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Cox-1 Gene-Based Identification and Molecular Phylogenetics of Horseflies

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Abstract— *The hematophagous flies of the family Tabanidae are involved in the transmission of various disease-causative agents such as protozoans, helminths, bacteria, and viruses. However, molecular research on this family is not conducted in Pakistan. This study seeks to investigate the molecular characterization of the horseflies, feeding on buffaloes of District Swat, Khyber Pakhtunkhwa, by targeting the mitochondrial partial Cox1 gene for the nucleotide identity/diversity and phylogenetic analysis. The Cox1 gene sequences were compared with NCBI databases using BLASTn. The analyses revealed two Tabanus species (Tabanus sp. 1 and Tabanus sp. 2) and one Atylotus species. Genetic comparisons found a close relationship: Tabanus sp. 1 (93.95%) to T. superjumentarius, Tabanus sp. 2 (94.55%) belong to T. bromius, and Atylotus sp. (93.97%) to A. agrestis. Species were identified at the genus level due to limited data availability. The nucleotide identity among the collected species was Atylotus sp. vs. Tabanus sp. 1 (90.7%), Atylotus sp. vs. Tabanus sp. 2 (89.4%), and Tabanus sp. 1 vs. Tabanus sp. 2 (90.1%). As expected, Tabanus sp. 2, clustered together with its congener, T. bromius forming a basal clade of all other Tabanus spp included in our phylogenetic analysis. This means that Tabanus sp. 2 and T. bromius have some similarities and both of them are from the Palaearctic region. However, Tabanus sp.1, though showing comparatively higher sequence identity with T. superjumentarius (94.55%) placed on a separate branch as a sister clade of the clade containing T. fontinalis and T. rubidus. T. fontinalis and T. rubidus are native to North America and their sequence identity with Tabanus sp.1 were 93.8% and 93.3%, respectively. Similarly, the phylogenetic tree placed our sequenced Atylotus sp. on a separate branch inside the clade uniting all Atylotus spp. included in our analysis containing A. agrestis as well. From our study, we concluded that the genus Atylotus is monophyletic while the Tabanus genus is may be polyphyletic or paraphyletic because as it does not include all descendants of a common ancestor. Molecular characterization of additional species of horseflies from Pakistani hosts will provide a clear image of their genetic inter-relationship and their phylogenetic affinities with other species recorded across the world. Such molecular study will provide a base for determining the prevalence of respective species and the development of their effective management strategies.*

Keywords— ***horseflies, PCR, phylogenetic tree, nucleotide identity and Cox-1 gene.***

I. INTRODUCTION

Horseflies are blood-feeding insects that belong to the Tabanidae family. Both humans and livestock suffer greatly from their itchy and painful bites. A range of infectious agents, including protozoa, helminths, bacteria and viruses, can be carried by and spread by these insects. Both veterinary and medical important must comprehend their part in the spread of disease [1].

The adult Tabanidae bug has to scrape and lick its mouthparts. It is a robust, muscular flyer. There are more than 20 families within Brachycera, with Tabanidae being the largest with about 4455 species spread across 144 genera. Some 1300 Tabanidae species are known to exist in the genus *Tabanus*. Horsefly females feed on blood by biting people and animals; this can cause anaemia and atopic dermatitis, among other health problems [2]. This family is found worldwide, with 415 species in Africa, 244 species recorded in India, 335 species in the Americas, 120 species in Malaysia, and over 100 species in Thailand [3]. Without a doubt, horse fly morphological traits aid in identification and classification. Nevertheless, depending solely on their morphological traits has some drawbacks [4]. Identifying dipteran species accurately can be challenging and potentially risky due to inadequate physical characteristics and taxonomic expertise. Consequently, molecular techniques such as DNA barcoding are commonly employed to facilitate species identification in this group[5]. The DNA barcoding of the mitochondrial COX-1 gene sequence enables the identification of rang arthropods, including tabanid flies [6]. This is more effective than morphological identification as this method allows genus/species level identification at any developmental stage, immatures or adults. DNA barcoding methods are widely adopted in bio-diversity studies owing to their ability for fast and accurate species identification [7]. DNA barcoding method PCR amplification of a target DNA of species. With DNA barcoding, the target species is identified by performing PCR amplification of their DNA[8]. The mitochondrial gene COX-1 is the most widely used target for this purpose, with the amplified region typically ~658 base pairs in size. This technique enables accurate species identification [9].

