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Research Article

DNA Barcoding Expands Reference Libraries through the Discovery of Novel Muscid Species in Quetta, Balochistan, Pakistan

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ABSTRACT

Muscid flies are biological vectors of several disease pathogens and are medically and forensically significant insects. This study presents a preliminary molecular assessment dataset comprising 2,195 specimens collected during the sampling period, including a subset of 283 specimens from the family Muscidae, representing 6 genera and 7 species across 3 subfamilies, resolved into 22 Barcode Index Numbers (BINs; 4 unique and 18 non-unique). The mitochondrial gene cytochrome c oxidase subunit I (COI; 658 bp) was employed to examine the genetic diversity among the seven muscid species. *Coenosia attenuata* was identified as the most common species, representing 83% of the collected specimens. According to barcode analysis, the maximum conspecific divergence was 1.43%, whereas divergence from the nearest neighbour species ranged from 7.41% to 13.55%. DNA barcoding of fly species showed a divergence of <2% compared to sequences available in the Barcode of Life Data Systems (BOLD), and a significant difference was observed between maximum intraspecific and minimum nearest-neighbour distances across all seven species. Neighbour-Joining cluster analysis demonstrated that all species formed monophyletic clusters supported by high bootstrap values. The dataset identified 22 BINs, with 7 species matching Barcode Index Numbers (BINs), including 5 novel BINs (BOLD IDs: ADZ8375, AEA1946, ADZ9134, AEA2865, AEA0284), reported here as new country records for Pakistan. Moreover, these results highlight the significance of COI gene sequencing in exploring insect diversification and contribute novel information to the DNA barcode reference library of a highly diverse and under-studied insect order.

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Introduction

Diptera, commonly known as true flies or two-winged flies, is a highly diverse order of Arthropoda with over 125,000 identified species globally; many of which play very important roles in animal health and environment (Bezeng et al., 2017). Muscid flies perform important ecological roles such as decomposition, predation and pollination, thus, are widely considered as agricultural and veterinary pests (Rochon et al., 2021). They include significant blood-feeding parasites, biological vectors of various disease agents and the species that parasitize domesticated animals and wildlife (Moon, 2019). The flies are medically and forensically important insects at worldwide, since the adult act as mechanical vectors and intermediate hosts to transmit various disease pathogens like *Enterocytozoon bieneusi* Desportes, *Encephalitozoon* spp., *Coxiella burnetii* Derrick and *Thelazia* spp. in cattle, dogs, rabbits, sheep and goats (Baldacchino et al., 2013); while larvae of house fly (*Musca domestica Linnaeus*) cause myiasis in humans and animals (Dehghani et al., 2012). Flies obtain the microbes on their surface by contact or while feeding on the animal waste, wounds and exudate and transfer to other animal by physical contact or by dislodging on fly grooming (Jacques et al., 2017). The bacteria like *E. coli* (O157:H7) even survive and proliferate in the mouthparts of these flies before being transmitted to livestock (Kobayashi et al., 1999). Moreover, the age of the larvae found on deceased bodies is considered as forensic indicator in estimating the interval between the death and post-mortem (Achint and Singh, 2021).

Genetic identification techniques like DNA barcoding have the potential to enhance the precision in the identification of plant, vertebrate and invertebrate animal species over traditional morphological methods (Sweeney et al., 2011). Moreover, it also facilitates in discovering new and cryptic species (Hajibabaei, 2012). Molecular characterization of animal hereditary resources using DNA barcode is crucial to reveal the genetic composition of the animals, understanding population structures, estimating their genetic parameters and securing the genetic patents (Montalvo-Sabino et al., 2022). The technology of short DNA segments based species identification was introduced in 2019 (DeSalle and Goldstein, 2019) that has been effectively applied in taxonomy, evolutionary biology and biodiversity research (Dmitrović et al., 2022; Koblmüller, S., 2023 Odah et al., 2023). Although, the mitochondrial gene cytochrome C oxidase subunit-I (COI) gene has proven as useful in the identification of dipterans of medical and veterinary importance such as black flies (Pramual and Adler, 2014; Onder et al., 2019), mosquitoes (Sumruayphol et al., 2016; Vadivalagan et al., 2017), sand flies (Contreras et al., 2014; Nzelu et al., 2015; Lozano-Sardaneta et al., 2020), tabanids (Morita et al., 2016; Nitiyamatawat et al., 2017; Changbunjong et al., 2018), haematophagous flies (Changbunjong et al., 2020) and stomoxys flies (Changbunjong et al., 2016), the Muscidae family has been studied very little from the perspective of DNA barcoding (Moon, 2019). Only few published reports used the COI gene for phylogenetic analyses including research on 17 species and three out-group species in genus *Thricops*, using COI, COII and tRNA leucine genes (Savage et al., 2004), four mitochondrial (12S, 16S, COI, Cytb) and four nuclear (18S, 28S, Ef1a, CAD) genes to understand the relationships in Muscoidea (Kutty et al., 2008). Simialry, COI was used to identify the forensically important fly species in China (Samerjai et al., 2019, Guo et al., 2014). All these studies included a limited number of species, each represented by a single individual, hindering a thorough species evaluation, limiting the analysis to closely related taxa as well as limiting the detailed calculation of intra-specific distances.

