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DNA barcoding reveals arthropod diversity and unveils seasonal patterns of variation in Quetta region, Pakistan

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Abstract

Deoxyribonucleic acid (DNA) sequencing is an emerging approach for revealing species diversity that has made species identification possible. This technique is an amazing and useful tool for studies in taxonomy, evolutionary biology, and biodiversity. Sequences of DNA can be used as genetic "barcodes" which could be used to identify all animals, including insects. True flies, belonging to the order Diptera, are widely distributed and crucial components of ecosystems worldwide. Despite the rich biodiversity of Pakistan, our knowledge of various insect groups particularly flies remains limited. The current study was conducted in Quetta, Pakistan, from June 2017 to May 2018, to use DNA barcoding technique for the determination of the diversity of flies (658 bp sequence from the 5′-end of cytochrome oxidase I). Our analysis focused on a specific region of the cytochrome c oxidase 1 (COI) gene, known as the barcode region which provides valuable information for inferring evolutionary relationships and identifying species. The obtained sequences of 2,195 fly specimens were then compared and matched against the Barcode of Life Data System (BOLD), which assigned the specimens to 309 Barcode Index Numbers (BINs), which operates as a counterpart in the BOLD database. Among the families identified, *Muscidae* was the most dominant, with 283 specimens, followed by *Cecidomyiidae* with 184 specimens, and *Ceratopogonidae* with 164 specimens. A total 82 number of species were identified with *Tricimba humeralis* with the maximum catch.

Keywords: Arthropod, BOLD, Barcode index number, Biodiversity, Cytochrome c oxidase I, DNA barcoding

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Introduction

Modern biodiversity research has taken deoxyribonucleic acid (DNA) barcoding more quickly due to its effectiveness in identifying specimens and identifying new species (Arain et al., 2011; Hebert et al., 2013; Hebert et al., 2004; Huemer et al., 2014; Kress et al., 2015; Ashfaq and Hebert., 2016). This platform has produced DNA barcode coverage for over seven lakhs and sixty thousand animal species by using the Barcode of Life Data System (BOLD) (DeSalle and Goldstein, 2019). Because the Barcode Index Number (BIN) system is an efficient species proxy that enables rapid assessment of species diversity, large-scale biotic assessments are now accessible (Ratnasingham and Hebert, 2007; Hausmann et al., 2013; Wirta et al., 2016; Ortiz et al., 2017). Morphological analyses have revealed limits of species that closely correspond with BINs (Huemer et al., 2014; Mitchell et al., 2020). They can be applied to identify cryptic species and to distinguish recently discovered species (Zhou et al., 2019; Ren et al., 2018), to figure out the distribution of species and evaluate the species richness in large samples, to analyze museum collections, and to evaluate the global and regional similarity of fauna (Braukmann et al., 2019; Pentinsaari et al., 2020; Ashfaq et al., 2017; Wilson et al., 2017; Ashfaq et al., 2018). Applied mitochondrial DNA barcoding is a novel method of species identification that classifies animal species using a standardized single molecular marker for mitochondrial gene cytochrome c oxidase subunit 1 (cox1 or COI) (Weigand et al., 2019).
DNA barcoding offers a very precise and impartial technique for species identification by relying on the distinct sequence of a standardized DNA region, usually the mitochondrial COI gene. DNA barcoding allows for rapid identification of species while morphological identification can be laborious and necessitates careful inspection and the expertise of taxonomists. DNA barcoding can identify cryptic species that are morphologically similar but genetically distinct, providing a more accurate reflection of biodiversity (Wilson et al., 2017). Broad biodiversity analysis is made possible by the application of DNA barcoding and technological developments in sequencing. Geographic location has, however, varied the intensity of research. As per www.boldsystems.org, Canada has an 8-fold higher BIN count (84,000) than Russia (11,000), despite Russia's being 1.7 times smaller. Analogously, Germany's BIN count (23,000) is 4× that of India (5,800), despite the latter being nine times larger (Savage et al., 2019).
DNA barcoding is an effective technique in entomology that makes it easier to identify and find new species within the order Diptera, also referred to as true flies. More than 150,000 species of insects have been identified in this diverse group (Hebert et al., 2016). Similarly, analysis of species richness in Diptera and Hymenoptera using DNA barcoding techniques revealed many overlooked species in these groups. Another study focused specifically on German Diptera and found that DNA barcoding successfully identified many species, including some that were previously taxonomically inaccessible (Moreinere et al., 2019; Government of Balochistan and IUCN Pakistan, 2000).
Balochistan province, which occupies 44% of Pakistan's total land area, is considered one of Pakistan's most important wildlife regions as it has a large number of resources, great endemism and wide range of species that represent worldwide biodiversity (Javed, 2019; Musakhel, 2008). Due to its substantial biodiversity and abundance of species, Balochistan is the traditional transition zone between the oriental and palearctic zoogeographical regions. Variations in climate and physical features create diverse ecosystems, habitats, and landscapes for a variety of life forms (Qureshi, 2012; IUCN, 1997). With respect to taxonomic identification, data of only few species of migratory birds, mammals and reptiles of Quetta, Kharan, Zhob, Nuskhi, Chagai, Lasbela, Loralai, and Hab districts are available (Ghalib, 1979; Syed, 2007; Tareen, 2017; Government of Pakistan, 2000). Many groups of diverse Taxa are poorly recognized, particularly insects, even though over five thousand insect species have been reported (Ward and Lariviere, 2004), due to the lack of taxonomists, no wide-ranging or taxonomically comprehensive assessments have been conducted and additionally, many insect species remain unidentified. Considering how challenging it is to execute standard morphological techniques (Pompeo et al., 2017; Scheffers et al., 2012; Moreinere et al., 2019).
The current study is significant as it represents the first study of any South Asian nation's insect fauna using DNA barcoding. The study provides a strong basis intended for a comprehensive fauna of Pakistan's insect species. Additionally, the data generated from this study contributes to the inclusive

DNA barcode allusion lending library, aiding future research, and facilitating species identification efforts worldwide. Enhancing DNA barcode accessibility in Pakistan, will help on the way to improve our understanding of the taxonomic makeup of the country's insect species and create a barcode mention lending library that will aid future routine eDNA and met-barcoding research.

Material and Methods

Specimen sampling

Insects were collected with a malaise trap from Quetta, Balochistan (30.1909 latitude, 66.9612 longitude, 1592 elevation) during 2017-2018 and stored at -20°C. All flies were sorted out from other insect orders based on key characteristics and labeled with a code for molecular identification (Van Emden, 1965; Jalali et al., 2015). Specimen information, including sequences, images, and collection data are accessible on BOLD (Barcode of Life Database) under the project GMTTC.

Morphological identification of collected samples (Family level)

All collected insects were morphologically identified at family level through the pictorial, linear and dichotomous taxonomic keys (Marshall et al., 2017).

Isolation of DNA, amplification by PCR, and Sequencing

The procedures outlined in the published protocols of the Canadian Centre (CCBD) for DNA Barcoding. were followed for DNA isolation, Polymerase chain reactions, and sequencing (Ivanova and Hebert, 2006; deWaard et al., 2019a). A 96-well microplate filled with 95% ethanol was used to suspend the DNA that was taken out of the legs of the sampled insects.

The COI gene implication was performed by using the set of primers (deWaard et al., 2019b; Ren et al., 2018; Posada-López et al., 2023; Pinto et al., 2023; Ralston and Crupper, 2018). This molecular marker has allowed advances in exploring the genetic diversity of the different insect species successfully (Ashfaq et al., 2014).

LepF1 (ATTCAACCAATCATAAAGATATTGG)
LepR1(TAAACTTCTGGATGTCCAAAAAATCA)
(http://www.dnabarcoding.ca/CCDB_DOCS/CCDB_PrimerSets.pdf).

