Original Article

Identification of Sand flies of the Subgenus *Larroussius* based on Molecular and Morphological Characters in North Western Iran

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Abstract

Background: The adult female sand flies (Diptera: Psychodidae) of the subgenus *Larroussius* are important vectors of *Leishmania infantum* (Kinetoplastida: Tripanosomatidae) in Meshkinshahr district, Northwest of Iran. Four *Phlebotomus* (*Larroussius*) species are present in this area, i.e. *Phlebotomus* (*Larroussius*) *kandelakii*, *P.* (*La.*) *major*, *P.* (*La.*) *perfiliewi* and *P.* (*La.*) *tobbi*. The objective of the present study was to identify and distinguish the females of *P. perfiliewi*, *P. major* and *P. tobbi*, in this district.

Methods: Adult sand flies were collected with sticky papers, CDC light traps, and aspirator in 2006. Individual sand flies of this four species from thirty different locations were characterized morphologically and by comparative DNA sequences analyses of a fragment of mitochondrial gene Cytochrome b (Cyt b) and nuclear gene Elongation Factor 1-alpha (EF-1α). PCR amplification was carried out for all three species *P. major, P. perfiliewi* and *P. tobbi* in the subgenus *Larroussius*.

Results: Phylogenetic analyses of *P. major* populations in this study displayed two different populations and genetic diversity. Spermathecal segment number, pharyngeal armature and other morphological characters of these three species were examined and found to present consistent interspecific differences.

Conclusion: According to our findings, the phylogeny of Cyt b and EF-1α haplotypes confirms the relationships between *P. major, P. tobbi* and *P. perfiliewi* as already defined by their morphological similarities.

Keywords: *Phlebotomus*, *Larroussius*, Cytochrome b, Elongation Factor-1α, Morphology, Iran

Introduction

The blood-feeding female of phlebotomine sand flies (Diptera: Psychodidae, Phlebotominae) include natural vectors of protozoa of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae), which are the parasitic causative agents of mammalian leishmaniasis (Killick-kendrick 1990). There are approximately 700 species of phlebotominae sand flies divided among 6 genera, of which only two, i.e. *Phlebotomus* in the old world (OW) and *Lutzomyia* in the new world (NW) are medically importance (Lane 1987, Lewis 1982, Lane 1993, Sharma and Singh 2008).

Only 10% of these Phlebotominae sand flies act as disease vector. Further, only 30 species of these are important from public health point (Sharma and Singh 2008). A total of about 21 *Leishmania* spp. have been identified to be pathogenic to human (Singh

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2006). However, recent studies based on molecular data have provided evidence that the genus *Phlebotomus* is not monophyletic (Depaquit et al. 1998). Furthermore, Rispail and Leger (1998a) have recently revised the definition of morphological characters states. In the Mediterranean Region, species of the subgenus *Larroussius* are the main vectors of *Leishmania infantum*, which cause Visceral Leishmaniasis (VL) in humans and dogs (Killick-kenrick 1990, Guernaoui et al. 2005). The subgenus is readily identified by the development long extension of the spermathecal neck in females (Rispail 1990).

Description of the subgenus *Larroussius* created by Nitzulescu (1931) with *P. major* Annandale, 1910 as the type species (Perfiliev 1968, Lewis 1982).

Among 27 species of Phlebotominae sand flies in the OW (Artemiev and Neronov 1984, Rispail and Leger 1998a, Rispail and Leger 1998b, Secombe et al. 1993), at least 12 species of the subgenus *Larroussius* are proven or probable vectors of leishmaniasis (Killick-kendrick 1990). The subgenus *Larroussius* is closely related to the subgenera *Transphlebotomus* and *Adlerius*, and the proliferation of species within *Larroussius* and *Adlerius* is probably recent (Rispail and Leger 1998b, Rispail and Leger 1991).

Although, identification of male specimens of *Larroussius* are not very difficult, but determination of some females of this subgenus has been considered impossible based on morphological characters.

Leger et al. (1983) showed that three sympatric species of *Larroussius* in Greece could readily be separated by the morphology of base of the spermathecal ducts.

