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First Report and Molecular Identification of *Sergentomyia babu babu* Annandale (1910) (Diptera: Psychodidae: Phlebotominae) in Iraq

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Abstract: This study aims to investigate the morphology and molecular identification of sandflies, one of the most significant disease vectors in Iraq, across the provinces of Nineveh Governorate (northern Iraq) during the period between May 2023 and June 2024. The male genitalia, female pharynx and spermatheca of the subfamily Phlebotominae were used as the basis for the morphological identification. For molecular identification, the conventional polymerase chain reaction (PCR) was used to amplify the mitochondrial cytochrome c oxidase gene subunit 1 gene, and the DNA sequences were analyzed and compared to isolates listed in the National Center for Biotechnology Information (NCBI). The findings showed that 8,355 sandflies (3,779 males and 4,575 females) and seven species were collected, belonging to the two genera: *Phlebotomus* and *Sergentomyia*. The genus *Phlebotomus* includes five species: *Phlebotomus papatasi*, *Phlebotomus sergenti*, *Phlebotomus alexandri*, *Phlebotomus kazeruni*, and *Phlebotomus tobbi*. As for the genus *Sergentomyia*, it includes two species: *Sergentomyia dentata* and *Sergentomyia babu babu*. The results of phenotypic and molecular identification showed that the *S. babu babu* was recorded for the first time in Iraq, as three isolates were documented in NCBI. The evolutionary tree and genetic divergence analysis confirmed the great genetic similarity between *S. babu babu* and the Indian and Pakistani species, which confirms the correctness of the identification. This study is the second in the country to address the molecular identification of the *Sergentomyia* species. So, our emphasis is on the necessity of coordinating efforts to perform additional morphological and molecular taxonomic investigations in order to accurately identify the sandflies in Iraq, particularly those belonging to the genus *Sergentomyia*.

Keywords: *Phlebotominae, Sandflies, leishmaniasis, Sergentomyia spp.*

1. Introduction

Insect-borne diseases represent a major public health problem worldwide. One of the most important vectors of these diseases is the sand fly, which belongs to the subfamily Phlebotominae and comprises blood-feeding insects associated with the transmission of many pathogens, such as *Leishmania*, viruses, and bacteria. Recent studies have indicated the possible role of sand fly species in the transmission of *Trypanosoma* parasites and *Onchocerca* worms. Given their substantial importance, sand fly species have been identified in endemic areas of both the Old and New Worlds, as these insects are associated with these diseases (Srisuton *et al*., 2019; Brilhante *et al*., 2020; Cecílio *et al*., 2022).

Sand fly taxonomy is a cornerstone of scientific research aimed at identifying groups of disease vectors and the pathogens transmitted by these insects. Traditionally, the use of phenotypic characters in sand fly taxonomy, which began in 1786, has been the main guide for species identification, but the use of genetic sequences provides an integrative and accurate approach for demarcating these species (Rodrigues & Galati, 2023; Galati & Rodrigues, 2023).

Species of *Sergentomyia* are found throughout the Old World but are particularly abundant in tropical Africa, the Middle East, Australia, and the Indian subcontinent (Theodor, 1948). Although the genus *Phlebotomus* in the Old World and the genus *Lutzomyia* in the New World are responsible for transmitting cutaneous and visceral leishmaniasis in endemic areas, recent evidence has emerged that members of the genus *Sergentomyia* may also be involved as vectors of *Leishmania* species to mammals (Maia & Depaquit, 2016). Experimentally, *S. baghdadi* has been shown to transmit both *Leishmania tropica* and *Leishmania major* in Iraq (Al-Mashhadani, 2006). In molecular studies, the DNA of *Leishmania* has been identified in *S. dentata,* but its species has not been identified in Turkey (Özbel *et al*., 2016).

Iraq is considered one of the endemic areas for leishmaniasis in the Middle East, with the disease being widespread across all governorates and more prevalent in warm and hot areas, especially in the southern regions of the country. For this reason, studies on the identification of sandflies have been numerous in those governorates, while fewer have been conducted in the northern governorates. Previously, three significant phenotypic surveys were conducted in Iraq, resulting in the identification of several sand fly species, including species from the genus *Sergentomyia*. However, the *S. babu babu* was not identified in any of these investigations (Adler & Theodor, 1929; Pringle, 1953; Abu Al-Hab, 1979). Regarding the molecular identification of the genus *Sergentomyia*, there are only two previous studies, the first conducted in Babylon Governorate, but without confirming the species (Al-Obaidi, 2021). The second is our previous study, during which we identified *S. dentata* at the molecular level for the first time in Iraq (Al Joary & Al Hamdani, 2024). Given the above and the health and veterinary importance of sand fly species, the present study was conducted in Nineveh Governorate, northern Iraq.