To address the challenges and limitations associated with the taxonomy of Muscidae, the present study employs molecular approaches for the identification of different Muscidae species using DNA sequences. Accordingly, this study aims to develop an innovative method for accurate species identification and the assessment of biodiversity among flies and other insect taxa. The resulting barcode library will serve as a valuable reference for future environmental DNA (eDNA) and metabarcoding studies. Therefore, this study represents the first comprehensive report based on the COI gene for the identification and classification of medically and economically important muscid flies.

Material and Methods

Study site

The research was conducted in Quetta (30°19′09.75″ N, 66°96′12.32″ E) and Baleli (30°16′41″ N, 66°54′34″ E). Quetta is the capital and largest city of Balochistan Province. It is the 10th largest city in the country, with a population exceeding 1.1 million as of 2017. Located in the southwest of Pakistan, Quetta is surrounded by

mountains on all sides. It lies at an average elevation of 1,680 meters above sea level and is recognized as the only major high-altitude city in the region, receiving an average annual rainfall of 212.9 mm.

Quetta is commonly referred to as the "Fruit Garden of Pakistan" due to the abundance of fresh and dried fruit orchards in its vicinity. The region experiences a cold semi-arid climate, characterized by a substantial temperature variation between summer and winter. The area supports diverse wildlife, including wild sheep, leopards, wolves, rabbits, hyenas, and wild cats. Avifauna in the region includes partridges, pigeons, eagles, hawks, vultures, and sparrows.

Collection of samples

A total of 2195 dipterans were collected via malaise traps (Renaud et al., 2012) at various study sites at BUITEMS from June 2017 to May 2018. This period was chosen to capture a maximum diversity of insects across different seasons of the region throught out the year. All specimens were immediately suspended in 95% ethanol in a 96-well microplate and stored at -20^{o}C until further analysis. The flies were distinguished from other insects based on their characteristic morphological features (Nihei and de Carvalho, 2009) followed by labelling with a code for molecular identification (Staubli et al., 2002). The specimen data including sequences, images and the collected information have been made accessible on the Barcode of Life Database (BOLD) under the project Global Malaise Trap Program of Centre of Biodiversity Genomics, Ontario, Canada.

Morphological identification of flies

The collected fly specimens were morphologically identified and segregated based on distinct anatomical characteristics using a Leica dissecting stereomicroscope, following the procedures described in our recent study (Ahmed et al., 2024). Identification was carried out using standard taxonomic keys and diagnostic features commonly employed for dipteran classification.

The identification of the collected specimens was based on a suite of morphological characters typical of the group. These included overall body size and shape, particularly the morphology of the abdomen, as well as coloration patterns of the head, thorax, and abdomen. Additional distinguishing features included the color and structure of the tarsi and palpi, and the morphology of the antennae (Dawah et al., 2020; Parchami-Araghi et al., 2020). Special attention was given to the presence of a plumose arista, which is a key diagnostic trait in many fly taxa.

Further taxonomic differentiation was achieved by examining the presence and arrangement of bristles (setae), including the characteristic frontal orbital setae and the presence of parafrontal setae in females, either confined to the upper half or extending along the entire length of the parafrontalia. The structure of the proboscis, typically retractile and flexible, was also considered. Wing venation patterns, particularly the sinuous subcostal vein and the configuration of vein R4+5, were carefully observed. In addition, the morphology of the lower calypter, which is often glossiform or posteriorly enlarged, and the presence of a setulose anepimeron were used as important diagnostic features.

Moreover, the presence of a calcar, defined as a posterodorsal seta on the hind tibia, was recorded as a distinguishing characteristic (Nihei and de Carvalho, 2009; Couri and Pont, 2018). These combined morphological traits enabled accurate identification and classification of the collected fly specimens to the appropriate taxonomic levels.

DNA extraction and sequencing

DNA extraction, PCR amplification and sequencing were performed at Canadian Centre for DNA Barcoding (CCDB), Centre for Biodiversity Genomics, Guelph, Canada by pursuing the published protocols (Ivanova et al., 2012; Hebert et al., 2018). DNA was extracted from legs of collected insect specimens, to avoid any possibility of amplification of DNA from endosymbiotic microbes present in gut of insects, which could result into incorrect interpretation of the data. The extracted DNA was quantified by 2% agarose gel electrophoresis (Ali et al., 2014a; 2014b) and amplification of the COI gene (Hebert et al., 2004) was performed by using specific set of primers (Ren et al., 2018; Hosseini-Chegeni, 2019) sequenced as:

LepF1: ATTCAACCAATCATAAAGATATTGG

LepR1: TAAACTTCTGGATGTCCAAAAAATCA

(available at: http://www.dnabarcoding.ca/CCDB_DOCS/CCDB_PrimerSets.pdf).

The PCR reaction was performed according to the manufacturer's instructions, as described previously (Dupain et al., 2016; Urbinati et al., 2016). The amplicons were separated using a 2% agarose E-Gel 96 system (Invitrogen Inc.) via gel electrophoresis, as described by Ali et al. (2014a, 2014b). Sequencing was carried out using SMRT sequencing on a Sequel system (Pacific Biosciences, Menlo Park, CA, USA), and the resulting sequences were submitted to the BOLD database to assign Barcode Index

Numbers (BINs) and to develop a reference library, as previously described (Ahmed et al., 2024). For phylogenetic analysis, the sequences were analyzed using MEGA-X (Tamura et al., 2011; Asghar et al., 2024).