The horsefly family (Tabanidae) contains 78 species divided into 2 subfamilies, and 10 genera in Croatia (species identification is mainly based on morphology). The most diverse genera are Tabanus (19 species), Hybomitra (7 species) and other genera. Morphological identifications were confirmed through DNA barcoding using the COX-1 gene, with 16 new Barcode Index Numbers (BINs) added to the Barcode of Life Database (BOLD) [10].

Phylogenetics is a common method in molecular biology and evolutionary biology. It uses molecular data sequences of amino acids or DNA — to explain the evolutionary relationships among a set of organisms. The approach has gained prominence due to the technological explosion of DNA sequencing and the accessing of sequence information for genes and proteins through publicly available internet dbs. The selection of a certain molecule for a phylogenetic analysis is not arbitrary; molecules evolve in various ways and rates [11].

They are an ecologically, and medically significant group, and evolutionary relationships within the Tabanidae family are still poorly understood[12]. The family Scionini includes a total of seven genera and is primarily distributed through the Southern Hemisphere, most notably in Australasia and South America. Both Bayesian and maximum likelihood approaches show strong monophyly of the Scionini but not of the genus Goniops, which occurs in the northern hemisphere [13].

Expanding the Characterisation of tabanid mitogenomes is essential for species identification, phylogenetic studies, and epidemiological research. Utilising existing mitochondrial genome databases, we used Illumina sequencing for decode of the mitochondrial genomes of six horsefly species[14]. Subsequently, we analyzed their evolutionary relationships with other species in the Tabanomorpha infraorder. The newly sequenced mitogenomes exhibited a consistent 37-gene circular topology typical of Tabanomorpha. Phylogenetic analysis unveiled non-monophyletic relationships within the genus *Tabanus* of horseflies [15].

The Tabanidae family has not been studied in Pakistan, particularly at the molecular level. To bridge this gap, we collected tabanid samples from the Swat district. The objectives were: 1) to identify horseflies using COX-1 gene sequences, 2) to compare nucleotide identity between the identified horseflies and their congeners. and 3) to investigate the phylogenetic relationship among the identified horseflies and their congeners.

II. MATERIALS AND METHODS

2.1 Sample collection and study area

A total of 250 tabanid samples were collected from various districts in Swat KPK, Pakistan, including Barikot, Kabal, Matta Khwazakhela, Babuzai, and Bahrain. These tabanid samples were physically collected while they were actively biting livestock to obtain blood. This method proved effective as it capitalized on the insects' preoccupation with bloodsucking, making them easier to collect. The samples were washed with saline solution four times and were identified using a standard taxonomic key outlined by Oldroyd in 1954 and further elaborated upon by Cameron in 2014a.