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2. Methods

Study Area, Collection of Sandflies, and Morphological Identification

Sand fly Specimens were collected during the period between May 2023 until June 2024 in nine provinces of Nineveh Governorate (northern Iraq), with a focus on areas known for leishmaniasis outbreaks: Mosul (43.159 ◦E, 36.349◦N), Alhamdanyia (43.399 ◦E, 36.190◦N), Al-Baaj (41.716◦E, 36.046◦N), Al-Hadar (42.734◦E, 35.575◦N), Sinjar (41.863◦E, 36.314◦N), Tel Kaif (43.120◦E, 36.490◦N), Tel Afar (42.402◦E, 36.362◦N), Al-shikhan (43.440◦E, 36.785◦N) and Makhmur (43.576◦E, 35.774◦N). The latitude and longitude of each study site were determined using Google Maps (Figure 1).

Photocatalysis-based Mosquito Killer light traps (China) were used to collect specimens, and the traps were installed at the study sites from 5:00 p.m. until 9:00 a.m. the next day, Baghdad time. Then, the specimens of sand fly were isolated from other insects using a dissecting microscope) Wild M7A, Switzerland), magnifying lenses, and at times with the naked eye, based on the initial characteristics of the sandflies, including their small size, gray color, large black eyes, raised wings, and long legs. When dead, the insect can also be distinguished by an inverted (L) letter (Pringle, 1953). Then, the specimens were separated by sex (male and female). The isolated specimens were preserved in test tubes containing 70% ethyl alcohol.

Prior to morphological identification, the method described by Dantas-Torres *et al*. (2014) was followed to prepare the specimens for microscopic examination, with the specimens being placed in potassium hydroxide solution for about 24 hours to make the internal structures more visible. The head and abdomen regions were then separated from the rest of the body and mounted on slides using Canada balsam. The morphological keys of the *S. babu babu* mentioned by Kakarsulemankhel (2004) were used for identification. The slides were examined using a Navite light microscope (China) at various magnifications, and then photographed using an RS500W digital camera (China).

