Phylogenetic characterization of two common sandflies, *Phlebotomus major* and *P. kandelakii,* in Inebolu District of the West Black Sea Region, Türkiye based on mitochondrial gene sequence analysis

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ABSTRACT

Phlebotomus major and *P. kandelakii* are members of the *Larroussius* subgenus, which includes important vector sand fly species. Most members of the subgenus *Larroussius* have the ability to transmit *Leishmania infantum*, the causative agent of visceral leishmaniasis. Here, we investigated the genetic diversity within each species collected from the West Black Sea Region of Türkiye using mitochondrial DNA markers, specifically cytochrome oxidase I (COI) and cytochrome b gene sequences (Cytb). A total of 1889 sand fly specimens were collected from the study area in June 2021 and August 2022; 1596 (84.49%) were identified as *P. major* sensu lato, and 253 (13.40%) were identified as *P. kandelakii*. Nine and four haplotypes of *P. major* were determined in the study area based on COI and Cytb sequences, respectively. Analysis of the phylogenetic datasets generated from our isolates and published isolates in GenBank revealed high haplotype diversities within *P. major* (COI = 0.933, Cytb = 0.714). For *P. kandelakii*, we detected four and three haplotypes within the COI and Cytb sequences, and the haplotype diversities were also high in the datasets, including our isolates and published isolates in GenBank (COI = 0.978, Cytb = 1.000). Pairwise mean genetic distances calculated from the COI and Cytb datasets were 0.4% and 1.4% for *P. major* and 1.0% and 0.2% for *P. kandelakii*, respectively, suggesting the absence of cryptic species. Phylogenetic analyses revealed three and two major clusters of the *Larroussius* subgenus in the COI and Cytb datasets, respectively. Our study contributes to molecular information for *P. major* and *P. kandelakii* distributed in Türkiye and provides valuable insights into the phylogenetic relationships among species within the subgenus *Larroussius.*

Introduction

The subfamily Phlebotominae of the order Diptera includes hematophagous insects that play a role in the transmission of various pathogens such as protozoa (*Leishmania* spp.), viruses (Toscana virus), and bacteria (*Bartonella* sp.) (10, 35). It has also been reported that they have a potential role in the transmission of nematodes in the Onchocercidae family (6). Their most important role

in human and animal health is the transmission of pathogens that cause diseases such as cutaneous (CL) and visceral leishmaniasis (VL), which affect millions of people each year (4, 34). Species identification of sand flies has therefore been undertaken in endemic areas since the discovery of their association with these diseases. Understanding sand fly ecology and host-parasite interactions is crucial for predicting future outbreaks and controlling existing ones (5). Furthermore, it is essential to establish the taxonomy and systematics of Phlebotominae in all areas of research aimed at controlling leishmaniasis (5).

Sand flies play a crucial role in the transmission of *Leishmania* parasites in the Mediterranean Basin. Approximately 20 species of sand fly are involved in this transmission, with the subgenus *Larroussius* containing the most significant vectors of *L. infantum* (14, 36, 45). One of the most important and widespread species of *Larroussius* is *P. major*, a member of the “Major Group." This group consists of morphologically similar species with mixed taxonomic status, geographic distribution, and vector potentials (21, 28). *Phlebotomus major* was the first species identified within the "major group" and was previously considered to be the only species in this complex (1). A thorough analysis of the morphological features of specimens from different biogeographical regions (2, 25, 30, 31, 41, 42) showed that *P. major* is a complex of species. Currently, six species with morphologically similar and largely allopatric names (*P. major, P. neglectus, P. notus, P. syriacus, P. wenyoni,* and *P. wui*) have been recorded within the *P. major* group (3, 13). However, the status of the species within this taxon is still unclear. *Phlebotomus kandelakii*, which is also common in several regions of Türkiye, has also been reported as one of the most common vectors of *L. infantum* in the north-east and north-west of Iran and Georgia (14, 33, 36, 45). However, data on the genetic diversity of this species is scarce, with only about 26 COI or Cytb sequences in GenBank (access date: November 25, 2023), mainly from Türkiye and Azerbaijan.

Mitochondrial genes have been widely used for Phlebotomine sandfly systematics (13) due to their slow evolutionary rate, which is interesting for population studies; they are haploids and easily amplified, in addition to their low recombination. The COI and Cytb genes have commonly been utilized for DNA barcoding and phylogenetic characterization of several sand fly species. They are appropriate markers for analyzing the genetic structure and phylogeny due to their high mutation rate (7, 13).

This study aimed to highlight the genetic diversity of *P. major* and *P. kandelakii*, the dominant sand fly species of the subgenus *Larroussius* in the study area, by analyzing the COI and Cytb gene regions and comparing them with the published haplotypes from other countries available in GenBank.