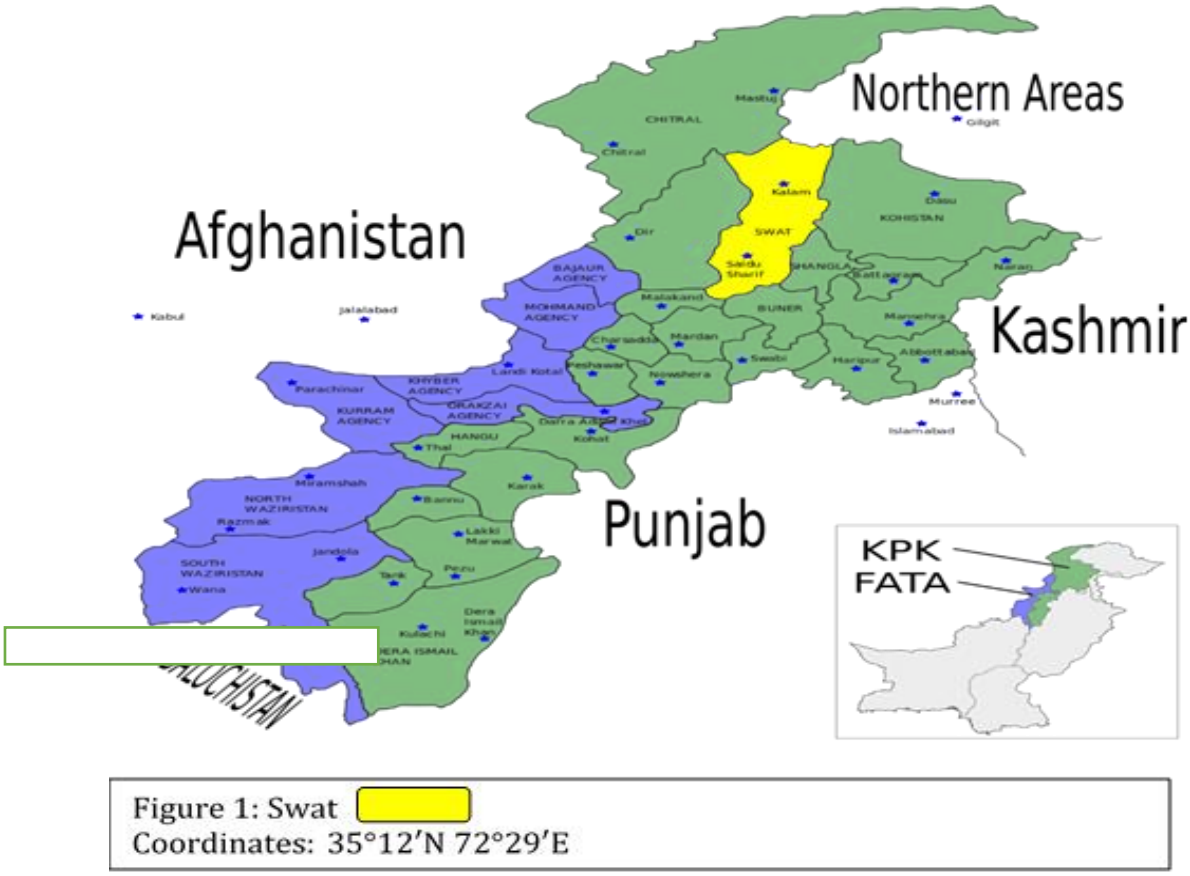

Fig.2.1. Map of study area (Swat).

2.2 DNA Extraction

Total genomic DNA was extracted from horse fly samples for molecular analysis using the Wizprep DNA extraction kit. After the material has been cleaned, extract genomic DNA using the Wizprep DNA extraction kit. To ensure a successful DNA extraction, strictly adhere to the kit instructions. Reagent addition, temperature incubation, and DNA purification are the steps. Make sure that the prescribed sequence and times are followed. The DNA was kept in −20 ◦ C for further analyses. Obtaining pure genomic DNA that is appropriate for later uses is the aim. Achieved DNA quality facilitates many uses, including sequencing and PCR.

2.3 Polymerase Chain Reaction (PCR) and Gel Electrophoresis

After DNA extraction the samples were subjected to a PCR machine, the LCO1490-HC02198 Primers (Votýpka *et al.,* 2019), and the master mix used for the Cox-1 gene in PCR amplification. PCR conditions, initial denaturation at 94°C for 5 min, followed by denaturation at 94°C for 30 sec, annealing at 40°C for 1 min and extension at 72°C , continued for 40 cycles. The amplification was extended for 10 minutes at 72°C, after which the PCR product was observed in 1% agarose gel with UV light under the Gel-Doc system.

Table 2.1 PCR Reagents and their volumes

Reagents	Concentration
ddH2O	10.0 µL
Master Mix	10.0 µL
Templet DNA	2.5 µL
Forward primer	0.50 µL
Reverse primer	0.50 µL
Total volume	25.0 µL

Table 2.2 Condition for gel electrophoresis

Requirements	Current	Time	Volume	Voltage
Eluted DNA	Adjust	25 minutes	3.5 µL	120 V

2.4 Bioinformatics analysis

With the use of the BLAST (Basic Local Alignment Search Tool) program, the query sequence of the COX-1 gene undergoes comparison with established sequences of the COX-1 gene within the NCBI. This software identifies resemblances between the new sequence and an archive of recognized sequences. Through these resemblances, BLAST can infer the probable species of the organism from which the sample originated.