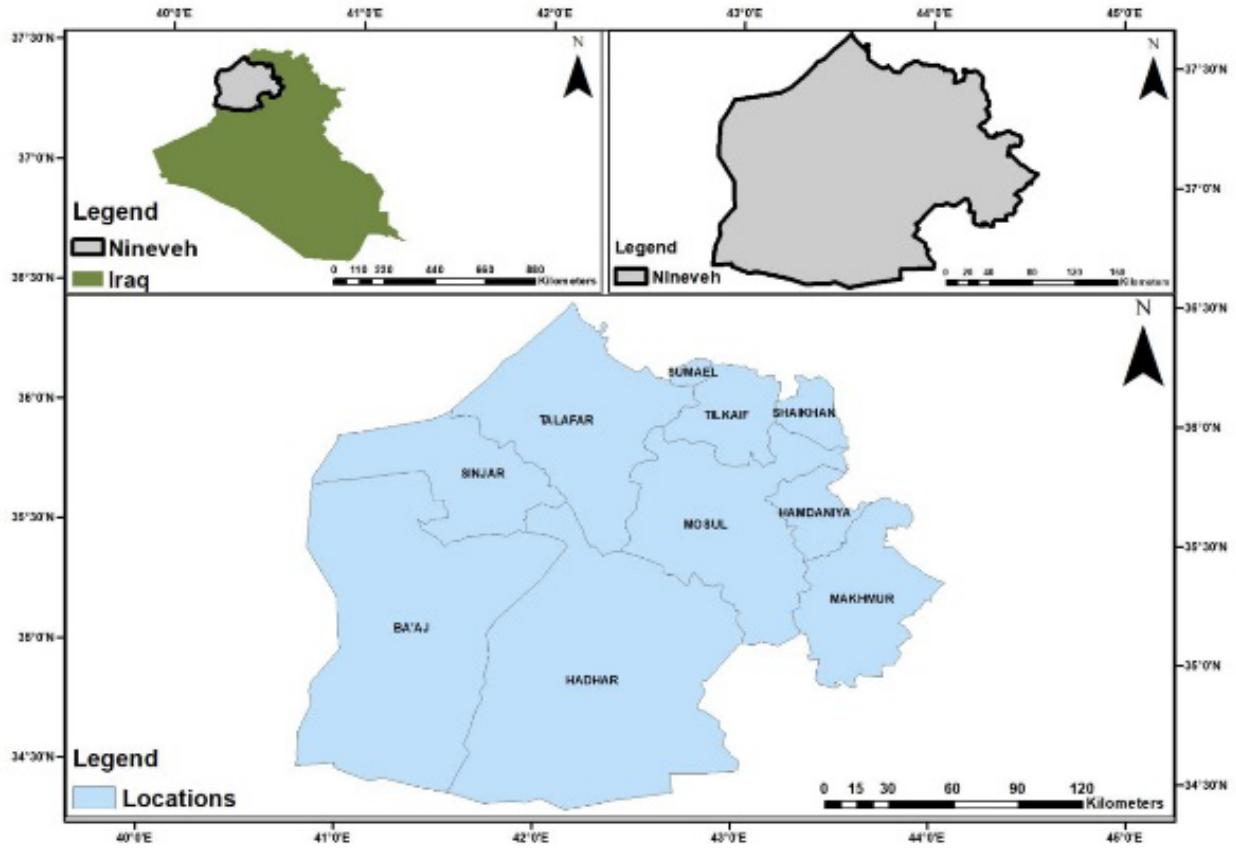

Figure 1. Map of study regions, designed using ArcGIS Pro3.0.2 program

Molecular Identification

The genomic DNA of sandflies was extracted following the manufacturer's instructions of Geneaid DNA Isolation Kit (Taiwan). The concentration and purity of the extracted DNA were measured using a BioDrop device (Finland) at a wavelength of 260 nm, and the purity of the extract was estimated from the 260/280 nm absorbance ratio. PCR with specific primers for amplification of the mitochondrial cytochrome c oxidase gene subunit 1 (*COX)* was performed using Taq Master Mix 2X (PROMEGA, USA) and the primers LCO 1490 (5-GGTCAACAAATCATAAAGATATTGG-3), HCO 2198 5-(TAAACTTCAGGGTGACCAAAAAATCA-3) designed by Macrogen (Korea) (Folmer *et al*., 1994). After preparing the PCR tubes, they were placed in a thermal cycler (BioRad, USA). The reactions were performed using the following program: 1 cycle of

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primary denaturation for 5 min and 94°C, followed by denaturation (40 cycles of 40 seconds at 94°C), annealing (40 cycles of 4 seconds at 52°C), and extension (40 cycles of 1 min and 72°C). Then, final extension (1 cycle of 5 min and 72°C), followed by cooling at 4°C. Gel electrophoresis (1% concentration) was conducted for PCR products and then examined under a gel documentation system (Bio-Rad, USA).

DNA Sequencing and Phylogenetic Tree

The positive PCR products that showed primer binding at 700 bp were sent to Psomagene company (USA) for sequencing of the *COX* gene by the Sanger method. After receiving the sequencing results, the high-quality sequences were matched with the global isolates documented in NCBI using the Blast program and recorded. For the purpose of constructing the phylogenetic tree of sandflies under study, the *COX* sequences were aligned with the corresponding species sequences documented in the NCBI of Old World sand flies, considering that Iraq is located within the Old World, using the MEGA 11 program and according to the following steps: alignment method (ClustalW), Test (Neighbor-Joining (NJ) tree, Model (Kimura-2 parameter) and Bootstrap *P* values (1000). Finally, evolutionary divergence was estimated using the Kimura-2 parameter model (K2P) and Bootstrap *P* values (1000) (Tamura *et al*., 2021).

3. Results

Sandflies Collection and Morphological Identification

In total, 8,355 sandflies were collected during the current study period, belonging to two genera: *Phlebotomus* and *Sergentomyia*. The genus *Phlebotomus* included five species: *Ph. papatasi* (males: 2471; females: 3111), *Ph. sergenti* (males: 1,227; females: 1,252), *Ph. alexandri* (males: 70; females: 203), *Ph. kazeruni* (Female: 5) and *Ph. tobbi* (males: 4; females: 2), While the genus *Sergentomyia* includes two species: *S. dentata* (1 male) and *S. babu babu* (males: 7; females: 2). Further details about molecular identification of the recorded species (except for *S. babu babu*) were addressed during our study (Al Joary & Al Hamdani, 2024). *Sergentomyia babu babu* (Annandale, 1910) was recorded for the first time in Iraq and in Nineveh Governorate. Nine specimens were found in three regions (Alhamdanyia, Al-Hadar, and Makhmur) during a two-month period (July 2023 and May 2024). The molecular identification confirmed the phenotypic identification of this species, which is briefly characterized as follows:

Male Genitalia

The stylets have four spines, two terminals and two subterminal. The paramere is long, with the basal and middle parts of its body extending downwards, with a ventral tubercle bearing three short spines; the body of the paramere has 8 to 10 long setae, gradually narrows anteriorly, and finally curves laterally like a bird's head. The aedeagus is long, straight, and highly pigmented, tapering to an almost colorless, rounded, and blunt tip; the genital pump is filiform with transverse striations.

