any preachers visit from other parts of the country to your village? 0= no 1=yes

GPS: Longitude |__|__|__| |__|__|__|__|__|__| Latitude |__|__| |__|__|__|__|__|__|

Remarks:

_____

_____

Thank you very much