2.5 Nucleotides identity

This alignment phase allowed meaningful and reliable comparisons to be made by ensuring that the sequences were placed correctly. To improve the quality of the analysis, the trimmed aligned sequences were trimmed using BioEdit. Trimming involved focusing on retained regions of the COX-1 gene and excluding only sequences that were not much related or comparable. Nucleotide identities were finally computed based on pairwise alignments among the sequences.

2.6 Phylogenetic tree

The identified horseflies and their relatives were examined for evolutionary adjacency by constructing a phylogenetic tree using MEGA 11.0 software (www.megasoft-ware.net/dload_win_beta). Sequences were initially aligned using ClustalW, which reliably matched homologous sites. The general-time reversible substitution model (GTR) was chosen for data as it is a correct and accurate representation of the rates of evolutionary change in sequences. Subsequently, MEGA 11.0 software was used to remove poorly aligned positions for better accuracy and more reliable results of phylogenetic trees. A phylogenetic tree was then generated based on the BI method to illuminate the evolutionary relationship among horseflies and from other species. The tree was visualised with Figtree v. 42 (http://tree.bio.ed.ac.uk/software/figtree/) as a graphical tree format for easier and clearer visualization. This paved the way for a greater understanding of the genetic diversity and evolutionary connections between horseflies and close relatives. These shocking findings were revealed after examining the combined capabilities of Figtree and MEGA 11.0, which demonstrated it is possible to better understand the phylogenetic history of the horseflies and their phylogenetic relationships within their genera.

III. RESULTS

3.1 Morphological Identification of Horseflies and Photography

By using a taxonomic key, it is possible to identify the *Atylotus* spp (Genus *Atylotus*) and *Tabanus* (Genus *Tabanus)* specimens (as described in the material method).

Fig.3.1. Entire specimen of Atylotus spp.

Fig.3.2 Head view of Atylotus spp.

Fig.3.3 Photograph of the entire specimen of Tabanus sp.

Fig.3.4 Head view of Tabanus spp.

3.2 Identification of Horseflies through the COX-1 gene sequence

In the present study, COX-1 gene partial sequencing was accomplished for three species; two of them from the genus *Tabanus* (designated as *Tabanus* sp. 1 and *Tabanus* sp. 2) and one belonging to the genus *Atylotus* (*Atylotus* sp.). Comparison of the gene sequences to databases of genetic information in the NCBI was carried out by using the Basic Local Alignment Search Tool (BLAST) program. However, due to limited sequence data, only the genera of these specimens could be identified, not the individual species. Notably, *Tabanus* sp. 1 exhibited a significant 93.95% similarity with *Tabanus superjumentarius* (Accession No. KM2433535). Notably, the genome of T. superjumentarius was sequenced in the United States for North Carolina State University before July 15, 2014 (Morita *et al.,* 2014). Additionally, Tabanus sp. 2 demonstrated a significant genetic similarity of 94.55% with *Tabanus bromius* (Accession No. MK941824), which was previously sequenced on May 16, 2019, at Eskisehir Technical University in Turkey (Sanal *et al.,* 2021). Moreover, *Atylotus* sp. showed a 93.97% similarity with *Atylotus agrestis* (Accession No. OL534387), which was sequenced earlier on November 17, 2021, in South Africa (Williams *et al*., 2022).

3.3 Nucleotide identity

The nucleotide names in among sequenced horseflies and their congeners are below. It is evident from table 3.1 that *Atylotus* sp. is 90.7% identical in nucleotide sequence to Tabanus sp. 1, whereas with *Tabanus* sp. and 2, the identity is a little bit lower- 89.4%. Nucleotide identity between *Tabanus* sp. 1 and *Tabanus* sp. 2 is 90.1%. These differences are evident in the distinct clades observed in the phylogenetic tree depicted in Figure 3.5 Upon thorough examination of Table 3.1, distinct patterns of nucleotide identity among the listed species emerge. For instance, *Tabanus* sp.1 exhibits a nucleotide identity of 93.8% with T. *fontinalis*, while with T. *rubidus*, the identity slightly decreases to 93.3%. In contrast, T. *fontinalis* and T. *rubidus* share a higher nucleotide identity of 94.0%. With a 94% nucleotide identity between T. *fontinalis* and *T. rubidus*, it suggests a shared ancestry between these two species, as indicated by their arrangement in the evolutionary tree diagram. Conversely, the nucleotide identities of *Tabanus* sp.1 with T. *fontinalis* and T. *rubidus,* at 93.8% and 93.3% respectively, slightly fall short of the 94% threshold. This positioning suggests that in the phylogenetic tree, *Tabanus* sp.1 is closely related to T. *fontinalis* and T. *rubidus*.