Female Spermatheca

An oblong, oval, thick-walled capsule in which the sperm ducts communicate through a common opening in the reproductive atrium.

Female Pharynx

The pharyngeal armature consists of 4 to 5 rows of scales with pointed posterior ends and longer basal spicules. Most of the pharyngeal armature is confined to the basal part of the pharyngeal bulb, and the lateral walls of the pharynx on the side of the bulb bear 5 to 8 long, sparsely arranged spines anteriorly (Figure 2,3) (Kakarsulemankhel, 2004).

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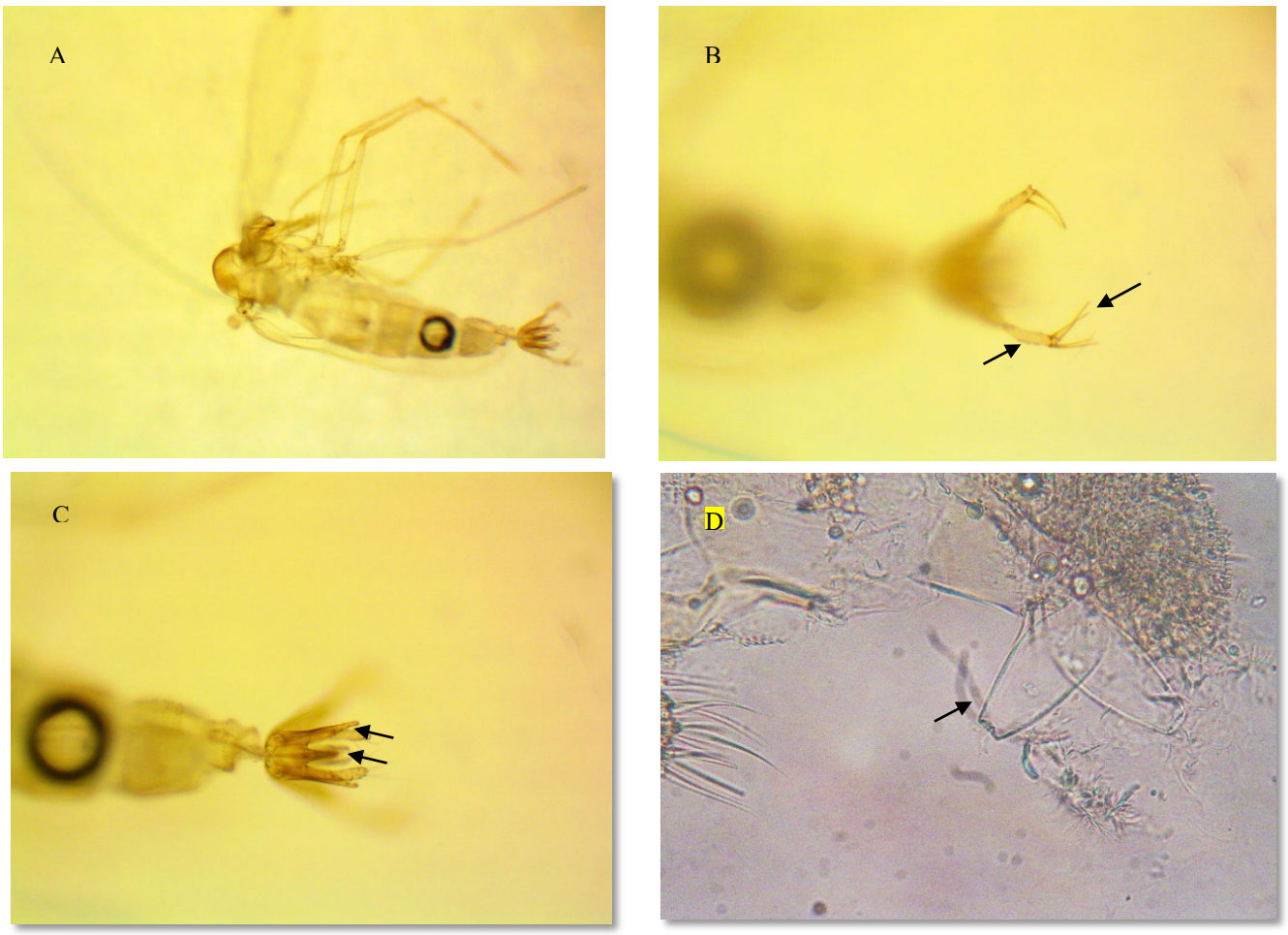

Figure 2. Phenotypic characters of male and female S. babu babu: A- Male (40X), B- Male genitalia (stylets with spines) (100X), C- Male genitalia (Aedeagus and paramere) (100X), D- Female spermatheca (640X).