The thermal cycler program included the initial step of 94℃ (1 minute), 5 cycles of 94℃ (30 seconds), annealing at 45-50℃ (40 seconds), and extension at 72℃ (1 minute), followed by 30-35 cycles of 94℃ (30 seconds), 51-54℃ for (40 seconds), and 72℃ (1 minute), with a last extension of (10 minutes) at 72℃. with a final extension at 72℃ (10 minutes), followed by an indefinite hold at 4℃. The extracted DNA was quantified on agarose gel. Amplicons were separated using an E-gel 96 system (Invitrogen Inc.) with 2% agarose. Sequencing of the samples was carried out by using Single Molecule, Real-Time (SMRT) sequencing on a Sequel platform (Pacific Biosciences, Menlo Park, CA, USA) following Hebert et al. (2013). The sequences of the CO1 genes produced were submitted to BOLD. Sequences were also interpreted on MEGA X (Tamura et al., 2011) for phylogenetics.

Data analysis

For identification and discrimination, the obtained sequences were compared to GenBank/BOLD sequences by using the tool BLAST on NCBI and BOLD. The sequence of Apis mellifera (OP034096) was retrieved from GenBank and revealed as an outgroup for the *dipteran* flies. The barcode index number (BIN) under BOLD, acquainted by (Ratnasingham and Hebert, 2013), allots a unique number to every species. The divergent species are mostly allocated to different BINs, however, this system encourages to arrange the data of specimens that do not have specific taxonomy. All the flies species of the present study have a Barcode Index Number assigned. The Multiple sequence alignment (MSA) of 32 sequences was conducted on MEGA X using the CLUSTALW function (Thompson et al., 1994). The NJ Tree with strong bootstrap of 1000 replicates based upon Kimura-2-Parameter was constructed and genetic distances were calculated with pairwise deletion using MEGA X. The current sequences were submitted in BOLD to analyze; the barcode gap, accumulation curve, distance ranks and histograms by using the effective tools of BOLD. The statically analysis was done by statistical software Minitab18**.**

Results

In the present study, a total of 8721 collected insect specimens were submitted for Sequel sequencing, and 6821 Sequences COI 5P sequences (78.2%) were recovered. The length of the recovered sequences varied and yielded a full length (>600 bp)

barcode in general barcoding weekly data and yielded 1039 BINs of all insects (GMTTC on BOLD). From all insect specimens, 2195 specimens (after initial screening by morphological characteristics) were recovered as dipteran specimens, (1911) Sequences and 309 BINS including unique and common BINs. In all dipteran specimens, 1045 (47.6%) were identified up to genus level while 83 (3.7%) species were identified.

Morphological characteristics of different families

External morphology of insect head (antennal segmentation), thorax (wings venation, colour of halter) and abdomen setae of all the species were observed and studied under a stereomicroscope (Leica ZOOM 2000). Families of different species were identified using published morphological keys (Table 1) (Marshall et al.,2017; Hackston, 2018).

General Barcoding: Weekly samples with geographical attributes

Insect samples of thirteen weeks were collected from Quetta, Balochistan (latitude 30.1909, longitude 66.9612 and Elevation 1592). The highest numbers of dipteran specimens, sequences, BINs, and species were observed during June 2017. There was a gradual decline in total specimens and species catch during the winter season of 2017. The data depicted again an increase peak of dipteran fly's samples with the gradual summer arrival in weekly samples of next year in 2018. The range of total numbers of specimen and identified families were observed (24-479), (2-29) in different weeks of 2017-2018 respectively. Similarly, the range of sequences were (2-30) and number of BINs were found (2-118) (Figure 1).

Table-1. Identification of Key characters of Different Families identified in order Diptera.

Number	Family Name	Key Characters	References
1	Muscidae	Thorax (red), or with only indistinct fine setulae, with a few scattered setae. Wing vein M1 usually straight or gently bent. Anal wing vein (A1) not curved, subcostal vein (Sc) usually curved towards costal vein (C) in distal 1⁄2. Hind tibia often with antero dorsal seta near middle.	(Marshall et al., 2017)
2	Choronomidae	Stout-bodied flies. Antenna short, not or only marginally longer than head (some Chironomidae with antennae the same length as head has fewer flagellomeres (4-7 rather than 9 or 10), usually conspicuously thickened; pedicel not enlarged and cup-shaped. Abdominal tergite 1 with or without fringe of elongate setae laterally. Wing usually broad.	(Marshall et al., 2017)
3	Ceratopogonidae	Wing vein M1+2 usually forked (= M-fork), but M-fork often indistinct; basal medial cross vein absent. Sub scutellum without longitudinal groove	(Marshall et al., 2017)
4	Phoridae	Wing with first few veins (radial veins) usually short, thick and crowded towards wing base; other veins weaker, unconnected by cross veins. Antennal pedicel usually hidden within post pedicle. Generally humpbacked thorax, bristly flies with distinctive habitus.	(Marshall et al., 2017)
5	Agromyzidae	Sub costa vein developed throughout its length, joining with vein R1 before reaching costa. At least 3 pairs of dorso centrals present; Halteres white or yellow.	(Hackston, 2018)
6	Sciaridae	Antenna usually long, with 14 flagellomeres Fore femur usually slender and less robust.	(Marshall et al., 2017)

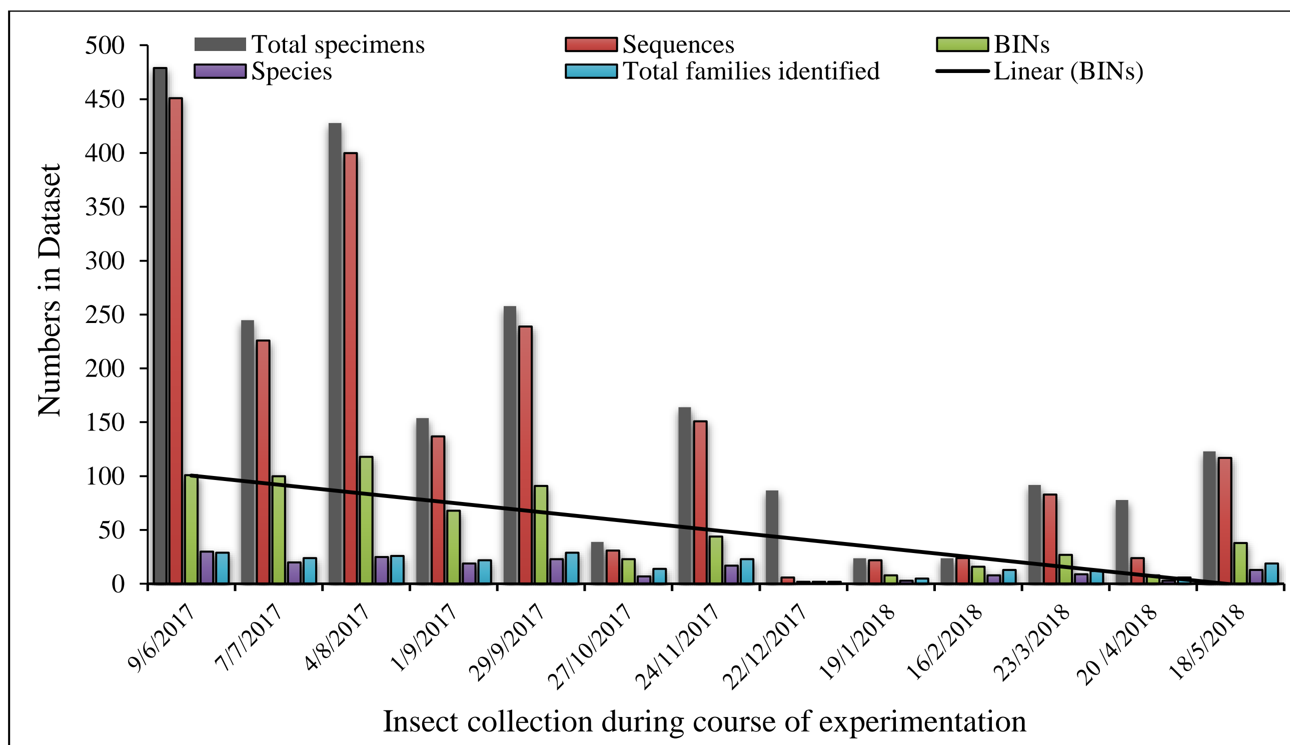


Figure-1. Standard barcoding results in week wise sampling (Diptera) of Quetta.