Killick-Kendrick et al. (1991) separated 13 species of *Larroussius* by the morphology of the base of the spermathecal ducts. Recent studies showed that molecular tools could help resolve phylogenetic relationships between species of subgenus *Larroussius* (Esseghir et al. 1997, Esseghir et al. 2000, Muccio et al. 2000, Pesson et al. 2004, Parvizi and Assmar 2007).

There are four species of subgenus *Larroussius* including *P. kandelakii*, *P. perfiliewi*, *P. major* and *P. tobbi* in Meshkinshahr district, north western Iran. The first species is a proven vector and the second is the probable vector of viscerotropic *Leishmania* spp. in this area (Rassi and Javadian, 1998, Rassi and Javadian 1999, Rassi et al. 2001a, Rassi et al. 2001b, Rassi et al. 2005).

The objective of the present study was to identify and distinguish the females of *P. perfiliewi*, *P. major* and *P. tobbi*. They are similar in morphological characters and indistinguishable. We analyzed the sequences of cytochrome b (mtDNA) and elongation factor-1α (nDNA) genes of these three species and compared with morphological characteristics. Complete phylogenetic information is available for these genes and they are useful for the study of molecular systematic and speciation.

Material and methods

Study area

Meshkinshahr District (48° 17′ N, 38° 15′ E) is located at 1890 masl in Ardebil Province, Iran. The district occupies the northern foothills of the Sabalan Mountains, which rise to an altitude 4881 masl. Temperature varies from -27 °C in winter to 41 °C in summer. The human population was 156141 in 2006 and the principal economic activity is sheep farming.

Sand fly sampling

Sand fly sampling was carried out from Jun-October 2006 (during the period of peak activity), in 30 villages distributed throughout the Meshkinshahr District. Sand flies were collected once every 15 d from indoor habitats (bedroom, stable, toilet, bath room, hen nest, hay loft and store room) and outdoor habitats (yard, rodent burrow, stone and wall

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crevices, fox burrows and dog kennel, rubble and riverbanks) using sticky papers, CDC light trap and Aspirator. Sand fly specimens were stored in 96% ethanol.

Dissection, Mounting and Morphological identification

After recording the sampling data and locations, sand fly specimens were washed in 1% detergent then twice in sterile distilled water. Each specimen was then dissected in fresh drop of sterile normal saline by cutting off the head and abdominal terminalia with sterilized forceps and single used mounted needles. The remainder of the body was stored in the sterile eppendorf microtubes.

Specimens were mounted in Puri's or Berlese's medium and identified using the identification keys of Theodor and Mesghali (1964), Perfiliev (1968), and Lewis (1982). Morphological characters, which used in this study, included pharyngeal armature, spermathecal segments number, length of spermathecal neck, palpal and ascoids formula. First and second are more important characters.

DNA extraction

DNA was extracted from the dissected thorax and attached anterior abdomen of individual sand flies using the method of Ish-Horowiz (Ready et al. 1991). In the 1.5 ml microtubes, the thorax plus anterior abdomen of each sand fly was frozen and defrosted twice to break up tissue using a sampler tips or pestel, with grinding mix. Then SDS mix was used to denature proteins associated with the DNA, then ice cold 8M KOAc was added to remove effectively the SDS proteins from solution. Cell debris and proteins were separated from the DNA by centrifugation then the DNA in the supernatant was precipitated over night at -20 °C in 96% ethanol. Following ethanol precipitation, the DNA was dissolved in 15µl 1X TE (10mM Tris-HCl, 1mM EDTA pH= 8.0) and stored at -20 °C.

PCR amplification of Cyt b and EF-1α

For Cyt b one pair primers were used. CB3-FC (forward) (5′-CA(C/T) ATTCAA-CC (A/T)GAATGATA-3′) with CB-R06 (reverse) (5′-TATCTAATGGTTTCAAAACA ATTGC-3′) to amplify an overlapping 3′ fragment of 499 bp without primers (CB3 fragment). Also for EF-1α one pair primers were used, EF-F05 (forward) (5′-CCTGG ACAT-CGTGATTTCAT-3′) with EF-F08 (reverse) (5′-CCACCAATCTTGTAGACATCCTG-3′) to amplify of 454 bp without primers.