Materials and Methods

Origin of the specimens of P. major and P. kandelakii: The specimens of *P. major* and *P. kandelakii* were collected in June 2021 and August 2022 from the populations distributed in the Inebolu district of Kastamonu province in the West Black Sea Region of Türkiye. Inebolu is located approximately 25 km from the 42 north parallel and 34 east meridian, which run through the north of Anatolia (Figure 1.). Inebolu generally has a

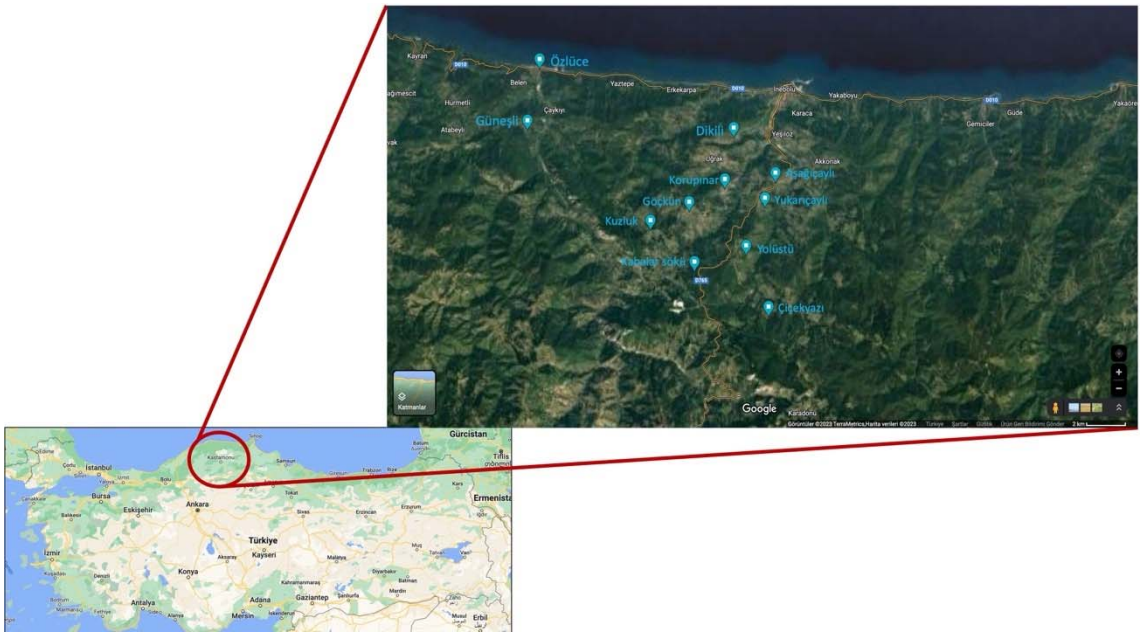

Figure 1. The map shows the Inebolu district of the West Black Sea Region, Türkiye. The blue pins indicate the collection sites of sand flies (Google maps was used to indicate the collection localities).

climate typical of the Black Sea Region, with the fog that occurs in the spring. Winters: mild and rainy; summer months: hot but not dry. It has a warm and mild climate with high relative humidity levels in all seasons, with the highest rainfall occurring between December and March. The average annual temperature of the district is 13.1 degrees, and the average annual rainfall is around 1000 mm. Inebolu is in a strategic position due to its geographical location and is the closest port to Anatolia (23).

For the collection of sand flies, CDC light traps (John W. Hock, USA) were placed in or near animal pens and in courtyards adjacent to houses in 11 locations in Inebolu district (Figure 1). They were set at each site before sunset, when sand flies are active for breeding and feeding, and collected before sunrise. Based on my personal observations, several animals, mostly cattle and also chickens and dogs, that might serve as hosts for the blood of sandflies were found in most of the collection sites.

Captured sand fly specimens were collected from the traps using a manual aspirator and preserved in tubes containing absolute ethanol. The specimens were then transported to the laboratory in a cold chain using ice boxes. Microscopic identification of each specimen was performed by head and genital morphology using an Olympus BX51 light microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP70 (Olympus, Tokyo, Japan) digital camera and imaging software cellSens Standard v.1.13 (Olympus, Tokyo, Japan) (2, 24, 28, 29, 39, 40). After identification, the body parts of the specimens were stored at -20 ºC for subsequent analyses.

Genomic DNA isolation and polymerase chain reactions (PCR): We included 5 specimens of *P. major* from each of the 11 collection sites, resulting in a total sample size of 55 for phylogenetic analyses. While the sample size of *P. kandelakii* included a total of 25 specimens due to the detection of low numbers in some collections, Each sand fly sample included in the survey was crushed to a fine powder using liquid nitrogen in pre-cooled microcentrifuge tubes with sterile pestles prior to DNA extraction. The PureLink™ Genomic DNA Mini Kit (Thermo Scientific, Waltham, MA, USA) was used to extract the total genomic DNA (gDNA) according to the manufacturer's protocol, with a final elution volume of 35 µl. The extracted gDNA was then quantified using a Qubit 3.0 fluorometer quantitation instrument (Thermo Scientific, Waltham, MA, USA) and stored at −20 °C for downstream applications.

For phylogenetic characterization, we targeted the COI and Cytb genes of the sand fly species. The partial 709 bp segment of the COI and 788 bp of the Cytb gene regions were amplified by PCR using LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (19), and CB1-SE (5′TATGTACTACCCTGAGGACAAATATC3′) and CB-R06 (5′TATCTAATGGTTTCAAAACAATTGC 3′) (37) primers, respectively, according to the described protocols (18, 35). Electrophoresed gels were analyzed using the Fusion FX Gel Documentation System (Vilber Lourmat, France).