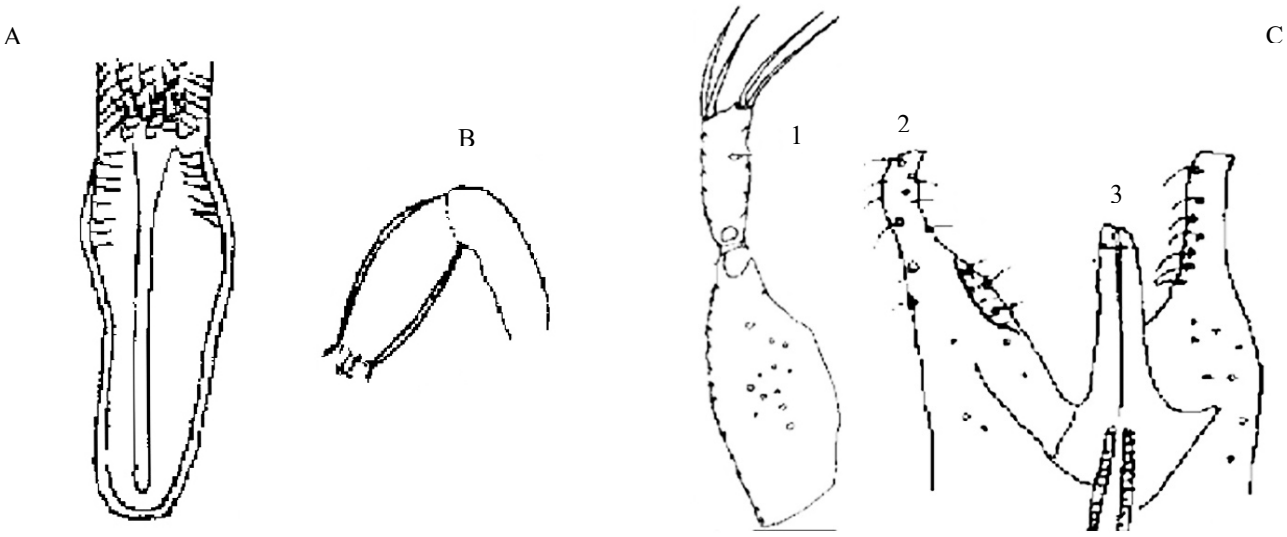

Figure 3. Diagram of phenotypic characters of male and female S. babu babu: A- Female pharynx, B- Female spermatheca, C- Male genitalia:1- stylets with spines, 2- paramere, 3-Aedeagus (Kakarsulemankhel, 2004).

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Molecular Identification

DNA Extraction and PCR Results

The genome of the sand fly specimens was extracted at high concentration (53.62 ng/µl) and purity (1.7 ng/µl), as shown in Figure 4. These were measured using the BioDrop device, which is highly efficient in estimating the concentration and purity of DNA, and is easy to use, inexpensive, and provides rapid results (Hameed & Hamed, 2023).

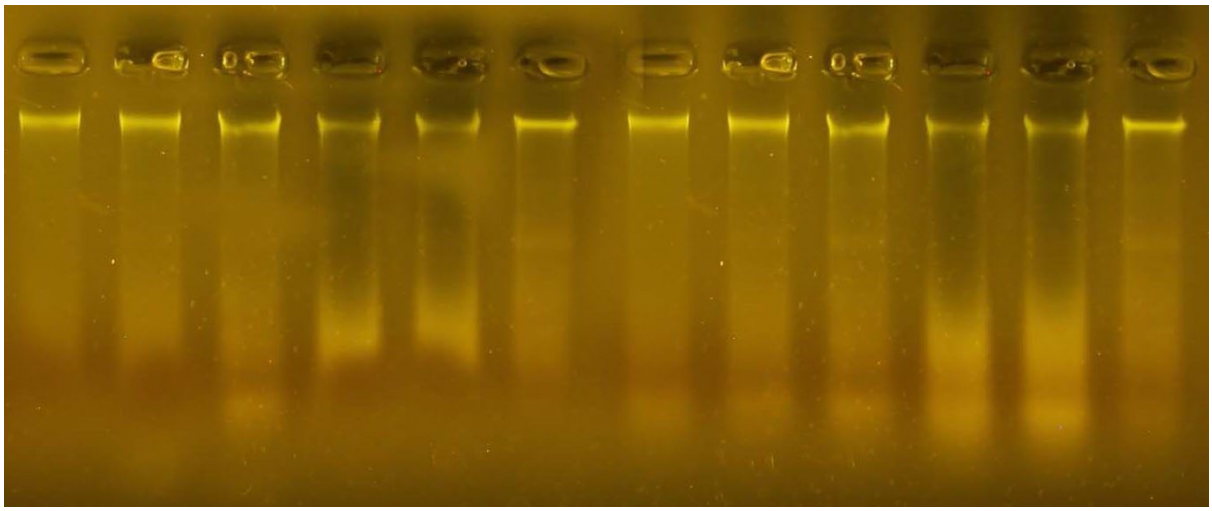

Figure 4. Whole genome extraction from sand fly specimens electrophoresed on 1% agarose gel.