Detail of identified BINs

A total of 2195 specimens were succumbed and identified into 1914 sequences matching 309 BINs. Dataset DS-DIPPAK26 can be downloaded from BOLD. In the current study Dipteran species were revealed by DNA barcoding, BINs detail can be found in BOLD, especially in Pakistan. Most of flies samples belonged to the family Muscidae and Cecidomyiidae. Some families existed in moderate numbers while few were found in very minimum numbers regarding taxa name, species, and numbers of BINs. Most prevalent families have been selected on the basis of specie abundance to give details of their 31 species such as Muscidae, Chironomidae, Ceratopogonidae, Phoridae, Agromyzidae and Sciaridae. All identified species were assigned BINs, indicating that they are either already present in Pakistan or were discovered for the first time in the current study according to https://www.gbif.org/occurrence (Table 2).

Total number of identified species

A total eighty-two species were identified in which maximum count of *Tricimba humeralis* was found (63) while *Delia platura* (52), *Physiphora alceae* (40), *Coenosia attenuata* (39), *Pollenia rudis* (36), *Pollenia pediculata* (35), *Psychoda alternata* (28) were counted. However, the number of *Lycoriella sativae* and *Crossopalpus aeneus* were (17) same and *Chironomus transvaalensis* (14), *Eristalinus aeneus* (11), *Chironomus dorsalis* were exist (10) in numbers of species. Furthermore, other species were observed less than ten numbers (Figure 2).

Table-2. Dipteran species revealed by DNA barcoding of Malaise trap samples collected in Quetta.

No.	Sample ID	Family	Genus	Specie name	Description of spp	BINs	Occurrence (Recent identification and Pakistan status) https://www.gbif.org
1.	BIOUG 49056-D02	Muscidae	*Atherigona Rondani,* 1856	*Atherigona orientalis* (*Schiner,* 1868)	Adults have a yellowish abdomen, a pair of black tiny dots in the dorsal abdomen and grey thorax (Malviya et al., 2015).	BOLD: AAF5305	International Barcode of Life project (iBOL) India and (iBOL) Pakistan in Current study

2	BIOUG 49028-H09	Sciaridae	*Bradysia Winnertz, 1867*	*Bradysia nomica (Mohrig & Roschmann,* 1996)	Little insect dark brownish color. Hypopygium is in place. The flagellomeres. Hairs on palpus. Comb setae on the front tibia (Babytskiy et al., 2019).	BOLD: ABW7972	International Barcode of Life project (iBOL) Australia and International Barcode of Life project (iBOL) Pakistan in Current study
3	BIOUG 49077-H09	Sciaridae	*Bradysia Winnertz,* 1867	*Bradysia ocellaris* (*Comstock,* 1882)	Body color is yellowish, with darker patches on the pleural sclerites. Hypopygium present place. The flagellomeres. Divided palpus that has two or more bristles (Mohrig, 2003)	BOLD: ABW7969	International Barcode of Life project (iBOL) Pakistan in Current study
4	BIOUG 49178-F03	Sciaridae	*Bradysia Winnertz,*1867	*Bradysia trivittata*	Little insects with a dark color. Hypopygium is in place.The Flagellomeres. Palpus's hairs. Fore tibia with setae (Babytskiy et al., 2019).	BOLD: ABA6488	International Barcode of Life project (iBOL) Canada and International Barcode of Life project (iBOL) Pakistan in current study
5	BIOUG 49028-C02	Chironomidae	*Chironomus Meigen,* 1803	*Chironomus dorsalis* (Goetghebuer, 1921)	Antennae plumose in both sexs,chewing mouthparts with short palps, Long, thin legs (da Silva et al., 2018).	BOLD: AAW4001	The InternationalBarcode of Life project (Pakistan) and current study
6	BIOUG 49077-G08	Chironomidae	*Chironomus Meigen,* 1803	*Chironomus kiiensis*	Plumose antennae (both sexes) Chewing mouthparts with short palps. Long slender legs (da Silva et al., 2018).	BOLD: AAD8162	The International Barcode of Life project (India) and International Barcode of Life project (iBOL) Pakistan in Current study
7	BIOUG 48255-B02	Chironomidae	*Chironomus Meigen,* 1803	*Chironomus transvaalensis*	Antennae plumose in both sexes, Mouth parts chewing, with brief maxiliary palps Long, thin legs (da Silva et al.,2018).	BOLD: AAW3995	International Barcode of Life project (iBOL)2019 south Africa and International Barcode of Life project (iBOL) Pakistan in Current study
8	BIOUG 49028-D01	Muscidae	*Coenosia Meigen,* 1826	*Coenosia attenuata* (Stein, 1903)	The fly is tiny, measuring between 2.5 to 4.0 mm in lengt (brownish grey in ground color, with frons parallel sided in both sex; male legs yellow and in female black. female head gray with two dark converging longitudinal stripes on the front (Hoebeke et al., 2003).	BOLD: AAD7633	International Barcode of Life project (iBOL) Spain and International Barcode of Life project (iBOL) Pakistan in Current study
9	BIOUG 49063-H10	Muscidae	*Mycophaga Rondani,* 1856	*Coenosia testacea* (Robineau-Desvoidy, 1830)	Antenna black, palpus entirely yellow in male (apically dark in female), legs yellow, tarsi black, two brown longitudinal stripes on scutum, female abdomen oval, dark with 2 spots (Parchami et al., 2020).	BOLD: ACR4672	International Barcode of Life project (iBOL) Germany and International Barcode of Life project (iBOL) Pakistan in Current study
10	BIOUG 49179-B12	Chironomidae	*Corynoneura Winnertz,* 1846	*Corynoneura arctica*(Kieffer, 1923)	Antenna with 10 flagellomeres, sternapodeme inverted V-shaped to U-shaped, and undeveloped inferior volsella (Tasdemir and Akyildiz, 2023).	BOLD: ACX4287	International Barcode of Life project (iBOL) Norway and International Barcode of Life project (iBOL) Pakistan in Current study
11	BIOUG 49178-G06	Sciaridae	*Corynoptera Winnertz,* 1867	*Corynoptera perpusilla* (Walker, 1848)	Antennal flagellomere 4. Front tibial organ arranged in a transverse row, shorter gonostylus with a relatively shorter apical tooth. Basic structure of the hypopygium (Menzel and Mohrig, 2000).	BOLD: AAU6595	International Barcode of Life project (iBOL) Australia and International Barcode of Life project (iBOL) Pakistan in Current study
12	BIOUG 49027-E09	Chironomidae	*Cricotopus Wulp,* 1874	*Cricotopus ornatus* (Meigen, 1818)	Tergite light or dark colour. All legs pale, or with dark sections. Tarsomeres of mid-and hind legs pale, dark (Drayson et al., 2015).	BOLD: AAP5926	International Barcode of Life project (iBOL) China and International Barcode of Life project