Sequence and phylogenetic analysis: The amplified fragments were sequenced bidirectionally with the PCR primers on the Sanger sequencing platform using the automated DNA sequencer ABI 3730XL (Macrogen Corporation, Korea). Paired nucleotide sequences of COI and Cytb from sand flies were processed and aligned using Geneious Prime 2022.1.1 software (http://www.geneious.com) in order to obtain a single consensus sequence. The obtained sequences were deposited in GenBank with the accession numbers OR511616-OR511628 for COI and OR520133-OR520139 for Cytb.

Using the BLASTn algorithm, the final sequences were searched in the GenBank database to compare fragments and generate the datasets for phylogenetic analyses of the related sand flies. The COI and Cytb datasets consisted of a total of 52 and 31 sequences, respectively. All sequences were aligned using MAFFT (22) through the plugin available in Geneious Prime. The parameters for MAFFT were set up as follows in Geneious Plugin as recommended (22); algorithm: “Auto”, scoring matrix: “200PAM/k=2”, gap open penalty: “1.53”, offset value: “0.123”. MEGA version 11 (49) was used to calculate the intra- and inter-specific genetic differences based on the Kimura-2-Parameter (K2P) distance model (25), as well as the ts/tv bias (R) in each codon. Haplotype number (K) and diversity (H), nucleotide composition, AT bias, and genetic diversity (π) indices were calculated using DnaSP v.5.1 (30). Neutrality tests, including Tajima’s D (48) and Fu’s F (16), were performed in DnaSP v.5.1.

Both maximum likelihood (ML) and Bayesian inference (BI) were used for phylogenetic analyses of sand fly isolates in the COI and Cytb datasets. The most appropriate DNA-substitution models for ML (GTR+G+I) and BI (GTR+G+I) phylogenetic analyses of the COI and Cytb datasets were selected based on Akaike’s information criterion (AIC) (42) and Schwarz's Bayesian information criterion (BIC) (46) algorithms, respectively, using jModeltest v.0.1.1 (42). Analyses were performed using PhyML (17) and MrBayes version 3.2.6 (20), respectively, through the plugins available with Geneious Prime. A bootstrap analysis was conducted in ML using 1000 replicates. For posterior probability calculations in BI, two Markov Chain Monte Carlo simulations were run

simultaneously for 10 million generations, with sampling every 200 generations. After discarding 25% of the initial trees in each run as burn-in, a majority consensus tree was constructed.

Results

Morphological identification of sand fly specimens: Based on the morphological identification results, the majority of the specimens 1596 (84.49%) and 253 (13.40%) out of the 1889 captured specimens were identified as *P. major* and *P. kandelakii*, respectively, in the study area. The detailed information about the collections and identifications is given in Supplementary Table S1. The morphological structures of the two species are shown in Figure 2. The remaining 40 specimens (%2.11) were morphologically identified as *P. papatasi, P. tobbi, P. sergenti, P. halepensis, P. alexandri*, and *Sergentomyia dentata* with 1, 3, 18, 15, 2, and 1 specimens, respectively.

COI and Cytb sequence analyses and divergence of sand flies: We successfully recovered the barcode sequences of the 658 bp region of the COI and the 711 bp region of the Cytb genes from the 55 specimens of *P. major* and 25 specimens of *P. kandelakii*. The absence of insertions, deletions, or stop codons in the COI and Cytb indicates that all sequences were functional mitochondrial products. Of the nine and four COI haplotypes detected in the study for *P. major* and *P. kandelakii,* respectively, seven from *P. major* and three from *P. kandelakii* were new to the respective sand fly species according to the blast searches in the GenBank and BOLD Systems databases. The remaining two *P. major* and one *P. kandelakii* haplotypes were previously reported from the Black Sea Region of Türkiye (Figure 3). COI-BSPmaj1 was the most common haplotype of *P. major,* comprising 21 out of 55 isolates. This was followed by the haplotypes COI-BSPmaj2 to COI-BSPmaj9, with 1 to 14 isolates within each haplotype (Figure 3). For the COI sequences of *P. kandelakii*, COI-BSPkan1 was the dominant known haplotype, with a total of 10 isolates. The new haplotypes COI-BSPkan2, COI-BSPkan3, and COI-BSPkan4 included seven, four, and four isolates, respectively (Figure 3). We identified three haplotypes (one known and two new) of *P. major* and three haplotypes (all new) of *P. kandelakii* based on the Cytb sequence analyses (Figure 3). The best-known haplotype of *P. major* is Cytb-BSPmaj1, representing a total of 37 isolates. The new haplotypes Cytb-BSPmaj2 and Cytb-BSPmaj3 included 10 and 8 isolates, respectively. Cytb-BSPkan1 was the dominant haplotype of *P. kandelakii,* represented by 20 isolates and the remaining two haplotypes, Cytb-BSPkan2 and Cytb-BSPkan3, were presented by three and two isolates, respectively (Figure 3).