In addition, the results in Figure 5 show the electrophoresis of the PCR products, where the primer used in this study bound to the 700 bp band of the *COX* gene region in the mitochondrial sand fly genome, which confirms the validity of using this primer in the current study.

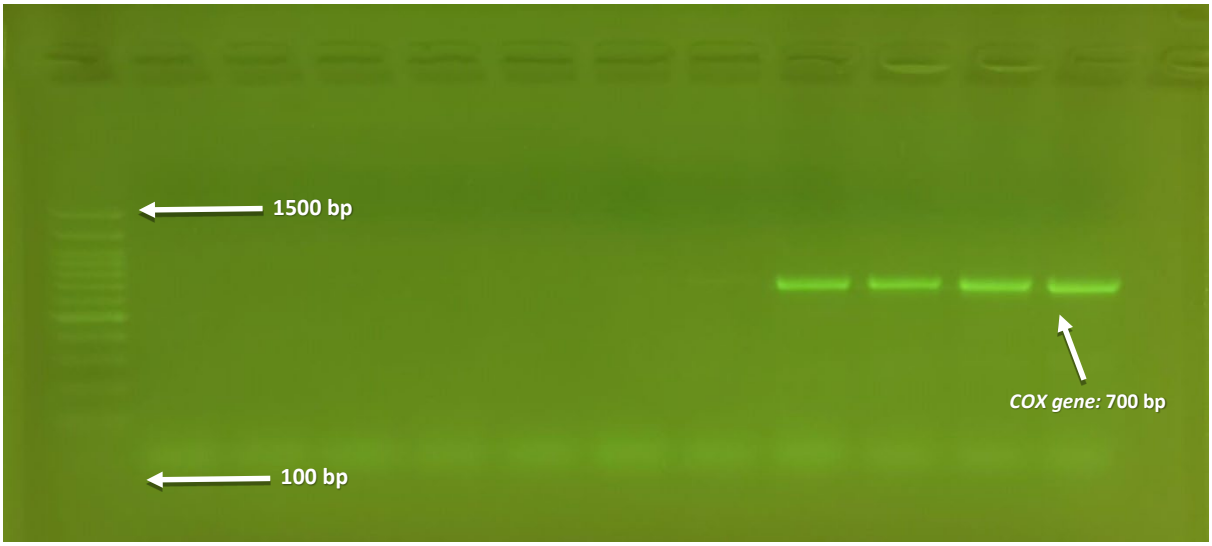

Figure 5. PCR amplification of the mitochondrial COX gene of sand fly specimens and primer binding with 700 bp PCR product separated by 2% gel electrophoresis.

Matching the Sequences of the Species Under Study and Recording Them in The NCBI.

The aim of conducting sequencing and matching the results with the isolates documented in the NCBI was to identify the species under study accurately and to determine the genetic variations and variability relative to global isolates, especially for species located within the Old World (Figure 6).

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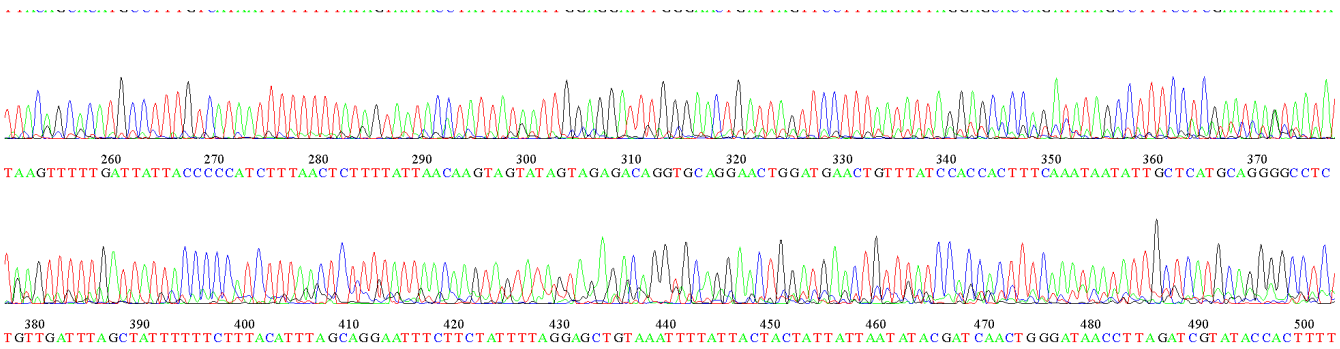

Figure 6. COX gene sequence analysis in the DNA-Sequencing test.