Data analysis

For identification and discrimination purposes, the obtained sequences were compared to GenBank/BOLD sequences by using BLAST (Basic Local Alignment Search Tool) on NCBI (National Center for Biotechnology Information) databse. In BOLD, each species has been assigned a unique Barcode Index Number (BIN), as described earlier (Ratnasingham and Hebert, 2013). The sequence alignment of seven sequences was performed on MEGA-X using CLUSTALW algorithm. The neighbour joining (NJ) tree with a bootstrap of 1000 replicates based on Kimura-2-Parameter (K2P) model was constructed and genetic distances were calculated with pair-wise deletion in MEGA-X. The sequences were submitted to BOLD for analysis using barcode gap, accumulation curves, rank distances and histograms using BOLD. The web version of Automatic Barcode Gap Discovery (ABGD) was used to construct distance ranks and distance histograms, by uploading the FASTA file. A barcode gap was identified as a measure of the accuracy of discrimination for each specimen. According to the barcode gap analysis, a species was considered distinct from its nearest neighbour (NN), if the maximum intra-specific distance was less than the distance to its NN sequence. The Barcode Gap Analysis (BGA) verifies that the greatest sequence divergence within individuals of a species or BIN must be less than the distance to the species or BIN's Nearest-Neighbour species, facilitating their clear differentiation.

Results

All identified muscid flies (n = 283) belonged to seven species representing six genera and four subfamilies of the family Muscidae. Among these, *Coenosia attenuata* (Stein, 1903) was the most prevalent species (81.3%), followed by *Lispe assimilis* (4.2%), *Musca domestica* (4.2%), *Helina clipes* (4.2%), *Coenosia testacea* (2.1%), *Lispe tentaculata* (2.1%), and *Atherigona orientalis* (2.1%) within the Muscidae dataset (Figure 1).

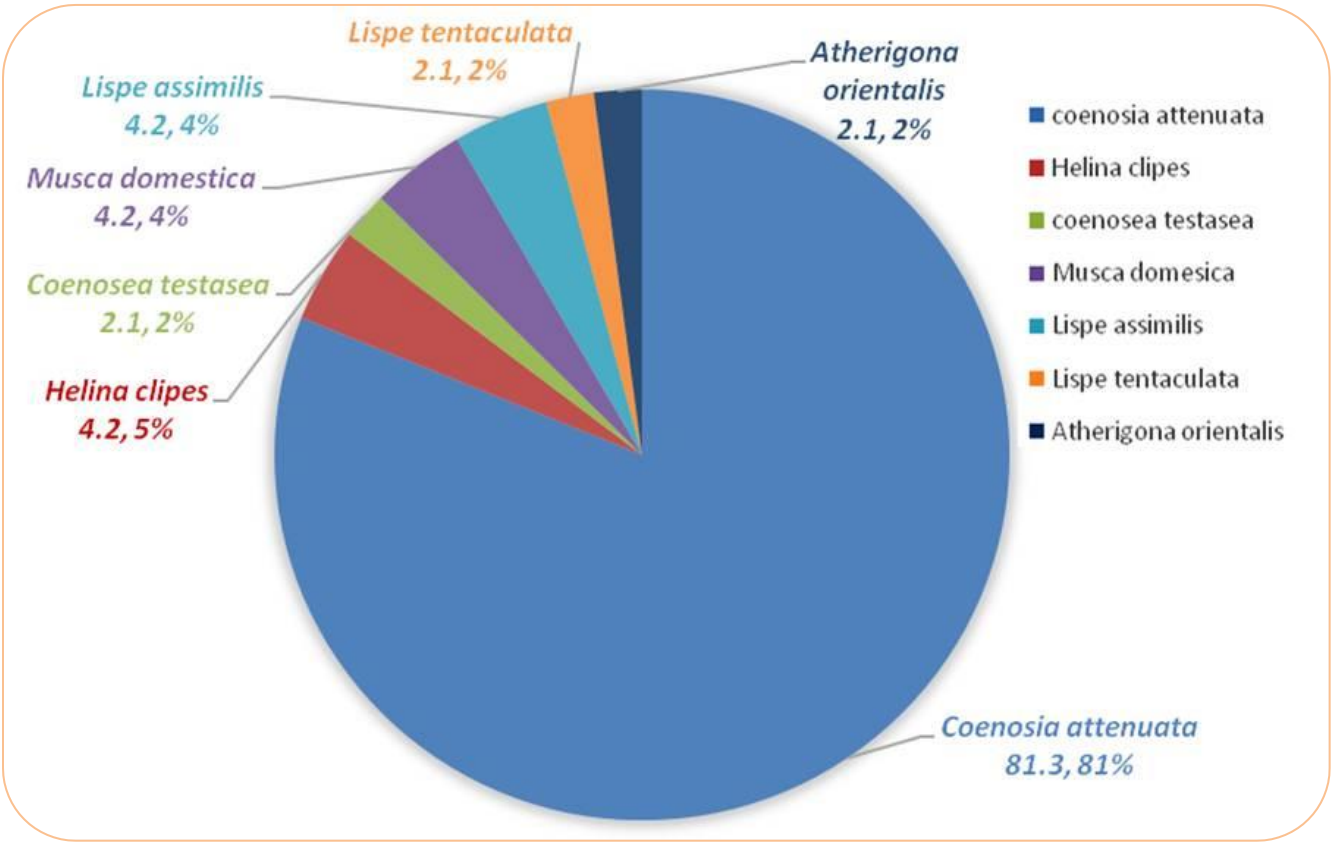

Figure 1. Pie chart of distribution of different fly species of family Mucidae. *Coenosia attenuata* has been found to be the most dominant (81.3%) among all the studied species.