The fragments of both COI and Cytb base compositions in the assembled datasets containing 51 and 31 sequences, respectively, showed significant variation with a total of 39.4% and 37.7% polymorphic sites among the species of the subgenus *Larroussius* of the genus *Phlebotomus*. The AT and GC composition of the entire datasets ranged from 68.4 to 64.4% and 31.6–35.6% for COI and 71.5 to 74.6% and 25.4–28.5% for Cytb, respectively, with an AT bias. The transition/transversion bias (R) was higher in the 2nd (19.59) codon position, followed by the 1st (4.39) and 3rd (1.64) codon positions for the COI dataset. While the R was higher at the 2nd (5.87) and 3rd (4.94) codon positions compared to the 1st (1.29) position for the Cytb dataset, The genetic diversity indices and the results of the neutrality tests for the COI and Cytb datasets are shown in Table 1. The overall haplotype and nucleotide diversities were 0.99 and 0.16 for COI and 0.99 and 0.16 for Cytb, respectively, among the *Larroussius* species, with low levels of haplotype and nucleotide diversity within each species. Tajima's D (48) and Fu's F (16) were significant ($P < 0.05$) only for *P. major* taxa.

The pairwise genetic distance matrix of the COI and Cytb gene regions among the *Larroussius* species is given in the supplementary tables (S2 and S3). The mean intraspecific genetic distance for the COI sequences of *P. major* was determined to be 0.4%, and our sequences showed 98.9% to 100.0% identity with isolates reported from the Middle Black Sea Region of Türkiye (GenBank accessions: OQ826546, ON093827, ON093829, and MN086538). The *P. major* haplotypes also showed 99.1% to 99.8% similarity with the *P. neglectus* isolates from the southern region of Türkiye (GenBank accession: OL352136) and Leros, Greece (GenBank accession: OL352154). The analyses of the COI sequences of *P. kandelakii* in the dataset showed a mean intraspecific genetic distance of 1.0%, and our haplotypes were found to be 96.7% to 100.0% identical to the haplotypes reported from the West Black Sea Region of Türkiye (GenBank accessions: ON093832, ON093833, ON093835, MN086479, MN086487, and MN086490).

Cytb sequence analyses of *P. major* revealed a mean intraspecific genetic distance of 1.4% in the dataset and our sequences showed 99.7% to 100.0% identity with haplotypes reported from several sites in the West Black Sea Region of Türkiye (GenBank accessions: ON097122, ON097127). The identified Cytb haplotypes were also 95.5% to 95.6% identical to the Iranian specimen (GenBank accession: GQ169334). Analysis of the *P. kandelakii* Cytb dataset revealed a low level of intraspecific genetic distance (0.2%) between the haplotypes identified in the study and published haplotypes from the West Black Sea Region of Türkiye (GenBank accessions: OQ846925, ON097120).

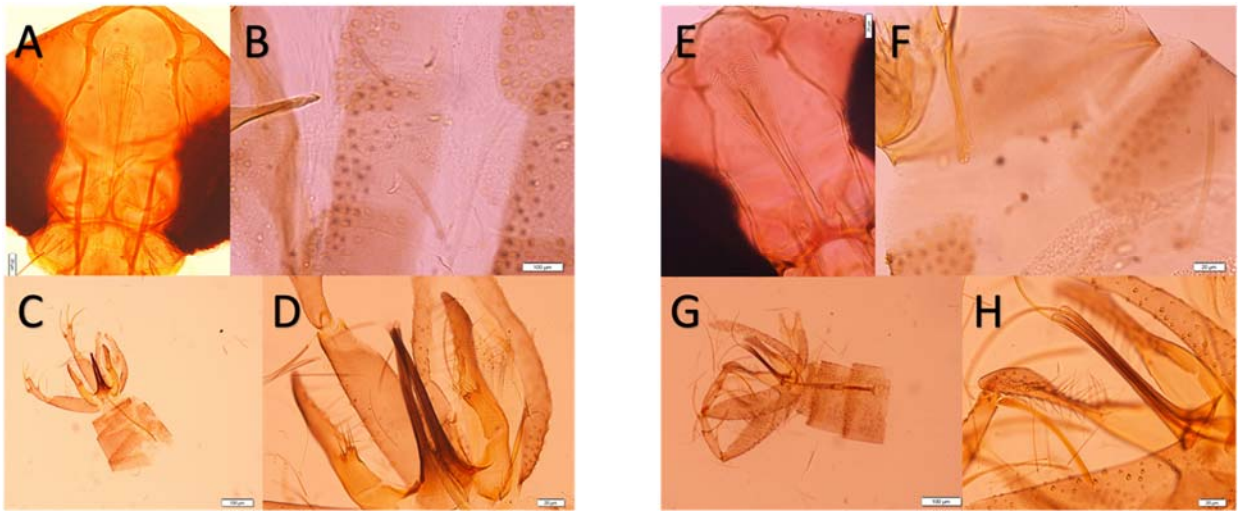

Figure 2. Morphological characteristics of *Phlebotomus kandelakii* and *P. major*. *P. kandelakii* female.
(A-B): pharynx and cibarium (A), spermathecal body (B); *P. kandelakii* male genitalia (C-D); *P. major* female (E-F): pharynx and cibarium (E), spermathecal body (F); *P. major* male genitalia (G-H).