When we examined the data for *Tabanus* sp. 2 and T. *bromius* we obtained a high nucleotide identity of 95%. genetic identity of *Atylotus* species, just as 94.0%, 92.6% and 93.6% of *Atylotus* sp. for A. *agrestis*, A. *fuscipes*, and A. *nigromaculatus*, respectively.

Table 3.1. The % nucleotide identity of identified species/and some congers.

S.No.	Names of Species	Nucleotide identity in %
1	***Tabanus* sp. 1& *Tabanus* Sp2.**	90.1
2	*Tabanus* sp. 1& *Tabanus fontinalis*	93.8
3	*Atylotus* sp. & *Atylotus nigromaculatus*	91.2
4	*Tabanus* sp. 1& *Tabanus rubidus*	90.3
5	*Tabanus* sp. 1& *Tabanus bromius*	94.9
6	*Atylotus* sp. & *Atylotus fuscipes*	88.8
7	*Atylotus* sp. & *Atylotus agrestis*	94.0
8	*Atylotus fuscipes* & *Atylotus nigromaculatus*	98.9
9	*Atylotus agrestis* & *Atylotus nigromaculatus*	98.9
10	*Atylotus agrestis* & *Atylotus fuscipes*	97.9
11	*Tabanus bromius* & *Atylotus nigromaculatus*	90.5
12	*Tabanus bromius* & *Atylotus fuscipes*	89.0
13	*Tabanus bromius* & *Atylotus agrestis*	90.3
14	*Atylotus* sp. & *Tabanus bromius*	90.7
15	*Tabanus rubidus* & *Atylotus nigromaculatus*	90.2
16	*Tabanus rubidus* & *Atylotus fuscipes*	89.5
17	*Tabanus rubidus* & *Atylotus agrestis*	90.5
18	***Atylotus* sp. & *Tabanus* Sp1.**	90.7
19	***Atylotus* sp. & *Tabanus* Sp2.**	89.4
20	*Atylotus* sp. & *Tabanus fontinalis*	89.8
21	*Atylotus* sp. & *Tabanus rubidus*	89.8
22	*Tabanus* sp. 1& *Atylotus agrestis*	91.4
23	*Tabanus* sp. 1& *Atylotus fuscipes*	90.0
24	*Tabanus* sp. 1& *Atylotus nigromaculatus*	91.2

25	*Tabanus* sp. 2 & *Tabanus fontinalis*	93.8
26	*Tabanus* sp. 2 & *Tabanus rubidus*	90.3
27	*Tabanus* sp. 2 & *Tabanus bromius*	94.9
28	*Tabanus* sp. 2 & *Atylotus agrestis*	89.8
29	*Tabanus* sp. 2 & *Atylotus fuscipes*	88.8
30	*Tabanus* sp. 2 & *Atylotus nigromaculatus*	89.4
31	*Tabanus fontinalis* & *Tabanus rubidus*	94.0
32	*Tabanus fontinalis* & *Tabanus bromius*	92.6
33	*Tabanus fontinalis* & *Atylotus agrestis*	89.8
34	*Atylotus fuscipes* & *Tabanus fontinalis*	88.6
35	*Tabanus rubidus* & *Tabanus bromius*	92.1
36	*Tabanus fontinalis* & *Atylotus nigromaculatus*	89.7

3.3 Phylogenetic relationship

The evolutionary tree depicted in Figure 3.5 contains nineteen (19) species and a single outgroup, *Sarcophaga ruficornis*, that represents the infraorder Muscomorpha, order Diptera. The primary clades observed in the phylogenetic tree are the *Tabanus* and *Atylotus* clades. The *Tabanus* clade includes numerous subclades and sister clades. *Tabanus* sp. 1 forms a robust clade together with the sister to the penultimate common ancestor of T. *fontinalis* (MZ769395) and T. *rubidus* (MT132391). T. *fontinalis,* and T. *rubidus*, both native to North America, are closely related if the evolutionary tree is any indication due to their proximal position in the same clade. Our sequenced *Tabanus* sp.1 displays a separate positioning from the T. *rubidus* (MT132391) species, likely attributed to prolonged geological isolation. This divergence signifies a substantial amount of genetic alteration, evident from the extended branch length observed in the phylogenetic tree. The separate position of Tabanus sp. 1 indicates that it diverged evolutionarily for possible adaptation to the unique extreme environmental conditions of KPK Swat