Morphological characteristics for the identification of muscid flies

Male individuals of *C. attenuata* were characterized by predominantly yellow legs, whereas the head and body were silvery-grey; females were predominantly brown. In both sexes, the antennae, palpi, and femora were yellow, and the cercal plate was distinctly elongated.

L. assimilis (Wiedemann, 1824) exhibited a ground-colored black head with a yellowish-white fronto-orbital plate. The antennae were black, except for the orange tip of the pedicel. The scutum and scutellum appeared yellowish-grey and dusted. The fore tibiae were dull yellow on the basal third, while the mid and hind tibiae were mainly orange-yellow, darkening only at the tips. The vein R_{4+5} was slightly curved forward before the apex.

In *M. domestica* (Linnaeus, 1758), the thorax displayed four narrow black longitudinal stripes, and the fourth longitudinal wing vein showed a distinct upward bend. The basal portion of the abdomen was yellow, particularly along the lateral margins. Males generally exhibited more extensive lateral yellow coloration than females. A dark longitudinal band was also present along the median dorsal region of the anterior abdominal segments.

H. clipes (Robineau-Desvoidy, 1830) possessed five sternites and occasionally exhibited strong, long setae, along with a short or elongated cercal plate. The katepisternal setae were arranged in an equilateral triangle, and the mid tibia lacked an anterodorsal seta. The surstylus could bear spines, and the wings showed dark clouding.

C. testacea (Robineau-Desvoidy, 1830) was characterized by black antennae; males had entirely yellow palpi, whereas females had apically darkened palpi. The legs were yellow, the tarsi entirely black, and the scutum bore two brown longitudinal stripes. Only one proepisternal seta was present. Males exhibited a cylindrical abdomen, with the first to third tergites yellow and the fourth to fifth tergites dark with paired spots. Females had an oval abdomen with entirely dark tergites bearing paired spots.

In *L. tentaculata* (De Geer, 1776), the scutum possessed only three strong dorsocentral setae and lacked a pruinose patch. In males, the fore tarsus ranged from yellow to dark in color, and the scutellum bore a few fine hairs ventrally at the apex. The tibiae were dark with distinctly yellow knees.

Adults of *A. orientalis* (Schiner, 1868) were small, yellowish-grey flies, with a body length of approximately 4 mm and a wing length of 2.5-3 mm. The head was nearly square in shape.

Genetic diversity of flies

The Barcode Index Number (BIN) system assigned 22 BINs (4 unique and 18 shared) to the 283 specimens analyzed (Table 1). Each specimen yielded a cytochrome c oxidase subunit I (COI) gene sequence of approximately 630 base pairs. Seven BINs corresponded to seven species recorded in BOLD, with the following process IDs: GMPQI019-19, GMPQA629-19, GMPQG010-19, GMPQC090-19, GMPQE1228-19, GMPQK046-19, and GMPQC094-19.

The barcode sequences were used to generate histograms of sequence divergence and ranked distance distributions (Figure 2a and 2b). These analyses revealed a clear barcode gap between intra- and interspecific variation (Table 2). Intraspecific divergence ranged from 0.0% to 1.43%, with a mean of 0.25%, whereas interspecific divergence ranged from 7.18% to 9.24%, with an average of 7.66%. Genetic distances within families ranged from 11.0% to 19.35%, with a mean of 14.81% (Table 3).

The mean and maximum distances to the nearest neighbor exceeded the corresponding intraspecific distances for all species (Figure 3). For each species, mean and maximum intraspecific divergences were compared with NN distances. Particular attention was given to cases where the NN distance was smaller than the maximum intraspecific divergence or where NN divergence was below 2%. However, barcode gap analysis confirmed that NN distances were consistently greater than maximum intraspecific divergences. The scatter plot further demonstrated that NN distances exceeded 5% for all species (Figure 3).

The species accumulation curve and BIN distribution across genera are presented in Figure 4, indicating ongoing species discovery at the sampling site. The curve did not reach an asymptote, suggesting that the current sampling was insufficient to capture the full diversity of muscid flies and that further investigation is required.

The mean nucleotide composition of COI sequences for the family Muscidae, as estimated using BOLD, was adenine (A) 29.22%, thymine (T) 39.23%, cytosine (C) 15.63%, and guanine (G) 15.92%. The A+T content (68.45%) was substantially higher than the C+G content (30.55%).

Table 1. The details of unique (n=04) and non-unique (n=18) BINs (n=22) of various species and genera of muscid flies on BOLD.