							(iBOL) Pakistan in Current study
13	BIOUG 49078-A01	Ceratopogonidae	*Culicoides Latreille,* 1809	*Culicoides gejgelensis* (Dzhafarov, 1964)	Wing patterned, with pale spot in distal part of each of cells r3, m and m and proximal parts of m and, second radial cell wholly dark; macrotrichia absent from basal cell. aedeagus rectangular-shaped, relatively long and thick (Matheu, 2014).	BOLD: ABX3080	International Barcode of Life project (iBOL) Turkey and International Barcode of Life project (iBOL) Pakistan in Current study
14	BIOUG 48256-G03	Ceratopogonidae	*Culicoides Latreille,* 1809	*Culicoides imicola* (Kieffer, 1913)	A medium-sized, dark brown species. Female with 3rd segment of maxillary palpus slender Wing with many, prominent pale markings, including large pale spots over-m cross- vein, just distal of 2nd radial cell, and at apex of cell R5; Dark brown. Legs brown (Glick, 1990).	BOLD: AAB8379	International Barcode of Life project (iBOL) Pakistan in Current study
15	BIOUG 48891-G05	Ceratopogonidae	*Culicoides Latreille,* 1809	*Culicoides oxystoma* (Kieffer, 1910)	Length of the wing, length of the space of the two sensilla up the eyes; length of the five flagellar segments (11 to 15) and eight basal flagellar segments (3 to 10). Pale spots under radial cells, pale spots in m1 that did not cross M2 (Slama et al., 2021).	BOLD: AAD1856	International Barcode of Life project (iBOL)India, Pakistan, and International Barcode of Life project (iBOL) Pakistan in Current study
16	BIOUG 49251-G02	Ceratopogonidae	*Dasyhelea Kieffer,* 1911	*Dasyhelea incisurata* (Remm, 1962)	Frontal sclerite broader than long, elliptical, pentagonal or slightly heart-shaped, with long slender ventral projection. Posterior margin of male sternite 9 is straight, arch-like or slightly concave. Female antennal flagellomeres elongate, bottle-shaped, with sculptured reticulations; flagellomere 13 with apical prolongation (Dominiak, 2012).	BOLD: AAN5169	International Barcode of Life project (iBOL) Pakistan in Current study
17	BIOUG 48392-G05	Muscidae	*Helina Robineau-Desvoidy,* 1830	*Helina cilipes* (Schnabl, 1889)	slender, brown-dusted species, 2 pairs of orbital setae, antenna black, arista plumose; palpus black (Sorokina and Pont Adrian, 2011).	BOLD: ACD1632	International Barcode of Life project (iBOL) Pakistan in Current study
18	BIOUG 49171-E05	Agromyzidae	*Liriomyza Mik,* 1894	*Liriomyza brassicae* (Riley, 1885)	Fronto-orbital setulae reclinate (backward pointing); a yellow frons and a bright yellow scutellum. The mesonotum is shiny black Subcosta becomes a fold distally and ends in costa separately (Malipatil et al., 2004).	BOLD: AAF6806	International Barcode of Life project (iBOL) Pakistan and Current study
19	BIOUG 49078-C02	Agromyzidae	*Liriomyza Mik,* 1894	*Liriomyza sativae* (Blanchard, 1938)	3rd antennal seg,small yellow. Male distiphallusWith slight constriction only between the apical and basal parts of the bulb. Basal section is not strongly curved. Pointed apex.femur bright yellow (Malipatil et al., 2004).	BOLD: AAF6797	International Barcode of Life project (iBOL) Pakistan and Current study
20	BIOUG 48413-F01	Muscidae	*Lispe Latreille,* 1796	*Lispe assimilis* (Wiedemann, 1824)	Head Ground-colour black. Fronto-orbital plate yellowish Antenna black, except tip of pedicel which is orange. Scutum and scutellum yellowish-grey dusted (Pont Adrian, 2019).	BOLD: AAZ1114	International Barcode of Life project (iBOL) Pakistan and Current study
21	BIOUG 48392-G09	Muscidae	*Lispe Latreille,* 1796	*Lispe tentaculata* (De Geer, 1776)	Posterior part of prescutal sulcus with dark or light yellowish-brown velutinous patchs. First fore tarsomere and straight phallus. Arista plumose, palpi abruptly expanded in apical half. (Ge et al., 2016)	BOLD: AAB8429	International Barcode of Life project (iBOL) Pakistan and Current study
22	BIOUG 49171-B05	Sciaridae	*Lycoriella Frey,* 1942	*Lycoriella sativae* (Johannsen, 1912)	Head dark brown to black. Face with fi ne 18–28 setae, arranged approximately radially. Thorax dark brown to black, with whitish yellow to brown setae	BOLD: ACM4797	International Barcode of Life project (iBOL) Pakistan in Current study

					Hypopygium about as tall as wide, pale to dark brown (Babytskiy et al., 2022).		
23	BIOUG 49027-H11	Phoridae	*Megaselia Rondani,* 1856	*Megaselia halterata* (Wood, 1910)	Adult phorids are 0.5–5.5 mm long with an enlarged thorax that gives them a characteristic humpbacked appearance. The hind femora are flattened and the major bristles of the head and legs are feathered (Gerhardt and Hribar, 2019).	BOLD: ABV8939	International Barcode of Life project (iBOL) Pakistan and Current study
24	BIOUG 49178-E10	Phoridae	*Megaselia Rondani,* 1856	*Megaselia sepulchralis* (Lundbeck, 1919)	Frons brown, Cheek with three to five bristles, Palps whitish-grey, Labrum pale yellow (Disney, & Russell-(Smith, A. 2014).	BOLD: AEV8824	International Barcode of Life project (iBOL) Pakistan and Current study
25	BIOUG 48405-A09	Muscidae	*Musca Linnaeus,* 1758	*Musca domestica* (Linnaeus, 1758)	Four dark stripes on the dorsum of the thorax and the pronounced upward bend in the fourth longitudinal wing vein (Crosskey and Lane, 1993).	BOLD: AAA6020	International Barcode of Life project (iBOL) Pakistan and Current study
26	BIOUG 49063-G06	Agromyzidae	*Phytomyza Fallén,* 1810	*Phytomyza thalictrella* (Spencer, 1981)	Length of the fronto-orbital setae, engths of the costal sectors.male and female genetalia (Lonsdale, 2021).	BOLD: ABX2176	International Barcode of Life project (iBOL) Pakistan and current study
27	BIOUG 49081-B12	Chironomidae	*Psectrocladius Kieffer,* 1906	*Psectrocladius schlienzi* (Wülker, 1956)	Head capsule yellow to light brown, occipital margin pale or darkened. Antenna 5-segmented. Mentum with one or two median teeth and 5 pairs of subequal lateral teeth (Chamutiová et al., 2020)	BOLD: AAU6327	International Barcode of Life project (iBOL) Pakistan in Current study
28	BIOUG 49027-B02	Chironomidae	*Rheotanytarsus Thienemann & Bause,* 1913	*Rheotanytarsus erignus*	Antenna with 12, 13 flagellomere. Apex of anal point pointed, spatulate, Abdomen banded evenly coloured. Base of anal point with a tuft of setae without setal cluster (Wang and Guo, 2004).	BOLD: ACR3994	International Barcode of Life project (iBOL) Pakistan and Current study
29	BIOUG 49171-D04	Sciaridae	*Scatopsciara Edwards,* 1927	*Scatopsciara curvilinea* (Lengersdorf, 1934)	Eye bridge 3 facets wide Legs yellowish; tibial organ of fore tibia small, with 4–5 bristles on a small lobe; spurs of middle tibia vary, one is long, the other strongly reduced; Hypopygium brown (Mohrig et al., 2016).	BOLD: ACG1189	International Barcode of Life project (iBOL) Pakistan and Current study
30	BIOUG 49251-F01	Chironomidae	*Tanytarsus Van Der Wulp*	*Tanytarsus formosanus* (Kieffer, 1912)	Head with large frontal tubercles; usually strong thoracic colorations anteriorly and dorsolaterally on scutum; wings with sparse setation. hypopygium: Spines in one row between well-developed anal crests; superior volsella tapered towards apex which is somewhat median elongated, with lateral microtrichia (Ekrem, 2001).	BOLD: ABY1131	International Barcode of Life project (iBOL) Current study and in Pakistan
31	BIOUG 43583-C02	Chironomidae	Tanytarsus Van Der Wulp	Tanytarsus volgensis (Miseiko, 1967)	Large Specimens aredarker. Smallest ones are lighter, Eyes black. Antenna, tentorium, scutal stripes, scutellum, postnotum, sternum, hypopygial apodemes and proximal leg segments, incl. femora and tibiae dark brown to black. Head capsule mouth parts, colour of thorax, tarsi and abdomen brown with a slight olive undertone. Wing and haltere pale brownish. (Giłka et al., 2018; Giłka and Gadawski, 2022).	NA	International Barcode of Life project (iBOL) in Current study