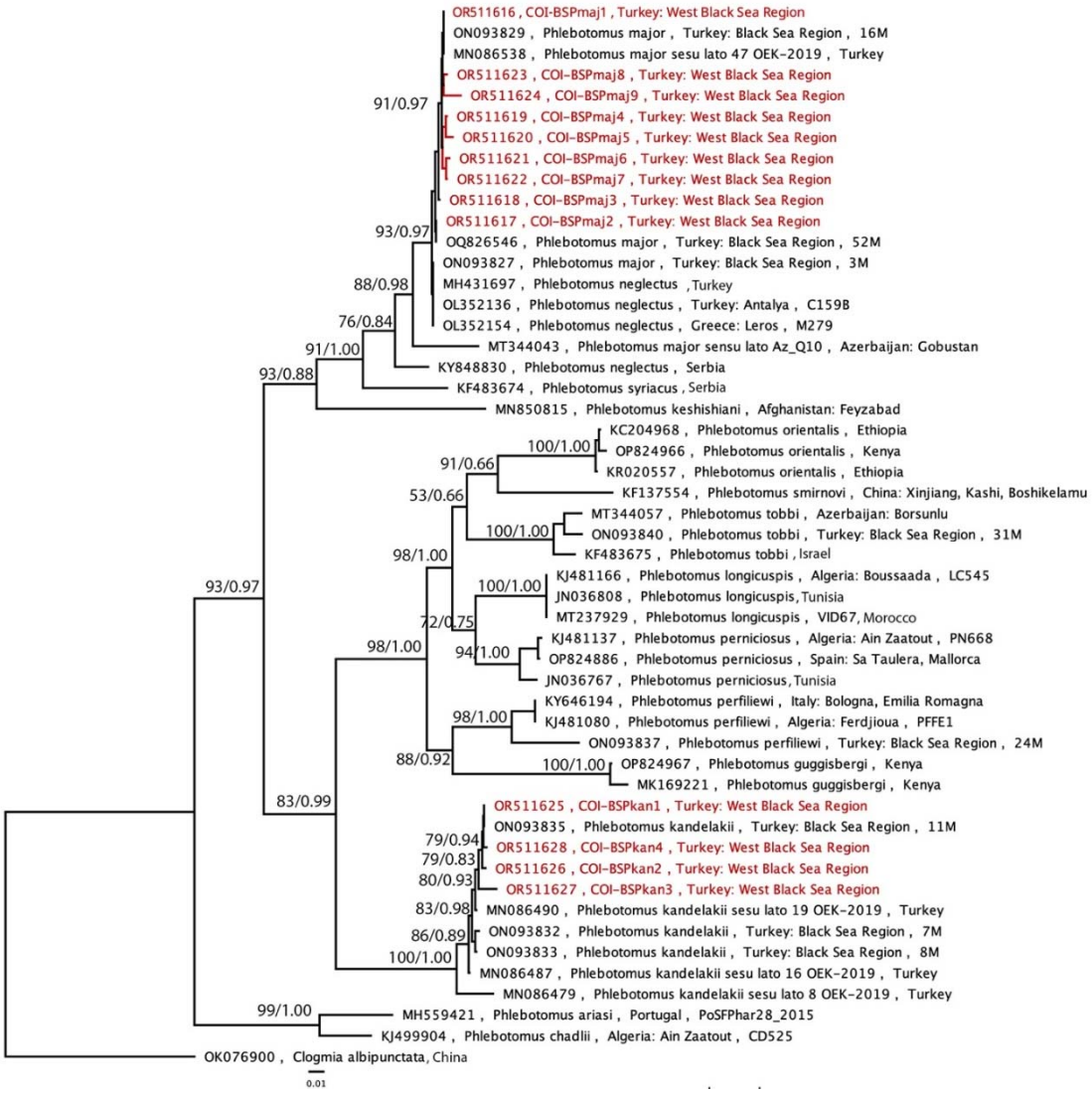

Figure 3. COI phylogenetic tree of the species of *Larroussius* subgenus including the haplotypes of *P. major* and *P. kandelakii* identified in the study (in red). Numbers at the nodes represent the bootstrap values (1000 replicates) and posterior probabilities, respectively. The sequences were given as GenBank accession number, country and isolate name if available. *Clogmia albipunctata* sequence was used as the outgroup. The scale bar represents 0.01% divergence.

Table 1. Summary of genetic diversity indices and results of neutrality tests (Tajima's D and Fu's F) in the mitochondrial COI and Cytb datasets of the species of *Larroussius* subgenus.