The PCR reaction conditions were identical for both Cyt b and EF-1α. 2µl 10X PCR buffer, 1.2 µl $MgCl_2$, 0.15µl primers (F and R), 2µl dNTPs, 1.5µl DNA with the reaction volume completed to 20 µl by distilled water, followed by initial denaturation of Cyt b at 94 °C for 3 min. PCR consisted of 35 cycles of denaturation at 94 °C for 30 sec, annealing1 at 40 °C for 30 sec, annealing 2 at 44 °C for 30 sec, extension at 72 °C for 90 sec and then final extension at 72 °C for 10 min. For EF-1α, initial denaturation at 94 °C for 3 min. PCR consisted of 35 cycles of denaturation at 94 °C for 30 sec, annealing at 48°C for 30 sec, extension at 72 °C for 30 sec and then final extension at 72 °C for 10 min.

Direct sequencing of PCR products

One hundred nanograms of each purified DNA sample was cycle-sequenced using an ABI Parsim® Big Dye™ Terminator cycle sequencing Ready Reaction Kit (version 2.0) and ABI 373/377 sequencing systems (ABI, PE Applied Biosystems), with 3.2 Pmol of the same primers that were used for PCR.

Aligning and phylogenetic analysis of DNA sequences

DNA sequences from both strands were aligned and edited using Sequencher Demo 4.7 and BioEdit softwares. Multiple or pairwise sequence alignments of DNA were used with CLUSTAL W PPC: Clustalw version 1.7. Phylogenetic analyses were done using

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Parsimony PAUP. Relationships were inferred based on genetic distances using the Neighbor Joining (NJ) option with default settings.

Results

Species composition of subgenus *Larroussius*

Out of the 1743 sand fly specimens collected, 660 specimens (37.9%) belonged to the subgenus *Larroussius*, including: *Phlebotomus* (*La.*) *kandelakii* (31%), *P.* (*La.*) *major* (1.5%), *P.* (*La.*) *tobbi* (1.5%), *P.* (*La.*) *perfiliewi* (1.7%) and *P.* (*La.*) *major* group (2.1%).

Aligning and phylogenetic analysis of *Larroussius* DNA sequences

PCR amplification of Cyt b and EF-1α was successfully achieved for all 3 species of the subgenus *Larroussius*.

Seven sequences for Cyt b and four for EF-1α were compared for *P. major*. Comparison of pairwise genetic similarity or score of sequences showed 87%-100% similarity for Cyt b and 96%-100% similarity for EF-1α. Also 2.7% genetic diversity for Cyt b and 2.8% for EF-1α was observed.

There were two haplotypes for Cyt b from seven sequences (Table 1) and for EF-1α, there were two haplotypes within four sequences (Table 2). The Neighbor-joining (NJ) phylogram for Cyt b showed two lineages, and each of these had subgroups with short branches. One of the lineages had one haplotype from sand flies from the same habitat (Stone crevices) but different locations (Ur kandi, Mueel, Agh daragh). The second lineage had one haplotype from sand flies from different habitats (Rodent burrow and Fox burrows) and locations (Ghurt tappeh, Alni, Niaz suee) (Fig. 1). In addition, phylogenetic tree for EF-1α showed two lineages and only one haplotype had subgroups. One of the lineages had one haplotype from sand flies from the same location (Ur kandi) but different habitats (Stone crevices, bedroom, and hen nest). The second lineage had one haplotype from one specimen in rodent burrow (Fig. 2).

Seven sequences for Cyt b and three for EF-1α were compared for *P. tobbi*. Comparison of pairwise genetic similarity of sequences indicated the 100% similarity for both Cyt b and EF-1α sequences. Genetic diversity and unique haplotype for Cyt b and EF-1α were not observed. For Cyt b and EF-1α one haplotype was obtained (Table 1 and 2). The Neighbor-joining (NJ) phylogram for both Cyt b and EF-1α showed one lineage for all specimens in the same location and habitat (Fig. 1 and 2).

Six sequences for Cyt b and three for EF-1α were compared for *P. perfiliewi*. Comparison of pair wise genetic similarity of sequences indicated 100% similarity for both Cyt b and EF-1α sequences. In this species genetic diversity for Cyt b and EF-1α were not observed (Table 1 and 2). Phylogenetic tree for both Cyt b and EF-1α showed one lineage for all specimens in the different location and habitats (Fig. 1 and 2). The Neighbor-joining (NJ) phylogram in combination of Cyt b and EF-1α for seven specimens showed two lineages for *P. perfiliewi* and *P. tobbi* and one lineage for *P. major* (Fig. 3).