The another subclade represents a distinct subgroup formed by *Tabanus* sp. 2 and T. *bromius* (MK941824), distinguishing them from other species. This topology clearly indicates that *Tabanus* sp. 2 deviates significantly from other members of its group. However, despite this distinction, both *Tabanus* sp. 2 and T. *bromius* (MK941824) are species endemic to the Palaearctic region, specifically located in the Swat district of KP province. Consequently, they share the same clade and exhibit a bootstrap score of 90, indicating a common ancestor. The sequenced *Tabanus* sp. 2 and T. *bromius* (MK941824) demonstrate considerable genetic variation from other members within the formed monophyletic group.

Atylotus represents another prominent clade comprising six species categorized into two subclades. The sequenced *Atylotus* sp. was grouped alongside A. *fuscipes* MW01386, A. *nigromaculatus* MW337211, and A. *agrestis* OL534387. A. *agrestis* and the *Atylotus* sp. series exhibit close linkage. A. agrestis has a wide distribution spanning the Palaearctic and Afrotropical regions, as well as China, Egypt, India, Saudi Arabia, and Sri Lanka. Similarly, *Atylotus* sp. is found in the Palaearctic region, aligning closely with A. *agrestis*. They cluster together in the phylogenetic tree, suggesting a strong evolutionary connection due to their shared geographic regions. The evolutionary tree constructed in this study highlights that although *Tabanus* sp. 1 and *Tabanus* sp. 2 belong to the same genus, they form distinct clades. Conversely, *Atylotus* sp. is grouped with *Atylotus* species within its own clade. Hence, from our study, we concluded that the genus *Atylotus* is monophyletic while the *Tabanus* genus is may be polyphyletic or paraphyletic because as it does not include all descendants of a common ancestor based on the provided phylogenetic tree.