Species	n	k	K	h (±SD)	Mean π	Tajima's*D*	Fu's *F*
COI gene							
P. major	15	33	11	0.933 ± 0.054	0.00848	-2.04*	-2.83*
P. kandelakii	10	26	9	0.978 ± 0.054	0.00969	-1.46	-1.96
P. ariasi	1	1	N	N	N	N	N
P. chadlii	1	1	N	N	N	N	N
P. guggisbergi	2	2	5	1.000 ± 0.500	0.0076	N	N
P. keshishiani	1	1	N	N	N	N	N
P. longicuspis	3	1	N	N	N	N	N
P. neglectus	5	2	20	0.400 ± 0.237	0.01216	-1.32	-1.23
P. orientalis	3	3	3	1.000 ± 0.272	0.00304	N	N
P. perfiliewi	3	2	25	0.667 ± 0.314	0.02533	N	N
P. perniciosus	3	3	11	1.000 ± 0.272	0.01114	N	N
P. smirnovi	1	1	N	N	N	N	N
P. syriacus	1	1	N	N	N	N	N
P. tobbi	3	3	19	1.000 ± 0.272	0.01925	N	N
Overall	52	172	31	0.994 ± 0.022	0.09330	0.04	0.07
Cytb gene							
P. major	7	4	31	0.714 ± 0.181	0.01308	-1.65*	-1.88*
P. kandelakii	5	5	4	1.000 ± 0.126	0.00225	-1.09	-1.11
P. ariasi	2	2	1	1.000 ± 0.500	0.00141	N	N
P. longicuspis	1	1	N	N	N	N	N
P. neglectus	4	4	8	1.000 ± 0.177	0.00587	-0.45	-0.44
P. orientalis	1	1	N	N	N	N	N
P. perfiliewi	4	4	32	1.000 ± 0.177	0.02981	1.46	1.70
P. perniciosus	3	3	16	0.667 ± 0.314	0.01502	N	N
P. tobbi	3	3	8	1.000 ± 0.272	0.00751	N	N
P. langeroni	1	1	N	N	N	N	N
Overall	31	27	206	0.985±-0.015	0.11652	0.54	0.76

n: number of sequences; k: number of variable sites; K: number of haplotypes; *h*: haplotype diversity; π: nucleotide diversity; N: value could not be calculated due to insufficient data ∗significant at the 0.05 level.

Phylogenetic analysis: Figures 3 and 4 show the consensus trees generated by maximum likelihood (ML) analyses with the corresponding nucleotide substitution models for the COI and Cytb datasets, which included the alignment of 52 and 31 nucleotide sequences, respectively. A similar topology for both gene regions was produced by the tree based on Bayesian inference (BI). Therefore, posterior probabilities were presented with bootstrap values on the ML trees. We used COI and Cytb sequences of a non-hematophagous insect, *Clogmia albipunctata,* within the same family (Psychodidae: Diptera) as an outgroup taxon to generate the dendrogram for phylogenetic analyses.

The COI phylogenetic tree revealed the presence of three major clusters within the subgenus *Larroussius*, supported by a bootstrap value of 83%-99% and a posterior probability of 0.88-1.00. The first cluster included *P. major*, *P. neglectus*, *P. syriacus,* and *P. keshishiani*. The phylogenetic resolution of this cluster was also supported by bootstrap values of 88%-93% and posterior probabilities of 0.88-1.00, except for the sister relationship between the clade containing the *P. neglectus* from Serbia (GenBank accession: KY848830) and the clade containing all *P. major* and *P. neglectus* haplotypes, which had a relatively moderate bootstrap (76%) and posterior probability (0.84) support. The second cluster was divided into two sub-clusters. The first sub-cluster included only *P. kandelakii* haplotypes from the Black Sea Region of Türkiye. The phylogenetic resolution in this sub-cluster was strongly supported, with a bootstrap value of 100.0% and posterior probabilities of 1.00. The second sub-cluster contained the monophyletic taxa consisting of

P. orientalis, *P. smirnovi*, *P. tobbi*, *P. longicuspis*, *P. perniciosus*, *P. perfiliewi,* and *P. guggisbergi*. The phylogenetic resolution of this sub-cluster was also supported by bootstrap values of 94%-100% and posterior probabilities of 1.00. The third major cluster was an outer taxon of the first and second major clusters and included the *Larroussius* species *P. ariasi* and *P. chadlii*. The phylogenetic relationships in this cluster had a high bootstrap value of 99% and a posterior probability of 1.00 (Figure 3).

Phylogenetic analyses of the Cytb dataset revealed two major clusters within the subgenus *Larroussius*, supported by bootstrap values of 89%-100% and posterior probabilities of 0.89-1.00. The first cluster was subdivided into two sub-clusters. The first sub-cluster was comprised of the *P. major* haplotypes and the second one included the *P. neglectus* haplotypes from several countries. The monophyletic resolution of both sub-clusters was well supported by bootstrap values of 94%-98% and posterior probabilities of 0.96–1.00, except for the sister relationship of the *P. major* Iran specimen (GenBank accessions: GQ169334), which exhibited a moderate bootstrap (61%) and posterior probability (0.70). The second cluster is also divided into two monophyletic sub-clusters and supported by bootstrap values of 89%-100% and posterior probabilities of 0.91-1.00. The first sub-cluster included *P. kandelakii* haplotypes and the second included *P. tobbi*, *P. langeroni*, *P. perniciosus*, *P. longicuspis*, *P. orientalis*, *P. perfiliewi,* and *P. ariasi*. *P. ariasi* was placed as an outer taxon within the second sub-cluster (Figure 4).