Overall, the high-quality sequences showed matching identity and identity with global isolates ranging from 94.42% to 98.59% (Table 1), and this resulted in the recording of three isolates in NCBI (accession numbers: PP856257, PQ056783, PQ063273) belonging to the species that were phenotypically identified, named YIMA in reference to the name of the authors.

Table 1. The accession numbers of *S. babu babu* isolates that were recorded and matched with different global isolates in NCBI.

No.	Isolates	Accession Numbers	Release Date	Matching with NCBI			
				Query Cover	Identity	Acc. Numbers	Country
1	*S. babu babu* isolate YIM32	PP856257	6-6-2024	100%	94.42	HQ585362	India
				100%	95.94	KY834515	Pakistan
				100%	95.43	MT472523	India
2	*S. babu babu* isolate YIM33	PQ056783	28-7-2024	100%	97.66	HQ585362	India
				100%	98.59	KY834515	Pakistan
				100%	98.36	MT472523	India
3	*S. babu babu* isolate YIM34	PQ063273	29-7-2024	100%	96.48	HQ585359	India
				98%	96.76	HQ585362	India
				99%	96.47	HQ585356	India

Phylogenetic tree and Evolutionary divergence

To determine the genetic proximity between the isolates recorded in the NCBI and to confirm the results of the molecular identification of the *COX* gene, an evolutionary tree was conducted with several closely related global isolates using the NJ tree and K2P model (Figure 7). The clades reflect the extent to which the isolates are related to each other. In general, the bootstrap values, shown as percentages at the key clade nodes, indicate the level of confidence or stability of the nodes observed in the tree. The group of isolates that are closely related are clustered together and supported by high bootstrap values (Aljubouri, 2024).

The isolates recorded during the current study were related to each other with a bootstrap value of 96%. Their association with the Pakistani and Indian isolates (Accession Numbers: KY834515, MT472523) with bootstrap values of 95, 98% respectively, indicates a very strong phylogenetic relationship. On the other hand, the three isolates showed evolutionary divergence values with global isolates ranging from 0.004 to 0.064, and the overall mean distance was determined to be 0.02 (Table 2). This low value indicates little evolutionary divergence between the Iraqi and global isolates, and also supports the results of the phylogenetic relationship.

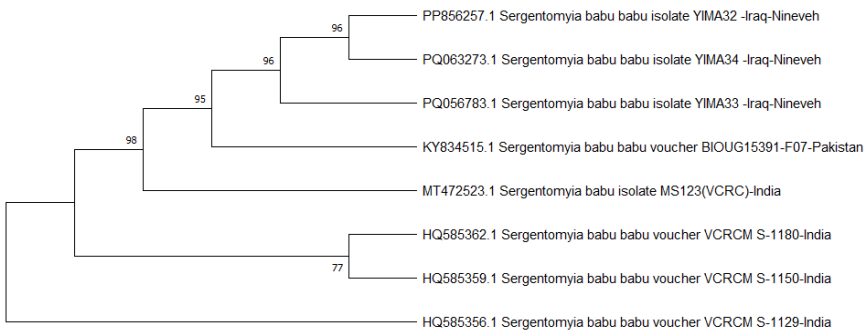

Figure 7. Phylogenetic tree of *S. babu babu* isolates and the Indian and Pakistani isolates documented in NCBI.

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Table 2. Evolutionary divergence (K2P) between *S. babu babu* isolates and the Indian and Pakistani isolates documented in NCBI.

No.	Isolates	1	2	3	4	5	6	7	8
1	*Sergentomyia babu babu* Isolate YIMA32-Iraq-Nineveh								
2	*Sergentomyia babu babu* Isolate YIMA33-Iraq-Nineveh	0.026							
3	*Sergentomyia babu babu* Isolate YIMA34-Iraq-Nineveh	0.021	0.005						
4	*Sergentomyia babu babu* Voucher VCRCM S-1180-India	0.058	0.024	0.030					
5	*Sergentomyia babu* Isolate MS123(VCRC)-India	0.047	0.017	0.026	0.011				
6	*Sergentomyia babu babu* Voucher BIOUG15391-F07-Pakistan	0.042	0.014	0.013	0.018	0.014			
7	*Sergentomyia babu babu* Voucher VCRCMS-1150-India	0.064	0.029	0.033	0.004	0.016	0.023		
8	*Sergentomyia babu babu* Voucher VCRCM S-1129-India	0.058	0.024	0.035	0.001	0.013	0.018	0.009	