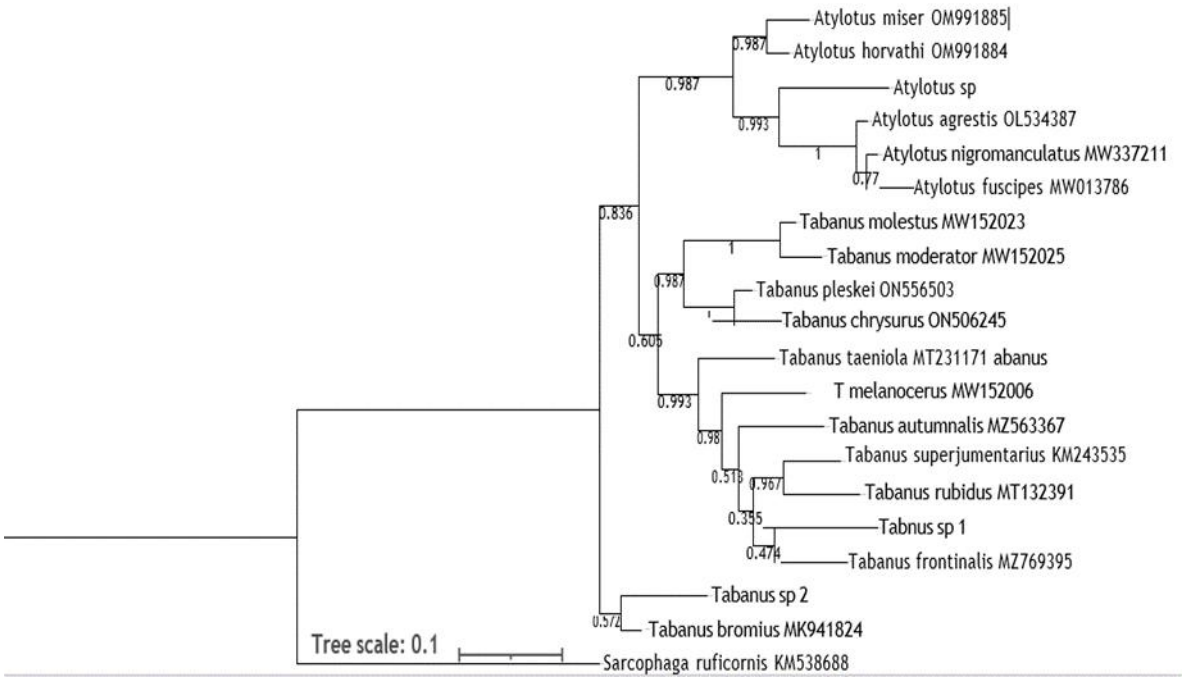

Fig.3.5. Phylogenetic relationship of collected species of horseflies and their congeners through BL method as a base on partial sequence of mitochondrial cox-1gene. Sarcophaga ruficornis is an outgroup organism from the infraorder Musco-morpha order Diptera.

Table 3.1. The species taxonomy and GenBank accession numbers included for phylogenetic analysis in this study. Bold se-quences indicate new sequenced species.

Name of species	Systematic position	Accession No.
***Tabanus* sp. 1**	Insecta, Diptera	XXXX
***Tabanus* sp. 2**	Insecta, Diptera	XXXX
***Atylotus* sp.**	Insecta, Diptera	XXXX
Tabanus fontinalis	Insecta, Diptera	MW013786
Tabanus rubidus	Insecta, Diptera	MT132391
Atylotus nigromaculatus	Insecta, Diptera	MW337211
Atylotus fuscipes	Insecta, Diptera	MW013786
Atylotus agrestis	Insecta, Diptera	OL534387
Tabanus bromius	Insecta, Diptera	MK941824
Tabanus melanocerus	Insecta, Diptera	MW152006
Atylotus miser	Insecta, Diptera	OM991885
Atylotus horvathi	Insecta, Diptera	OM991884
Tabanus chrysurus	Insecta, Diptera	ON506245
Tabanus pleskei	Insecta, Diptera	ON556503
Tabanus moderator	Insecta, Diptera	MW152025
Tabanus molestus	Insecta, Diptera	MW152025
Tabanus autumnalis	Insecta, Diptera	MZ563367
Tabanus superjumentarius	Insecta, Diptera	KM243535
Sarcophaga ruficornis	Insecta, Diptera (Muscomorpha)	KM538688

IV. DISCUSSION

In this study, samples were collected from Swat, and their DNA were amplified by PCR targeting the COX-1 gene. Following amplification, sequencing of the COX-1 gene revealed that *Tabanus* sp.1 and *Tabanus* sp.2 exhibited sequence identities of 93.95% and 94.55% with T. *superjumentarius* and T. *bromius*, respectively. Moreover, Atylotus sp. showed 93.97% sequence identity to A. agrestis. According to [6], the specimens were originally identified based on morphology as Ha. t*urkestanica*, C. *vanderwulpi,* C. *dissectus,* T. *chrysurus,* T. *pleskei* and *Hy*. sp. were known only to genus because species-specific information was not available. When amplified cox1 sequences were compared with sequences deposited in GenBank (MT231188, OM991886, OM991887, NC062705, NC062705, and MT410834), identities of 93.5%, 93.8%, 96.2%, 95.62%, 96.48%, and 96.15% were observed, respectively.