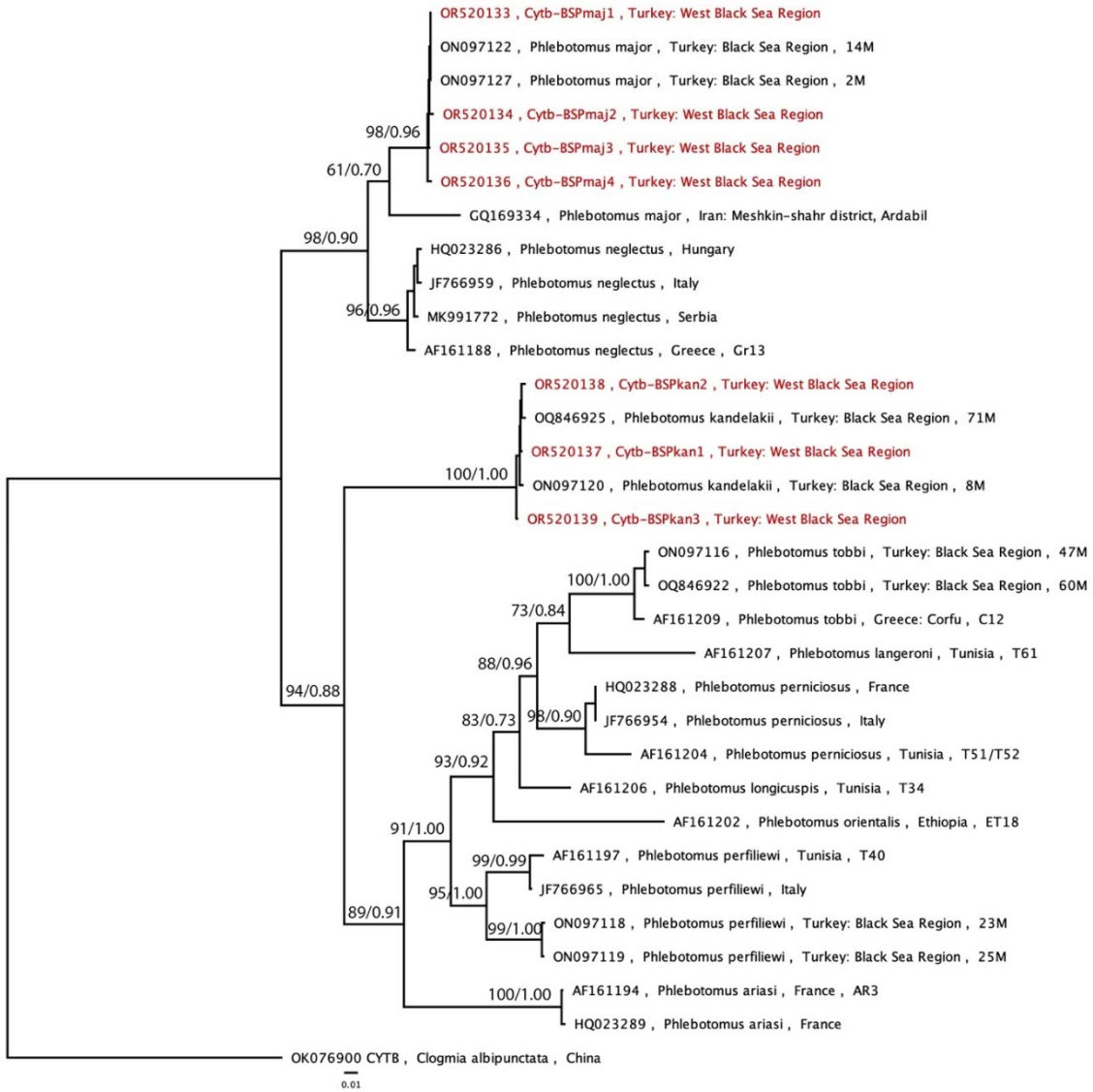

Figure 4. Cytb phylogenetic tree of the species of *Larroussius* subgenus including the haplotypes of P. major and P. kandelakii identified in the study (in red). Numbers at the nodes represent the bootstrap values (1000 replicates) and posterior probabilities, respectively. The sequences were given as GenBank accession number, country and isolate name if available. *Clogmia albipunctata* sequence was used as the outgroup. The scale bar represents 0.01% divergence.

Discussion and Conclusion

The members of *Phlebotomus major* s.l. are one of the most prevalent sand fly species in almost all geographical regions of Türkiye (21, 38). The members of this complex are known as competent vectors of *L. infantum* and also have an overlapping distribution with the endemic area of VL around the world (21, 28, 33, 45). The major group of *Larroussius* has had a complex taxonomy since its first description by Annandale (1910) and currently six species are recognized within this taxon, including *P. major, P. neglectus, P. syriacus, P. wui, P. notus*, and *P. wenyoni* (3, 13). The females of *P. major* s.l. are mainly distinguished from other *Larroussius* species mainly by the shape of their pharyngeal armatures and this typical morphological character was observed in all *P. major* s.l. specimens in our study. The male morphological characters, including the aedeagus, palpal formulae, length of the style to coxite, and pharyngeal armature, are the only known characters used for the identification of the corresponding six species of major complex (3, 29). The morphological analyses of the male specimens of *P. major* s.l. in our study revealed the same characteristics of the aedeagus, the length of the style to coxite, and the pharyngeal armature as those of the species *P. major*. Nevertheless, the taxonomic situation of this complex is not yet resolved and the status of the described species as valid or conspecific within taxa is still unclear.