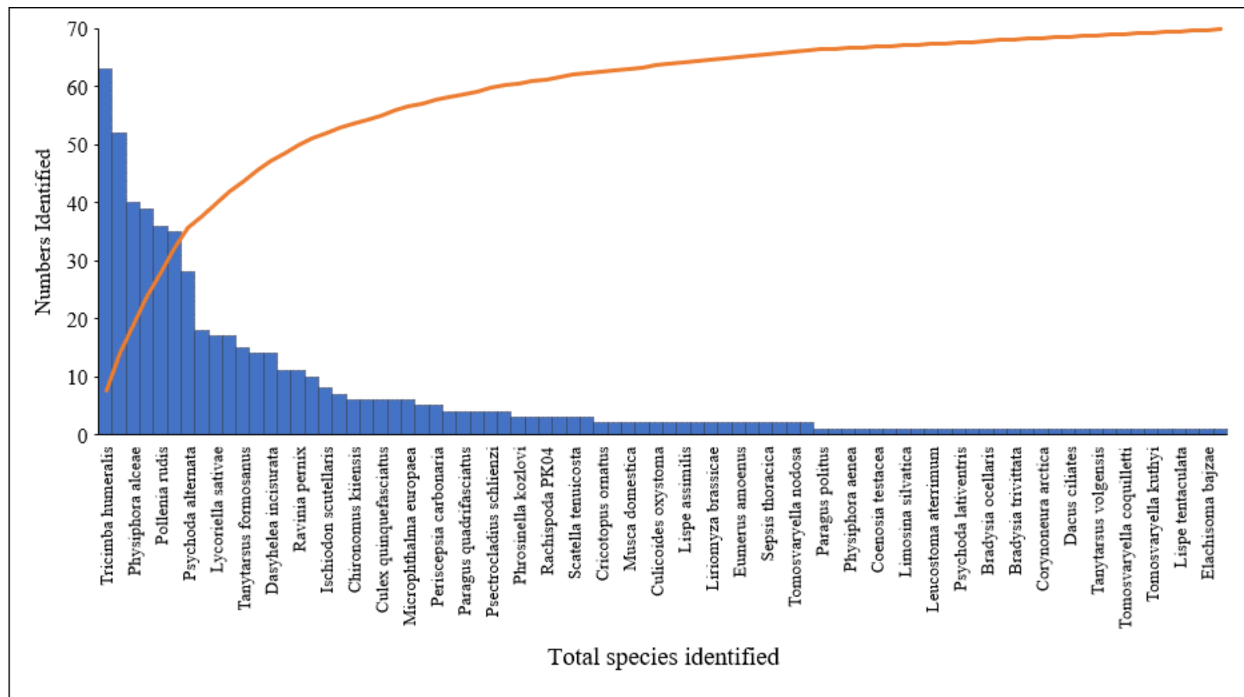


Figure-2. Total number of species identified and their abundance.

Temporal distribution of dipteran species in general barcoding via Venn diagram

To analyze the distribution of dipteran specimens throughout the sampling duration, the time of sample collection was observed into 4 categories according to different months and seasons. Results of temporal distribution of dipteran species in general barcoding showed the number of species in January-March was (17) with five elements included exclusively in January-March. However, in April-June the number of species were (40) with 12 elements included exclusively in "April-June similarly in July-September the number of species were (53) with 25 species included exclusively in July and September Furthermore in moth of October-December (27) with 5 common species included exclusively in October-December (Figure 3).

Identification of different families of order Diptera

A total of 2195 specimens have been identified in 41 families. Family Muscidae and Cecidomyiidae were the most dominant with (283, 184) specimens respectively. The turn down families were observed such as ceratopognidae, (164) Chironomidae (134), Phoridae (132), Agromyziae (108), Sciaridae (81), Chloropidae (78), Anthomiidae (73), Polleniidae (71), Syrphidae (63), Sareophagidae (61), Scatopsidae (50). Families with the least specimen containing were Calliphoridae, Haleomyzidae, Limoniidae, Mythicomyiidae, Tabanidae and chyromiidae with only 1 specimen as shown in Figure 4. The counts of specimens for each family indicate their relative abundance in the collected dataset. This information is valuable for understanding the composition and distribution of dipteran families, which can contribute to further research and conservation efforts related to these insect groups, according to maximum frequency to lowest frequency.

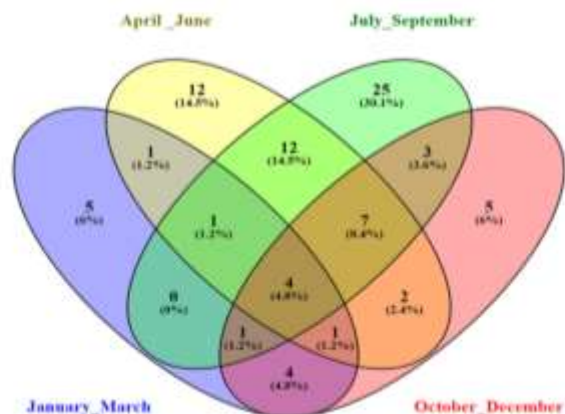
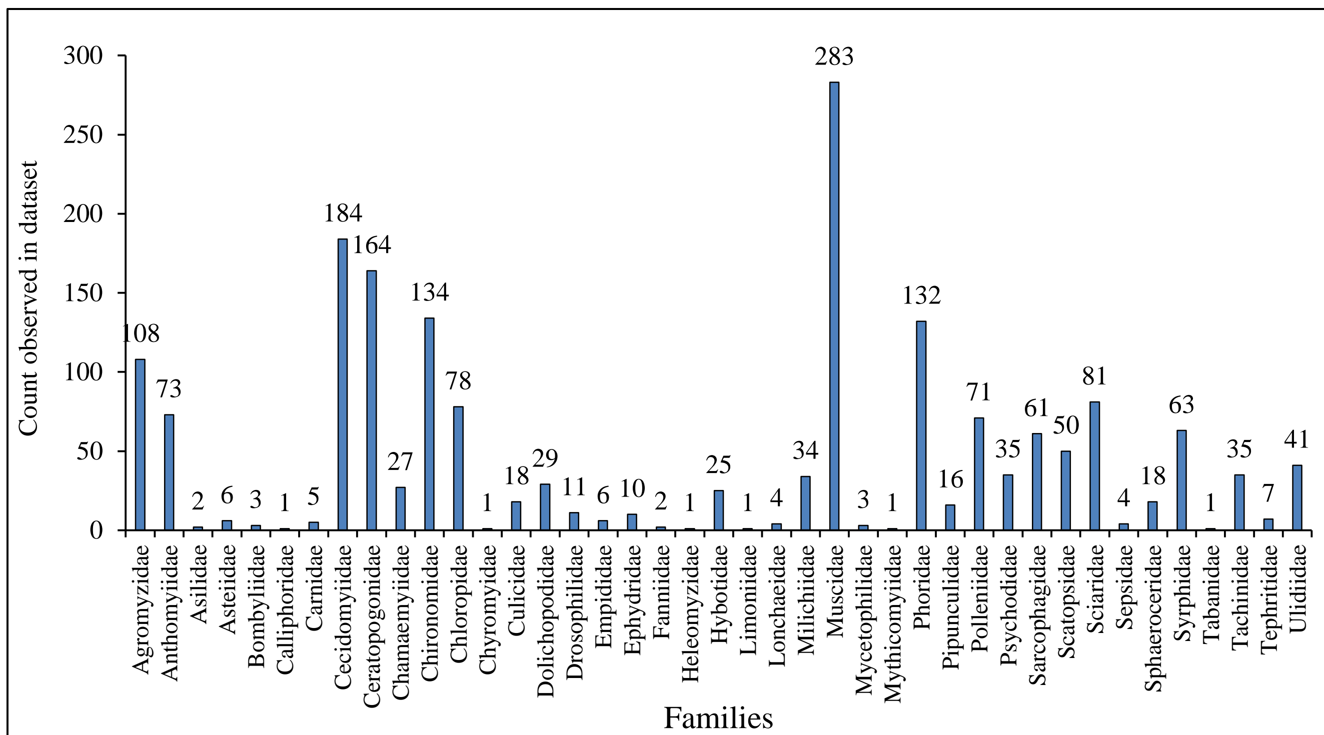


Figure-3. Venn diagram (showing unique and



shared species).