A. Unique Bins = 4 (Members = 05)			
BIN	Members	Taxa	Count in dataset
BOLD:ADZ8375	2	Muscidae	2
BOLD:ADZ9134	1	Muscidae	1
BOLD:AEA0284	1	Lispe	1
BOLD:AEA1946	1	Muscidae	1
B. Non-Unique Bins having 18 (Members = 20790)			
BIN	Members	Taxa	Count in dataset
BOLD:AAA6020	719	Musca domestica	2
BOLD:AAB8429	562	Lispe tentaculata	1
BOLD:AAD7633	2956	Coenosia attenuata	39
BOLD:AAF5305	262	Atherigona orientalis	1
BOLD:AAZ1114	535	Lispe assimilis	2
BOLD:ABW5492	8816	Lispe	15
BOLD:ABW5571	295	Lispe	68
BOLD:ABX0288	881	Atherigona	67
BOLD:ABX4632	2993	Atherigona	30
BOLD:ACA4002	29	Lispe	2
BOLD:ACD1632	49	Helina cilipes	2
BOLD:ACG0670	63	Limnophora	2
BOLD:ACJ5809	387	Atherigona	1
BOLD:ACK3262	1198	Atherigona	15
BOLD:ACP4053	153	Musca	5
BOLD:ACR4672	516	Coenosia testacea	1
BOLD:AEA1372	374	Musca	2
BOLD:AEA2865	2	Muscidae	1

Table 2: Barcode gap analysis of the flies of Muscidae family.

Order	Family	Species	Mean	Max	Nearest	Nearest neighbour	Distance
Diptera	Muscidae	*A. orientalis*	N/A	0	C. testacea	GMPQK046-19	11.00
Diptera	Muscidae	*C. attenuata*	0.27	1.43	*C. testacea*	GMPQK046-19	7.41
Diptera	Muscidae	*C. testacea*	N/A	0	*C. attenuata*	GMPQA265-18	7.41
Diptera	Muscidae	*H. clipes*	0	0	*C. attenuata*	GMPQA367-18	13.44
Diptera	Muscidae	*L. assimilis*	0.46	0.46	*L. tentaculata*	GMPQC094-19	8.97
Diptera	Muscidae	*L. tentaculata*	N/A	0	*L. assimilis*	GMPQA406-19	8.97
Diptera	Muscidae	*M. domestica*	0.62	0.62	*C. attenuata*	GMPQA265-18	13.55

Barcode gap analysis showed that maximum intra-specific distance for all species was less than its NN distance. N/A = not available, the species is a singleton.

Table 3. Percentage K2P sequence divergence at the COI barcode region among species

Label	n	Taxa	Comparisons	(%) Minimum distance	(%) Maximum distance	(%) Mean distance	(%) SE distance
Within species	45	4	744	0.00	1.43	0.25	0.00
Within genus	43	2	41	7.18	9.24	7.66	0.01
Within family	48	1	343	11.00	19.35	14.81	0.00

The above table compared the genetic distance with >2 specimens, among the 2 genera with two or more species and within 1 family with two or more genera.

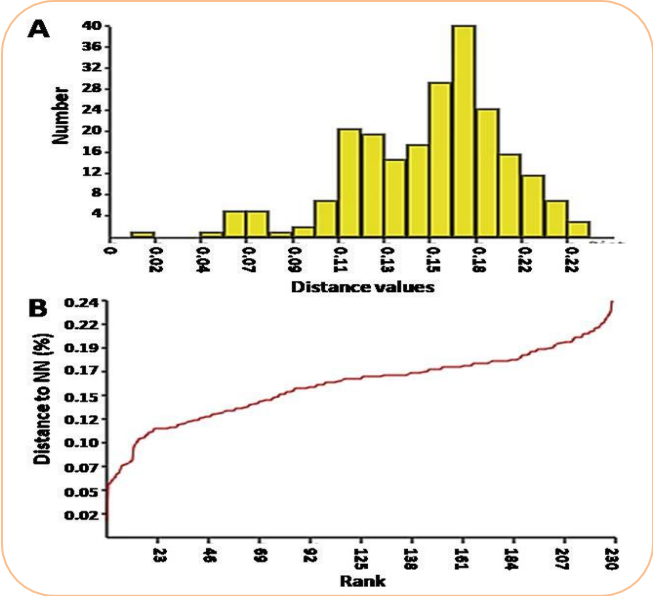

Figure 2. Histograms of the distances (A) and ranked (pair-wise) distances (B) among nucleotide sequences of 22 species. The barcode gap analysis for 22 BINs of Muscid flies from Quetta, Pakistan generated by using the tool Automatic Barcode Gap Discovery (ABGD).

Neighbor-Joining (NJ) tree and genetic distances of the family muscidae

A total of 50 sequences were selected for phylogenetic analysis of the mitochondrial COI gene (658 bp) to assess genetic diversity among muscid fly species. Evolutionary relationships were inferred using the Neighbor-Joining method based on the Kimura 2-parameter (K2P) model implemented in MEGA X software.

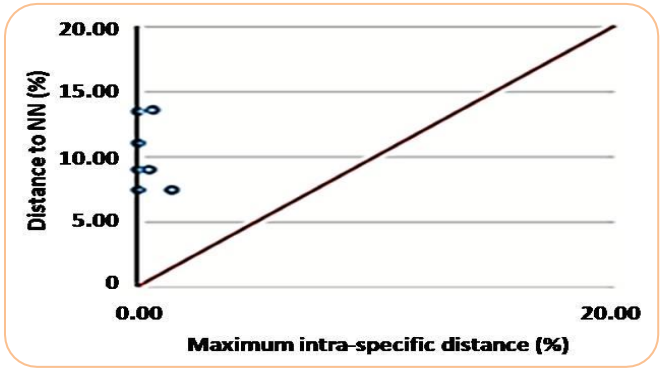

Figure 3. Scatter plot of the nearest neighbor (NN) distance of all the species. The plot shows that the NN distance was more than 5% of all species. The genetic divergence of all the flies increased with the ranking of taxonomy without overlapping between the conspecific and congeneric distances.