Both mitochondrial COI and Cytb gene fragments are considered to be valid molecular markers for distinguishing several sand fly species, such as *P. chinensis, P. stantoni, P. papatasi, P. sergenti, P. ariasi* and *P. tobbi* (7, 9, 46). DNA barcoding using these mitochondrial markers has been widely used to characterize and identify sand flies (11, 15, 18, 26, 27, 31, 41). The barcoding gap between the species in most phlebotomine taxa was considered to contain sufficient sequence diversity for species delimitation (7, 8, 31, 34). In the current study, both mitochondrial markers were evaluated for their ability to discriminate between the species of the subgenus *Larroussius* using the characterized sequences of *P. major* and *P. kandelakii* and the sequences of related taxa within the subgenus. Considering the major complex, a low level of intraspecific genetic distance (0.2% to 0.9%) was found between *P. major* COI sequences. However, some of the *P. neglectus* sequences from different parts of Türkiye and Greece (OL352154, OL352136, and MH431697; Figure 3) showed interspecific genetic differences <0.9, with the *P. major* sequences indicating an overlap with the intraspecific difference. On the other hand, the Serbian isolate of *P. neglectus* (KY848830; Figure 3) showed 3.2% to 4.0% intraspecific genetic differences with *P. major*. The only available COI sequence of *P. syriacus* from Israel (KF483674; Figure 3) also showed a 5.9% to 7.2% difference with *P. major* sequences. All these data suggest that the COI sequence can be used as a DNA barcode to distinguish *P. syriacus* from *P. major* and *P. neglectus*. It appears that the overlap between the COI sequences does not allow *P. major* and *P. neglectus* to be distinguished. However, the overlapping sequences of *P. neglectus* from Türkiye and Greece could also be related to misidentification. In fact, the Serbian isolate of *P. neglectus* has a genetic difference of more than 3.0% from *P. major*, which serves as a suitable barcode gap between the species. Further data based on the combination of morphological and molecular analyses is needed to clarify the efficiency of COI-based barcoding within this complex. With regard to the Cytb sequence analyses, it appears that *P. neglectus* differs from *P. major* with 5.2% to 8.0% genetic distance and the phylogenetic tree clustered the isolates into monophyletic clades (Figure 4). Even though the *P. major* haplotypes from Türkiye were close to each other with an overall identity of 99.8%, the Iranian *P. major* isolate showed a mean genetic difference of 4.5% from the Turkish haplotypes and formed a separate clade in a phylogenetic tree (Figure 4). There is also a lack of information on the Cytb sequence characterization of other members of the major complex in GenBank. While the data obtained in our study provides initial evidence for the usefulness of Cytb-based barcoding to distinguish the members of the major complex, the lack of sequence information from other species within the taxa limits the comprehensive evaluation of Cytb barcoding.

The second widespread sand fly species in the study area is *P. kandelakii*, which is also capable of transmitting *L. infantum* (14, 33, 45). Data on this species of COI and Cytb sequence diversity were limited, with only a few sequences mainly from different regions of Türkiye. The phylogenetic analyses of the COI and Cytb datasets clearly indicated a monophyletic taxon for this species with an overall identity of 99.0 for COI and 99.7% for Cytb among the haplotypes of *P. kandelakii*. Both COI and Cytb trees clustered the *P. kandelakii* haplotypes in a separate clade closer to the cluster comprised of *P. orientalis, P. smirnovi, P. tobbi, P. longicuspis, P. perniciosus, P. perfiliewi,* and *P. guggisbergi* rather than the cluster of major complex species.

Although we determined several haplotypes of *P. major* and *P. kandelakii* based on COI and Cytb sequences, the overall haplotype and nucleotide diversities were low for both species. Different statistical tests have been utilized to test the selective neutrality and population growth of several organisms (44). We used two frequently used tests to analyze population growth that have variable power: Tajima's D-test and Fu's F-test (43). The outputs indicated negative values for both *P. major* and *P. kandelakii* with P-values < 0.05. This result might indicate an excess of recently derived haplotypes of both species and an excess of low-frequency polymorphism in the

populations, which was also observed in other sand fly populations in different regions, such as *P. sergenti* (12).

In conclusion, our results contribute to the current knowledge of the species' genetic diversity in the subgenus *Larroussius* of sand flies. We also provide further data on the utility and usefulness of COI and Cytb barcoding for delimiting species within this subgenus, focusing on the two widespread species, *P. major* and *P. kandelakii*. Further investigations with large-scale samplings from different regions using both morphological and molecular approaches are proposed to clarify the genetic diversity and taxonomic status of the members of the major complex.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

GKK, GZP, MA, DP, KY, GY, and SU planned and carried out the field samplings. GKK, MA, KY, YO, ST, ZO, and AI contributed to the preparation of slides and morphological identifications. GKK, OD, AY, MA, and GY carried out the DNA extraction, PCR, and sequencing. GKK, AY, AC, and GZP contributed to the molecular and phylogenetic analyses. GKK, YO, and AI provided a conception of research, methodology, and supervision. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

Data Availability Statement

Accession numbers given by NCBI for *P. major* and *P. kandelakii* isolates identified in this study (https://www.ncbi.nlm.nih.gov/) are OR511616-28 and OR520133-9.

Ethical Statement

This study does not present any ethical concerns.

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