Figure-4. Bar chart of different families identified, and their number of specimens recovered.

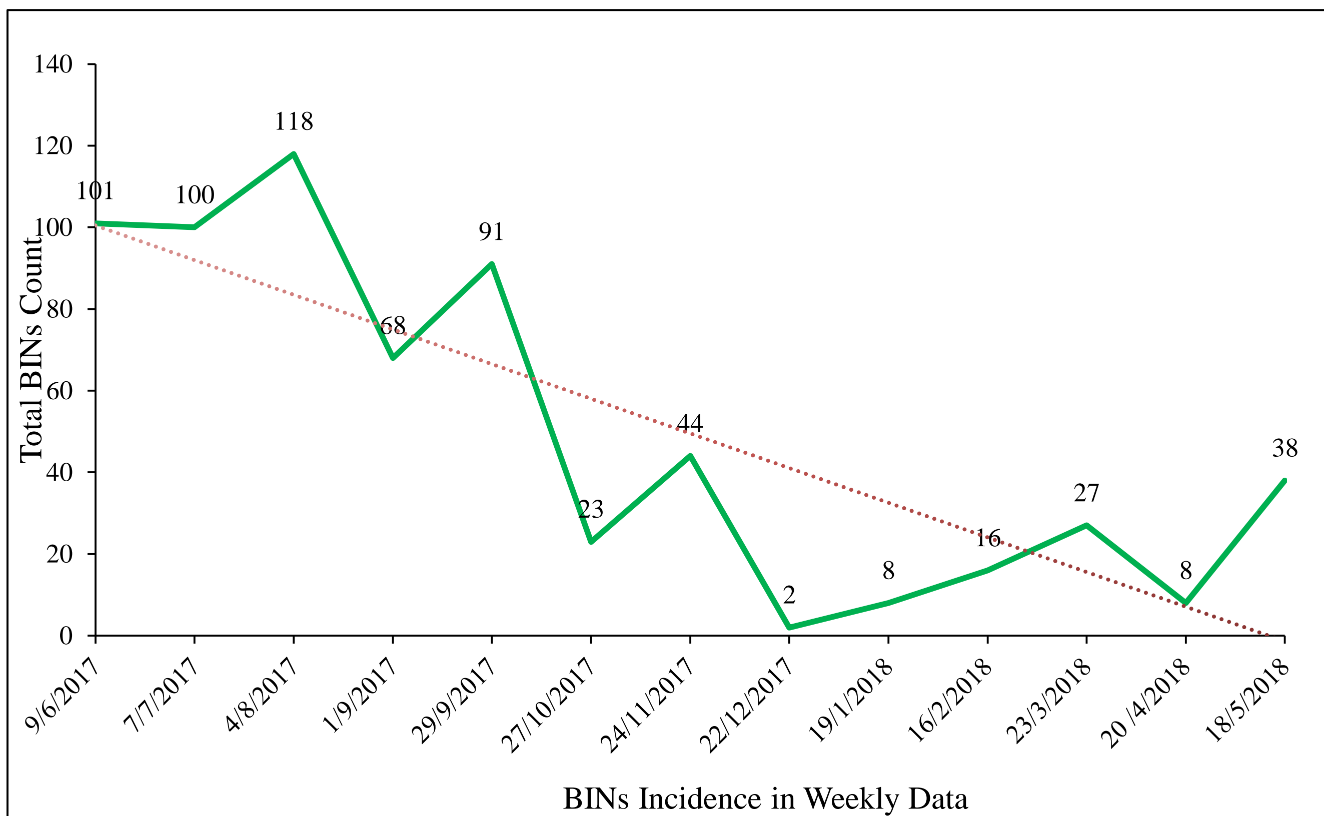


Figure-5. BINs incidences in weeks.

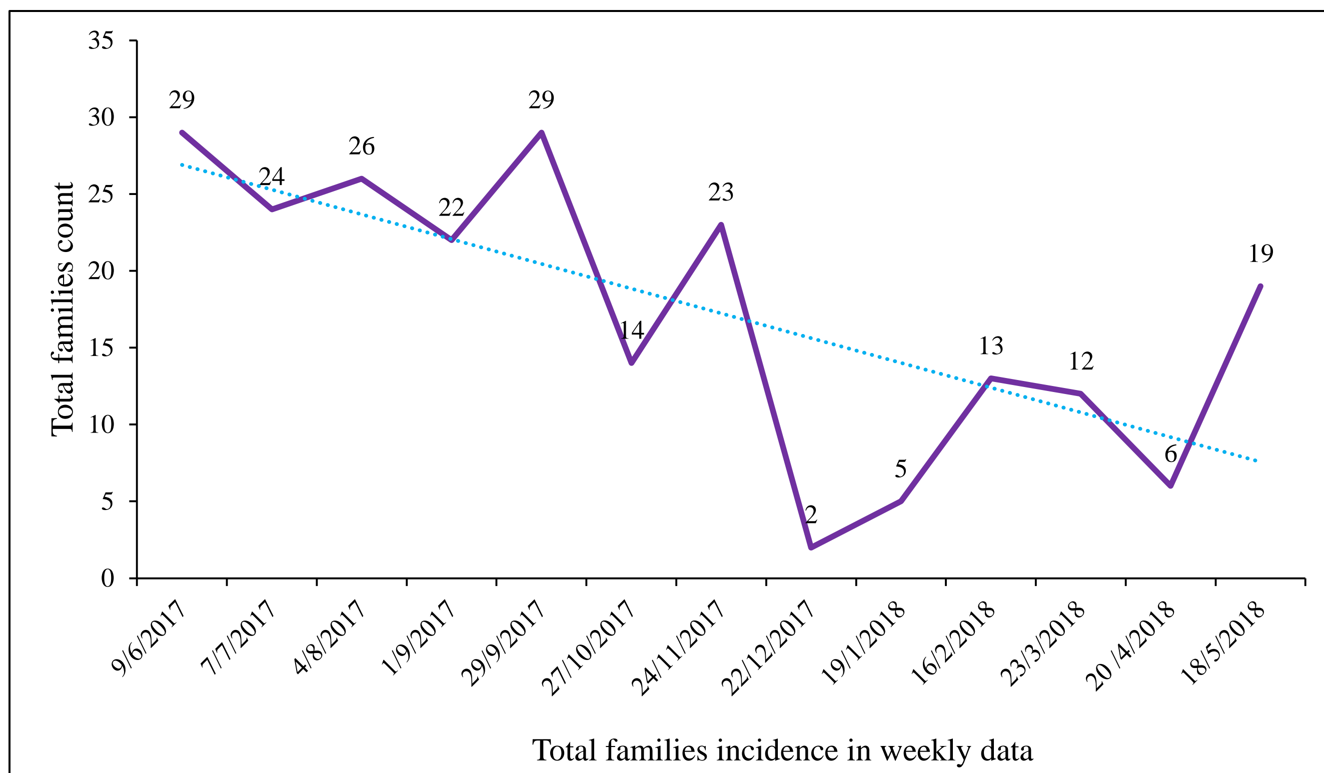


Figure-6. Total identified families in weeks.

Detail of Dipteran specimens

Among all weeks highest Dipteran Specimens 479 obtained were maximum 9/6/2017 in June while minimum 24 were observed in February 16/2/2018 week data. Among all weeks, the highest Dipteran sequences obtained were maximum 452 in June (9/6/2017) while minimum 6 were observed in December (22/12/2017) week data. Among all weeks highest dipteran BINs obtained were 118 in (4/8/2017) in June while minimum 2 were observed in (22/12/2017) December week data as shown in Figure 5. Among all weeks highest Dipteran Species were 34 obtained in (9/6/2017) in June while minimum 2 were observed in (22/12/2017) month of December. Among all weeks total families identified were maximum 29 obtained in (9/6/2017and 29/9/2017) while minimum 2 were observed in (22/12/2017) week data (Figure 6).