The phylogenetic relationships within the family Muscidae were reconstructed as an NJ tree, revealing distinct monophyletic groupings among the analyzed taxa (Figure 5). The dataset included representatives from six species and one genus: two sequences of *M. domestica*, two of *H. cilipes*, three of the genus *Lispe* (one *L. tentaculata* and two *L. assimilis*), forty specimens of *Coenosia* (39 *C. attenuata* and one *C. testacea*), and two species of the genus *Limnophora*.

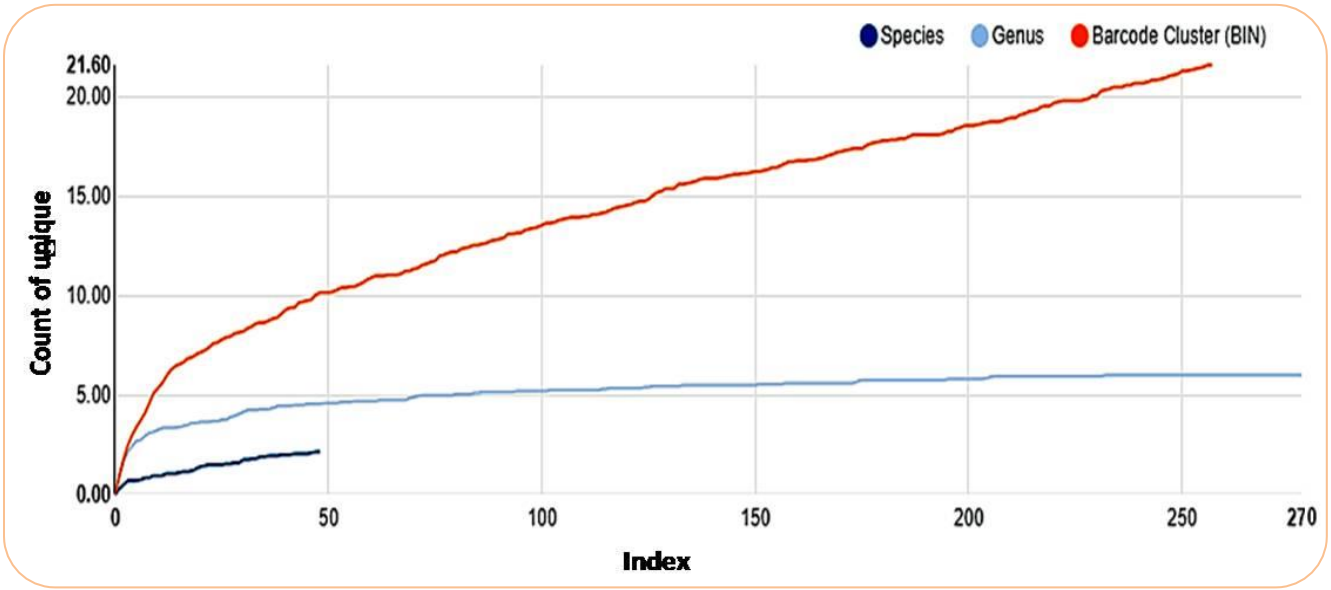

Figure 4. The accumulation curve of the family Muscidae. The accumulation curve failed to reach an asymptote, hence, muscid flies requires further investigations, as the present estimation is not being sufficient.