4. Discussion

The sandfly fauna of Iraq has received some attention from taxonomists. Newstead (1920) described specimens collected in southern Iraq during World War I (Newstead, 1920). Adler and Theodor (1929) constructed an extensive epidemiological survey of sandflies and Oriental sore (leishmaniasis) in Palestine, Syria, and Iraq; in Iraq, their study was limited to the urban areas of Baghdad, Basra, and Mosul, where they were able to record six species of the genus *Phlebotomus*. Pringle (1953) recorded 12 species of sandflies during his survey of most governorates of Iraq in the fall of 1949 and throughout 1950, four of which belonged to the genus *Phlebotomus* and eight to the genus *Sergentomyia* (Pringle, 1953). Abu al-Hab in 1979 recorded sixteen species across all governorates of Iraq, six of which belonged to the genus *Phlebotomus* and ten to the genus *Sergentomyia* (Abu al-Hab, 1979).

The genus *Sergentomyia*, a genus within the subfamily Phlebotominae (Theodor, 1948), has a variable distribution in Iraq, being more prevalent in the central and southern regions of the country (Al-Mayali & Al-Hassani, 2017), while its distribution is lower in the northern regions, as observed in our previous study in Nineveh Governorate (Al Joary & Al Hamdani, 2024). It was not identified in Erbil Governorate in 2022 during the study carried out by Rasool *et al*. (2024). Therefore, our current study also observed a low distribution of *Sergentomyia* species in general and the species *S. babu babu* in particular. The *S. babu babu* belongs to the subgenus *Parratomyia*, which includes species previously recorded in Iraq, such as *S. baghdadis* and *S. palestinensis* (Pringle, 1953; Abu Al-Hab, 1979). It is also more common in countries such as India (Shah *et al*., 2023) and Pakistan (Rasheed *et al*., 2023).

Given that sandflies are significant disease vectors, understanding the genetic diversity of these species is important for ecological monitoring. Many species may become resistant to insecticides as a result of gene flow between them, which could affect control programs (Pathirage *et al*., 2021). Overall, there are few studies on the molecular identification of *Sergentomyia* species throughout the Old World (Rodrigues & Galati, 2023). The *COX* gene is the most widely used molecular marker for studying sandflies despite their diversity, as it offers a substantial and rapid benefit in identifying species. It can also be used in cases where phenotypic identification is difficult, in addition to its benefit in suggesting and analyzing the population structure of the sandfly's community (Grant *et al*., 2021) and to correctly identify females from several genera, including the genus *Sergentomyia* (Srinivasan *et al*., 2014).

The primer used in this study (LCO1490 and HC02198) was first described by Folmer and used to amplify the *COX* gene in the mitochondrial DNA of 11 invertebrate phyla. Initial comparisons of the use of the *COX* primer showed that it generates informative sequences for phylogenetic analyses at the species and higher taxonomic levels. Since then, this primer has been used to detect the *COX* gene in sand flies and is among the most widely used primers for this purpose, which is why it was used in the current study (Folmer *et al*., 1994).

Regarding the results of evolutionary divergence, the findings of the current study are consistent with many previous studies. In India, Kumar found that the evolutionary divergence values among eight sand fly species, seven of which belonged to the genus *Sergentomyia* and one to the genus *Phlebotomus,* ranged between 0.031 and 0.207 (Kumar *et al*., 2012). In Brazil, Rodrigues found that the genetic divergence values between the sand fly species recorded in their study ranged from 0.008 to 0.177 (Rodrigues *et al*., 2018). In Jordan (west of Iraq), the genetic divergence between sand fly species belonging to the genus *Phlebotomus* ranged from 0.0853 to 0.2692 (Mukbel *et al*., 2024).

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5. Recommendations

The current study recommends the re-identification of the species of the genus *Sergentomyia* in Iraq using both phenotypic and molecular methods rather than relying on either approach alone. This is deferred as future work using a dual approach to take into account both methods simultaneously.

6. Conclusion

The current study addressed an important disease vector, the sand fly, in Iraq, a country known for its outbreaks of leishmaniasis, and recorded for the first time in Iraq a new species belonging to the genus *Sergentomyia*, confirming this through molecular identification, which represents an integrative approach in the classification of species. The results of phenotypic and molecular identification demonstrate that the *S. babu babu* was recorded for the first time in Iraq, as three isolates were documented in NCBI. The evolutionary tree and genetic divergence analysis confirmed high genetic similarity between *S. babu babu* and the Indian and Pakistani species, which confirms the correctness of the identification. This study is the second in the country to address the molecular identification of the *Sergentomyia* species. Our emphasis is on the necessity of coordinating efforts to perform additional morphological and molecular taxonomic investigations to accurately identify the sandflies in Iraq, particularly those belonging to the genus *Sergentomyia*.

7. Acknowledgements

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