One-way analysis (ANOVA) of total Diptera specimens

One way analysis (Table 3) was performed to compare total recovered Diptera specimen, sequence, BINs, and species with seasons. Results indicated that in the community structure of Diptera, total identified BINs were significantly affected by seasonality (month) indicating seasonal temperature is an important factor in growth and reproduction in all insects.

Table-3. One-way analysis (ANOVA) of total dipteran specimens, sequence, BINs, and species with season.

Relation	R-sq(adj)	R^2-sq	P -Value
Total Diptera Specimens versus Season	24.50%	43.37%	0.146*
Sequences versus Season	20.90%	40.68%	0.176 *
BINs versus Season	54.11%	65.59%	0.018**
Specie versus Season	29.01%	46.76%	0.114*
Total identified families versus Season	20.02%	40.02%	0.184*

Evolutionary relationships of taxa (Interspecific analysis of Identified species by phylogenetic tree)

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions

reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA X.(Figure 7 & 8).

Genetic distances

The number of base substitutions per site between sequences are shown in figure 8. Analyses were conducted using the Kimura 2-parameter model. This analysis involved 32 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 575 positions in the final dataset. The overall genetic distance is calculated as 0.23%. Evolutionary analyses were conducted in MEGA X.

[1] *Helina cilipes* (BIOUG49179-C02) (BOLD:ACD1632), [2] *Lispe assimilis* (BIOUG48409-B10) (BOLD:AAZ1114), [3] *Lispe tentaculata* (BIOUG48392-G09) (BOLD:AAB8429), [4] *Musca domestica* (BIOUG48405-A03) (BOLD:AAA6020), [5] *Coenosia attenuate* (BIOUG49027-A03) (BOLD:AAD7633), [6] *Coenosia testacea* (BIOUG49063-H10) (BOLD:ACR4672), [7] *Atherigona orientalis* (BIOUG49056-D02) (BOLD:AAF5305), [8] *Dasyhelea incisurata* (BIOUG49251-G02) (BOLD:AAN5169), [9] *Culicoides imicola* (BIOUG49082-B09) (BOLD:AAB8379), [10] *Culicoides gejgelensis* (BIOUG49078-A01) (BOLD:ABX3080), [11] *Culicoides oxystoma* (BIOUG49056-A04) (BOLD:AAD1856), [12] *Corynoneura arctica* (BIOUG49179-B12) (BOLD:ACX4287), [13] *Cricotopus ornatus* (BIOUG43583-C01), [14] *Rheotanytarsus erignus* (BIOUG49027-B02) (BOLD:ACR3994), [15] *Chironomus kiiensis* (BIOUG49087-G01) (BOLD:AAD8162), [16] *Chironomus dorsalis* (BIOUG49029-G01) (BOLD:AAW4001), [17] *Chironomus transvaalensis* (BIOUG48891-H11) (BOLD:AAW3995), [18] *Psectrocladius schlienzi* (BIOUG49178-E12) (BOLD:AAU6327), [19] *Tanytarsus formosanus* (BIOUG49251-F01) (BOLD:ABY1131), [20] *Tanytarsus volgensis* (BIOUG43583-C02), [21] *Megaselia sepulchralis* (BIOUG49178-E10) (BOLD:AAG3235), [22] *Megaselia halterata* (BIOUG49027-H11) (BOLD:ABV8939), [23] *Liriomyza brassicae* (BIOUG49171-A02) (BOLD:AAF6806), [24] *Liriomyza sativae* (BIOUG49078-C02) (BOLD:AAF6797), [25] *Phytomyza thalictrella* (BIOUG49063-G06) (BOLD:ABX2176), [26] *Lycoriella sativae* (BIOUG43584-G09), [27] *Bradysia nomica* (BIOUG49056-D04) (BOLD:ABW7972), [28] *Bradysia trivittata* (BIOUG49178-F03) (BOLD:ABA6488), [29] *Bradysia ocellaris* (BIOUG49077-H09) (BOLD:ABW7969), [30] *Scatopsciara curvilinea* (BIOUG49171-D04) (BOLD:ACG1189), [31] *Corynoptera perpusilla* (BIOUG49171-F01) (BOLD:AAU6595), [32] *Apis mellifera* (OP034096)

Phylogenetic Inference (Interspecific Analysis of 32 species identified (including outgroup)

This study initiates the interspecific analysis, among 31 species belonging to six different families of the order Diptera and an outgroup to show the evolutionary history of Diptera. Phylogenetic tree was constructed by applying the Neighbour joining tree method. Kimura-2- parameter (K2P) model was used with 1000 bootstrap support. The cluster analysis revealed that all the clades were monophyletic. The monophyly of 31 species were arranged in six clades with a bootstrap value ranging from 7 to 99% and a basal branch formed the root of the phylogenetic tree. Dipteran families that were constituted for the observation of interspecific analysis were Muscidae, Ceratopogonidae, Chironomidae, Phoridae, Agromizidae and Sciaridae. Species included in the interspecific analysis *were L.assiimilis, C.attenuata, M. domestica, A.orientalis, C.testacea, L. tentaculata* and *H.cilipes* (Muscidae); *D.incisurata, C.imicola, C.gejgelensis, C.oxystoma (Ceratopogonidae); C.arctica, R.erignus, C.kiiensis, C.dorsalis, C.ornatus, C.transvalensis, T.formosanus, P.schlienzi, T.volgensis* (Chironomidae); *M.sepulchralis, M.halterata (Phoridae); L.brassicae, L.sativae, P.thalictrella (Agromizidae); L.sativae, B.trivittata, B.nomica, B.ocellaris, S.curvilinea, C.perpusilla (Sciaridae).* To examine the ancestor of diptera *Apis mellifera* (honey bee) belonging to order Hymenoptera is studied forming the root of the NJ tree and appeared as an outgroup at the basal branch of the tree. Hence, Diptera is among the most ancient pollinators of flowering plants and a Cretaceous era is a significant period for them in which angiosperms rose (Bertone et al., 2008). Flies are the second most important

pollinators to the bees and other hymenopteran relatives. Thus, Diptera is closely related to Hymenoptera. The first clade of the phylogenetic lineage arises from a family Ceratopogonidae (*C. imicola*) corresponding to the cluster of family Sciaridae with a bootstrap support ranging from 29% to 98%. The ancestral branch is corresponded to all the descendants of the order Diptera with strong to weak bootstrap support. High bootstrap values indicate uniform support. 100% bootstrap value suggested that all the significant characters are closely related in a group (Holmes, 2003). Poor bootstrap appeared on the nodes of the phylogeny indicated the fact that the data set might contains few argued characters (Soltis and Soltis, 2003). The evolutionary scale bar is 0.050%. All the species were accurately assigned to their locations with the same branch lengths (Figure 7 & 8).

Figure-7. Interspecific analysis of 31 species (with outgroup as a root On basis of Nucleotides sequences as a matrix

	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]	[21]	[22]	[23]	[24]	[25]	[26]	[27]	[28]	[29]	[30]	[31]	[32]
[1]																																
[2]	0.2																															
[3]	0.2	0.1																														
[4]	0.2	0.2	0.2																													
[5]	0.1	0.1	0.2	0.1																												
[6]	0.2	0.2	0.2	0.1	0.1																											
[7]	0.2	0.2	0.2	0.1	0.1	0.1																										
[8]	0.2	0.2	0.3	0.2	0.2	0.2	0.2																									
[9]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2																								
[10]	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.3																							
[11]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2																						
[12]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.2																					
[13]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.2																				
[14]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.2																			
[15]	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.3	0.3	0.3	0.2	0.2	0.2	0.2																		
[16]	0.2	0.3	0.3	0.2	0.2	0.3	0.3	0.3	0.3	0.3	0.2	0.3	0.2	0.2	0.2																	
[17]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2																
[18]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.3	0.2	0.2															
[19]	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2														
[20]	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.3	0.3	0.3	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2													
[21]	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2												
[22]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.1											
[23]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.3	0.2	0.2	0.2	0.2	0.2	0.2										
[24]	0.2	0.2	0.2	0.1	0.2	0.1	0.1	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.1									
[25]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.3	0.3	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.2								
[26]	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.3	0.2	0.2	0.3							
[27]	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.3	0.3	0.2	0.3	0.2						
[28]	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.3	0.2	0.3	0.2	0.3	0.2	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.2	0.2	0.3	0.2	0.2	0.2	0.2					
[29]	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.3	0.2	0.2	0.3	0.2	0.2	0.2				
[30]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.3	0.2	0.2	0.3	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2			
[31]	0.3	0.3	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.2		
[32]	0.4	0.3	0.4	0.3	0.4	0.4	0.4	0.4	0.3	0.4	0.3	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.3	0.4	0.3	0.4	0.4	0.4	0.4	0.4	0.4	0.4	

Figure-8. Estimates of evolutionary divergence between species sequences (including outgroup as root).