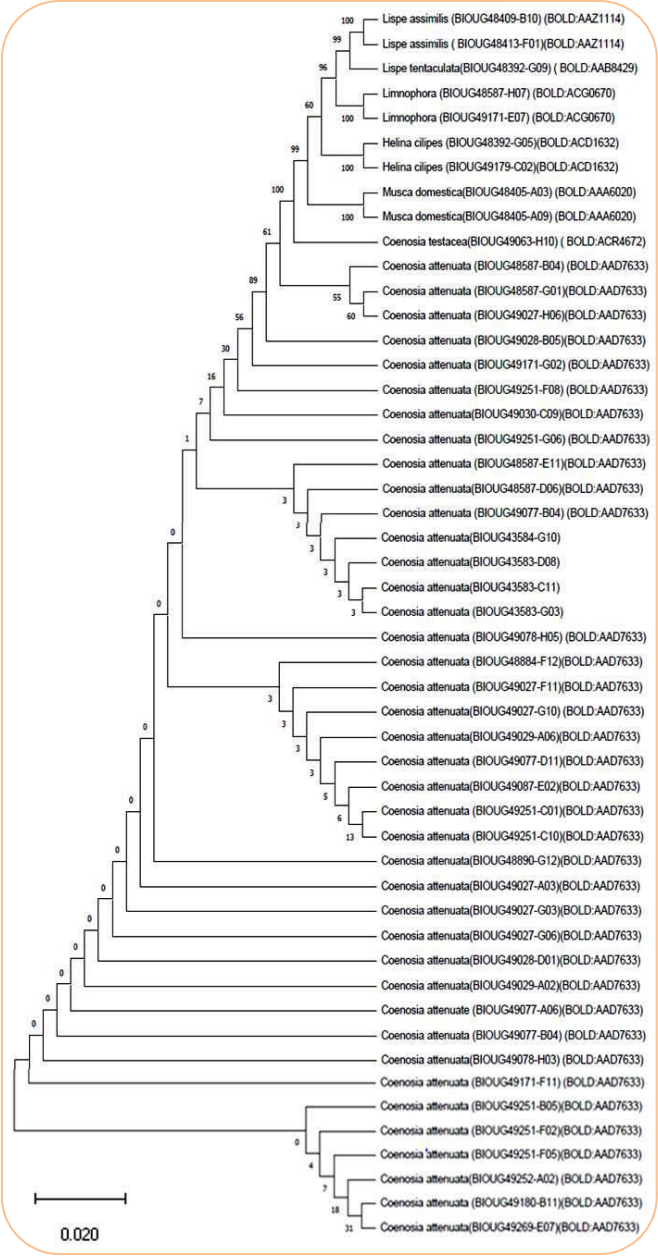

Figure 5. The origin of evolution of muscidae family as derived using NJ tree. 1000 bootstrap support reflects the association of analyzed flies and represents them in the clades. The bootstrap values are substituted outside the tree branches. Evolutionary divergence was calculated to be 0.020%. The genetic distances were examined on the K2P (Kimura-2- parameter) model and the analysis was done on MEGA X.

The phylogeny was primarily structured around *C. attenuata*, which formed the dominant lineage. *C. testacea* showed clustering patterns relative to *M. domestica*, with bootstrap support values ranging from low (0%) to high (100%) across *C.* specimens. Most other taxa appeared as descendants within the broader *C.* lineage.

M. domestica clustered closely with *H. cilipes* and members of the genus *Limnophora*, with moderate to high bootstrap support (60-100%). The genera *Helina* and *Limnophora*, belonging to the same subfamily, grouped together as expected.

The uppermost clade consisted of the genus *Lispe*, where *L. tentaculata* was closely related to *L. assimilis*, supported by 100% bootstrap value, with an evolutionary divergence of 0.020%. Intraspecific variation across all examined taxa ranged from 0.01% to 0.20%, with an overall mean genetic divergence of 0.05.

Discussion

Muscid flies cause skin injuries, hamper growth, and transmit pathogenic viruses, bacteria, helminths, and cestodes; thus, they are significant factors affecting the health and economics of wildlife and domestic animals (Moon, 2019). For species identification and evolutionary research across different insect orders, DNA barcoding is considered one of the most advanced techniques. As part of this research project, we recently reported 82 identified fly species belonging to 41 families of the order Diptera out of 2,195 collected specimens (Ahmed et al., 2024). In the current study, we have described and identified species of the family Muscidae, among which five are reported for the first time in Pakistan. *C. attenuata* was the most abundant species in the family Muscidae, likely reflecting the natural population structure and ecological dominance of this species in the sampling area rather than sampling bias.

The study focused on the creation of a barcode library for muscid flies (Diptera) collected from Quetta (Pakistan). Based on DNA barcoding, the specimens showed similarities with flies from China (8%), India (12%), and Bangladesh (13%) (Ashfaq et al., 2022). Nine species of the Australian blowfly genus *Chrysomya* (Robineau-Desvoidy, 1830), namely *C. cisuralis, C. varipes, C. semimetallica, C. saffranea, C. rufifacies, C. nigripes, C. megacephala, C. latifrons,* and *C. flavifrons*, were identified through COI gene sequencing (Kim et al., 2021). Likewise, the common bottle fly (*Lucilia sericata*) has been identified in India (Archana et al., 2016), and sand flies comprising 12 species under two genera, *Phlebotomus* and *Sergentomyia* (Psychodidae: Phlebotominae), were identified in Peru (Nzelu et al., 2015), Colombia (Gutiérrez et al., 2014), and Brazil (Pinto et al., 2015) using genetic

markers, wherein *Phlebotomus argentipes* appeared as the predominant species. The same techniques facilitated the identification of flesh flies, i.e., *Sarcophaga tibialis* (Macquart, 1851) and *S. cultellata*, in Spain (Arnaldos et al., 2015), whose immature stages were used in forensic investigations to estimate the post-mortem interval (Meiklejohn et al., 2011).

The capability to discriminate intra-specific and inter-specific variations is key to DNA-based species recognition methods. Extensive taxonomic sampling enhances intra-specific distance estimates while reducing inter-specific divergence bias. For the COI gene to serve as a valid identifier, intra-specific divergences must be <3%, and inter-specific divergences must be >3% (Wells and Sperling, 2001). In this study, intra-specific divergences ranged from 0.0% to 1.43% with a mean of 0.27%, while divergences among species within a genus ranged from 7.41% to 9.24% with a mean of 7.70%. COI sequence variation across congeneric species was nearly ten times higher (maximum 9.24%) than within-species differences. The mean intra-specific distance observed in the current study (0.27%) and the maximum value (1.43%) are consistent with previous reports (Sehrawat et al., 2014), which documented intra-specific sequence divergences ranging from 0 to 1.54%. Moreover, studies on 13 forensically important dipteran fly species in Germany reported intra-specific variation ranging from 0 to 1.17% and inter-specific variation between 1.17% and 15.21% (Boehme et al., 2012). However, a relatively high mean intra-specific divergence of 2.5% was recorded in *Halyomorpha picus* (Kaur and Sharma, 2017), while a proposed threshold of 3% intra-specific divergence has been suggested for species identification in mayflies (Blažek et al., 2017; Suh et al., 2019). Similarly, inter-specific divergence ranging from 3% to 13% and intra-specific divergence from 0% to 2% were reported among several Chinese muscid fly species (Xiong et al., 2012), whereas 33 muscid species from China exhibited intra-specific divergence ranging from 0% to 7.4% and inter-specific divergence from 4.7% to 17.7% (Ren et al., 2018). Comparable low intra-specific genetic divergence values were reported in birds (0.27%) (Dalén et al., 2017), marine fish (0.39%) (Ude et al., 2020), mayflies (0.11%) (Suh et al., 2019), and bats (0.06%) (Mota et al., 2022). Likewise, a mean intra-specific distance of 0.4% was reported in true bugs (Jung et al., 2011). The difference between the highest intra-specific genetic distance and the lowest inter-specific genetic distance is referred to as the barcoding gap (Chapple and Ritchie, 2013), which was observed in the present study and is consistent with previously reported findings (Mongkolphan et al., 2023).