The findings of this study revealed distinct genetic variances between *Tabanus* sp.1 from KP Swat and the indigenous species of North America, T. *fontinalis* and T. *rubidus*. Although T. *fontinalis* and T. *rubidus* exhibit 94% identities at the nucleotide level, *Tabanus* sp. 1 has nucleotide identities of 93.8% and 93.3% to T. *fontinalis* and T. *rubidus.* This slight divergence suggests a genetic distinction of *Tabanus* sp. 1 from the native North American species (T. *fontinalis* and T. *rubidus*), likely attributable to significant geographical separation over time. According to [16].In contrast to species inhabiting the Nearctic and Neotropical regions, *Tabanus* species exhibit genetic uniqueness as they originate from the Afrotropical region. The nucleotide alignment exhibited interesting patterns as reported by [17] specimens of *H. lurida* exhibited close matches with those from the Nearctic and Neotropical regions, whereas specimens of both T. *par* and A. *agrestis* from Saudi Arabia were more similar to populations in the Afrotropical region than the Palearctic. Notably, molecular research unveiled a heightened level of genetic resemblance between tabanid specimens from Saudi Arabia and those from other species worldwide. This study uncovered that *Tabanus* sp. 2 and T. *bromiu*s share an astonishing 95% similarity at the nucleotide level, which suggests a remarkable affinity between the two species especially, as alluded to above as they are both present in the Palaearctic. In contrast, 94.0% of nucleotide identity was found between the sequenced *Atylotus* sp. and A. *agrestis*, indicating a close genetic relationship of the two. The other two were A. *agrestis* species that are widely distributed; the Palaearctic and Afrotropical A. agrestis was previously identified from China, Egypt, India, Saudi Arabia, and Srilanka. Nucleotide identity of Saudi Arabian specimens of A. *agrestis* (MW243943), H. *lurida* (MW265638) and T. *par* (MW238412) was investigated by [17]. The results showed characteristic identity patterns: the sequence of A. *agrestis* from Saudi Arabia proved from 92% to 99% nucleotide similar to tabanid isolated from other regions. The nearest tabanid isolates from varied regions such as the Central African Republic (CAR) (MK396308), India(KM111678), Gabon(MK396326), Thailand (MG426116) and the USA (KM243535) ,estimated a highest nucleotide identity of 91% to 92.80% with the T. par sequence of Saudi Arabia.

In the present study, the phylogenetic relationship of Tabanus sp. 1 clade and T. *frontinalis* and T. *rubidus* and the *Tabanus* sp. 2 clade and *Tabanus bromius* were studied. Similarly, the *Atylotus* sp. clade was examined alongside A. *agrestis*, A. *fuscipes*, and A. *nigromaculatus*. Hence, from our study, we concluded that the genus Atylotus is monophyletic while the Tabanus genus is may be polyphyletic or paraphyletic because as it does not include all de-ascendants of a common ancestor based on the provided phylogenetic tree. In a previous cladistic study by[6] , Maximum Likelihood (ML) analysis was utilized to construct a phylogenetic tree. The findings from the analysis consistently supported the delineation of six recognized branches. It was observed that Hy. sp. grouped within these branches alongside other species belonging to the genus *Hybomitra*, such as H. *astur*, H. *lurida*, and H. *bimaculata*. Moreover, the research indicated H. *turkestanica* is a member of the genus *Haematopota*, and T. *pleskei* and T. *chrysurus* fall under the genus *Tabanus*. Moreover, six *Atylotus* horseflies were virtually huddled on a single branch, while C. dissectus and C. vanderwulpi horseflies were embedded on the branch of the *Chrysops* horseflies. This study confirms the current taxonomic classification and supports the idea that all genera are monophyletic. COX-1 sequences obtained from genera in three tribes of the Tabanidae family (*Atylotus, Hybomitra*, *Tabanus*, *Haematopota* and Philoliche), and from the Chrysops genera were used for ML and BI phylogenetic analyses, according to . All COX-1 sequences found in tabanids are clustered with their congeners. The monophyly of the family Tabanidae was strongly supported by both topologies[6]. 1. Monophyly Tabanidae are monophyletic as previously proposed [18, 19]. The above results coincide with earlier studies showing that based on genetic data (nuclear (28S) and mitochondrial (COX-1) genes) and by morphology (genitalia and external features) Tabanidae is a monophyletic group[6].

V. CONCLUSION

The molecular study of Tabanid from Pakistan which indicates the two genera *Atylotus* and *Tabanus* with 93.95% similarity between *Tabanus* sp. 1 and T *superjumentarius* (Accession No. KM2433535), while *Tabanus* sp. 2 shares

94.55% sequence identity with T. *bromius* (Accession No. MK941824). Conversely, Atylotus sp. shares a sequence identity of 93.97% with *Atylotus agrestis* (Accession No. OL534387). The nucleotide divergences between known species and their congeners were 89.4-94.55%. Our phylogenetic analysis delineates two genetically distinct clades corresponding to the *Tabanus* and *Atylotus* genera. Based on our findings, we concluded that the genus *Atylotus* is monophyletic while the *Tabanus* genus is may be polyphyletic or paraphyletic because as it does not include all descendants of a common ancestor.

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