Discussion

The most reliable approach for classifying insect species has been taxonomy that makes use of morphological traits. But when knowledge is scarce and external characteristics are harmed due to inappropriate specimen treatment, morphological identification becomes difficult. Because of the diversity of the Order Diptera, it is challenging to identify species just by morphological means using taxonomic keys. As a result, an additional, broadly useful technique is required to supplement the current approaches for identifying insects. In current study we explored the applicability of mitochondrial cytochrome C oxidase subunit 1 (COI) gene-based DNA barcoding as an alternative tool beside morphology to identify true flies (Jinbo et al., 2011).

A new method for discovering and studying the evolution of various insect groups is DNA barcoding. By far, this study is the first attempt to retrieve DNA barcode information from a varied collection of Insects. Despite using a set technique to analyze samples from different insect families, sequence recovery success was good. This is a significant finding because it shows that even the most basic analytical procedures can effectively quantify the species diversity of all insect lineages. Based on the availability of taxonomic literatures, a thorough inventory of true flies from Pakistan's northernmost regions was created (Fatima and Yang, 2022) but could only reveal 16 families and 153 species of Azad Jammu & Kashmir and Gilgit-Baltistan. Such studies could be a base line for future studies of DNA barcoding in Pakistan.

The development and production of DNA barcode library of Diptera is detailed study of insects' barcoding in Quetta, Balochistan. All obtained 2195 sequences of dipteran flies, which were submitted to

BOLD, presenting 309 BINS (82 species). Cluster analysis revealed all BINs and species investigated produced a monophyletic cluster. A similar study in Germany has revealed the mitochondrial DNA records of 24 families (5200 BINS) of Diptera and submitted on BOLD. According to a study on insect inventories, nearly 75% of the total specimens of dipterans were collected in Sweden using Malaise traps (Karlsson et al., 2020), which is like our study.

Diptera is a diverse order in the world of insects. In current study identification of all dipteran specimens were further identified into 41 families while a similar study in Germany. Dipteran diversity revealed 111 families, which contains 40,753 individual records (Kotrba, 2020; Braukmann et al., 2019). DNA barcoding has also been used in different similar studies specific families within Diptera. Such as Ståhls et al. (2009) used COI barcodes to identify hoverflies (Syrphidae) on Lesvos Island, Greece, and found that DNA barcoding was effective in distinguishing between different species. Silva et al. (2013) analyzed the applicability of DNA barcodes for delimiting species in the genus Labrundinia within the family Chironomidae and found that it was an excellent tool for species identification and resolving taxonomic conflicts used DNA barcodes to uncover potential species within the *Tanytarsus curticornis* and *Tanytarsus heusdensis* species complexes in the family Chironomidae (Lin et al., 2018).

Muscidae was the most dominant diversified family (283 specimens) of the total count. (Mazumdar et al., 2021) leading to Cecidomyiidae (N=184), Certopognidae (N=164) Chironomidae (N=134), Phoridae (N=132) and Agromyzidae (N=108). All families have more than 100 specimens (Michelle et al., 2021). Our results indicate that diversity for 5 major Dipteran families (Muscidae, Cecidomyidae, Ceratopognidae, Chironomidae, and Agromyzidae) were mostly higher in number of specimen catch in General barcoding (Brandon-Mong et al., 2018). Primer binding errors may be the cause of low sequence and species recovery in certain families. These mistakes may cause the species richness of insect groups with poor recovery to be underestimated. (Hebert et al., 2016)

Other variables could also reduce the ability of barcodes to distinguish between species, which would impact diversity estimations. Examples of these variables include poor lineage sorting (Mallo and Posada, 2016), recent speciation, and co-amplification of pseudogenes such as Wolbachia (Smith et al., 2012). Detail of 31 species (belonging to the most abundant families) their scientific names, morphological description, matching BINs on BOLD and its Occurrence (Recent identification and Pakistan status acc to https://www.gbif.org) has been shown in Table 2.

According to the temporal distribution of this study, Venn diagrams provided an efficient way to visualize the intersection of BINs between consecutive months Nazareth et al. (2016) and clearly showed 17 species during duration of 3 months average such as January to march (Venny, 2.1) in April to June, we observed total 40 species, July to September 53 species were obtained while October to December showed 27 species which is similar to (Plant et al., 2011). The abundance and variety of insects gathered for our investigation changed over the course of the collecting period. Temperature increases from March to June and decreases from September to December were correlated with the trend of larger and more varied catches earlier in the year compared to the later months. (Ashfaq et al., 2018; Geiger et al., 2016; Brandon-Mong et al., 2018).

Both the nucleotide sequence and the specific nucleotide percentage have been analyzed in the current study since these elements are crucial for understanding the diversity across various species. All samples were examined to determine the average percentage of each nucleotide for the COI gene segment under study. This demonstrates conclusively that the nucleotide sequences have a larger A+T content than G+C. Insect mt DNA is distinguishable by its higher A+T frequency (Lunt and Hyman, 1997). All BINs were identified from which one 138 were singletons, while 171 were concordant BINs with record counts (Chonticha and Pairot, 2019).

Current research revealed a significant interaction between BINs and morpho-species without any BIN differences. Most species were discovered to be BIN concordant, meaning that all the barcodes from specimens of these species combined to generate a single BIN that contained only barcodes from that species. There was no BIN discordance (Hawlitschek et al., 2017).

This study initiates the interspecific analysis, among thirty-one species belonging to six different families of the order Diptera. Phylogenetic tree was constructed by applying the neighbor joining tree method. Kimura-2- parameter (K2P) model was used with 1000 bootstrap support. The cluster analysis

revealed that all the clades were monophyletic. High bootstrap values indicate uniform support. Nearly all the characters significant for a group agree that it is a group if the bootstrap value of that group is close to 100% (Holmes, 2003). Zero percentage bootstrap appears on the nodes of the phylogeny, considering the assumption that the data set contained few characters that were in dispute. Bootstrap values are low due to the few characters that each node is supported by Soltis and Soltis (2003) the evolutionary scale bar is 0.050%. All the species were accurately assigned to their locations with the same branch lengths.

Beside taxonomic identification, DNA barcoding has proven to be a valuable tool in the study of Diptera, allowing for the identification and discovery of species within this diverse insect order. It has been used successfully in various studies to identify overlooked species, resolve taxonomic conflicts, and uncover potential new species. The current survey represents a first step towards building an inventory for the insect fauna of Quetta, Pakistan. However, challenges and limitations still exist, and further research is needed to improve the accuracy and applicability of DNA barcoding in Diptera.

Conclusion

The genetic variations and the construction of NJ tree assess the DNA Barcoding effectiveness to specie-level recognition of the order (Diptera). DNA Barcoding is a successful method for the exploration of unstudied species of Pakistan. This study recommended DNA barcodes for distinguishing organisms. Additional attempts are required to make the barcode library more accessible.

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Contribution of Authors

Ahmed HA: Conceived idea, conducted the experiment, collected & analyzed data and prepared the final draft for submission
Ahmed N: Analyzed the collected data and revised the first draft
Ahmed SS & Saddozai S: Contributed in draft preparation
Rais A: Helped in manuscript writing and revisions
Sani IA & Shahid D: Helped in data analysis
Khan S: Literature review, final formatting, proofreading and manuscript submission

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