Of the 22 Barcode Index Numbers identified in the dataset of 283 flies, eight were singletons (with eight record counts) and 14 were concordant BINs. A strong correspondence between BINs and morpho-species, without any BIN discordance, was observed in the present analysis. The Barcode Index Number system (Ratnasingham and Hebert, 2013) represents a significant development, as BINs provide strong evidence for the genetic distinctiveness of species. In this study, a distinct BIN was assigned to all 22 members belonging to various species and genera, confirming a pattern observed in other taxa (Table 1). In the current study, Kimura 2-Parameter (K2P) divergences were <2% in all 50 flies from Quetta (Pakistan), with a distance range of 0.01-0.20%. Muscid fly species were clearly separated and clustered with high bootstrap support values (>95%), consistent with previous findings (Achint and Singh, 2021). Bootstrap values approaching 100 indicate strong support for groupings, whereas values near 0 suggest weak phylogenetic signal or insufficient character resolution (Soltis and Soltis, 2003).

Despite the relatively small sample size, the results highlight the utility of DNA barcoding in identifying muscid flies from the previously unexplored region of Quetta, Pakistan. The average nucleotide composition of the studied COI gene fragment was A (29.22%), T (39.23%), C (15.63%), and G (15.92%), indicating that A+T content was higher than G+C. A higher A+T frequency is a characteristic feature of insect (Diptera) mitochondrial DNA, as reported previously (Zhang et al., 2021). Similarly, analyses of mitochondrial COI genes across various Arthropoda classes have consistently revealed AT-rich nucleotide compositions (Barbhuiya et al., 2020). The species accumulation curve (Figure 4) failed to reach an asymptote; this non-asymptotic trend indicates that the current sampling effort did not fully capture the complete diversity of muscid flies in the region, emphasizing the need for extended sampling across additional locations and seasons.

Conclusions

Our results demonstrate that a well-populated reference library not only facilitates the association of conspecific specimens and the detection of identification errors that may exist in taxonomic work, but also contributes to the

taxonomic workflow by discovering morphologically distinct taxa. Our findings further demonstrate the effectiveness of DNA barcoding in identifying muscid flies from a previously unexplored location in Quetta, Pakistan, despite the limited sample size in this study. The results of DNA barcoding in the current study, supported by the authentication of modern genome sequencing, strengthen the database library of organisms. This research encourages the molecular recognition and classification of organisms according to their eco-morphological and geographical factors. The current dataset provides a useful reference for further work and will serve as a foundation for investigations into community ecology, species interactions, and large-scale faunal shifts in the order Diptera associated with climate change. These opportunities highlight the importance of thorough investigations of target taxa in conjunction with the common goal of utilizing focal geographic regions and different standardized markers to better understand biodiversity.

Notably, the study has uncovered five novel species that have never been reported previously in Pakistan, showcasing the potential of DNA barcoding to reveal undocumented biodiversity within muscid flies. Moreover, the research highlights a higher A+T content characteristic of insect mitochondrial DNA, reaffirming the conserved nature of the COI gene across diverse taxa and thereby contributing significantly to the development of a comprehensive barcode reference library, which is essential for enhancing the accuracy of environmental DNA and metabarcoding studies. The establishment of such a library would be a critical step in documenting the biodiversity of diverse taxa in under-studied regions and, consequently, facilitating future ecological and conservation research. Future studies will encourage the deposition of DNA sequences, alongside morphological and ecological data, into publicly accessible databases such as BOLD and GenBank, which will significantly enhance the capacity of the global scientific community to identify and study muscid flies.

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Author's contributions

HAA conducted the experiments, performed the data analysis, and prepared the initial draft of the manuscript. NA conceived and supervised the research project, guided the experimental design, and critically reviewed and revised the manuscript for intellectual content. AR, SA, RU, and BM provided critical evaluation of the study. HMA and SSA contributed to the editing and refinement of the manuscript. AQ assisted in improving the scientific clarity and overall presentation of the work. All authors read and approved the final version of the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

Sustainable Development Goals Targeted

SDG 9: Industry, Innovation and Infrastructure
SDG 14: Life below Water
SDG 15: Life on Land

Policy Addressed

1. Convention on Biological Diversity (CBD, 1992)
2. Nagoya Protocol (2010)
3. Global Taxonomy Initiative (GTI)

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