Identification of the female *Larroussius* species using morphological characters

In the present study, the morphological characteristics of the three species female of *Larroussius* were described as follows:

Phlebotomus major and *Phlebotomus neglectus*

Palpal formula: 1, 4, 2, 3, 5 and the formula of ascoids: 2/3-8, 1/9-5. Pharyngeal armature has occupied ⅓ of pharynx space and punctiform, arranged in several rows and anterior elements have serrated margin (Fig .4). Spermatheca with 14-16 segments and length of spermatheca neck ⅔ of spermatheca capsule (Fig. 5).

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Phlebotomus tobbi

Palpal formula: 1, 4, 2, 3, 5 or 1, (2, 4), 3, 5 and the formula of ascoids: 2/3-8, 1/9-15. Pharyngeal armature has occupied over ½ of pharynx space and punctiform, arranged in several concave irregular rows of large dots and anterior part of pharynx have a fine dots (Fig. 6). Spermatheca with 11-13 segments. Length of spermatheca neck as long as spermatheca capsule (Fig. 7).

Phlebotomus perfiliewi

Palpal formula: 1, 4, 2, 3, 5 and the formula of ascoids: 2/3-9, 1/10-15. Pharyngeal armature has occupied over ½ of pharynx space and punctiform, arranged in several concave regular rows of large dots (Fig. 8). Spermatheca with 17-20 segments. Length of spermatheca neck ½ of spermatheca capsule (Fig. 9).

Identification of the male *P. major* and *P. neglectus* using morphological characters

Among 26 male specimens in this species 17 specimens of *P. major* (65.4%) with 20-30 ventrally directed and long hairs stand densely on coxite and Palpal formula 1, 4, (2, 3), 5 (Fig. 10 and 11). In 9 specimens of *P.neglectus* (34.6%) with less than 20 ventrally directed hairs, widely spaced and sparser and Paplal formula 1, 4, 2, 3, 5 (Fig. 12 and 13).

Discussion

According to the results of previous studies, the vectors of viscerotropic *Leishmania* spp. in OW mainly belong to the subgenera *Larroussius*, *Adlerius*, *Euphlebotomus*, *Synphlebotomus* and *Paraphlebotomus*. In the subgenus *Larroussius*, *P.perniciosus* in Algeria, France, Italy, Malta, Spain, *P.ariasi* in France, Spain, Italy, *P. perfiliewi* in Italy, East Mediterranean, North of Africa, Greece, Azerbaydzhan, Tunisia, *P. tobbi* in Cyprus, East Mediterranean, Sicil, *P. kandelakii* in Afghanistan, Russia, and *P. neglectus* in Greece, Albania, Portugal (Killick-kendrick 1990). In North West of Iran *P. kandelakii* and *P. perfiliewi transcaucasicus* are proven and probable vector of viscerotropic *Leishmania* spp. (Rassi et al. 2005).

Table 1. Sequences comparison of Cyt b-mtDNA gene in the subgenus *Larroussius*

Species	Total Sequences	Pairwise genetic similarity (%)	Total aplotype	Unique haplotypes	Genetic diversity (%)
P. major	7	87 – 100	2	0	2.7
P. tobbi	7	100	1	0	0.3
P. perfiliewi	6	100	1	0	0

Table 2. Sequences comparison of EF-1α-nDNA gene in the subgenus *Larroussius*

Species	Total Sequences	Pairwise genetic similarity (%)	Total aplotype	Unique haplotypes	Genetic diversity (%)
P. major	4	96-100	2	0	2.8
P. tobbi	3	100	1	0	0
P. perfiliewi	3	100	1	0	0

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Table 3. Collected sand flies of the subgenus *Larrossius* in different location and habitats in Meshkinshahr District, Iran

Species	Code No.	Sex	Location of capture	Altitude of capture (masl)	Habitat	Date of capture	Method of catching	Type of Gene	Genbank accession number
P.major	MSH-1201	Male	Agh daragh	1390	Stone crevices	05.08.06	SP	Cyt b	GQ169331
P.major	MSH-297	Male	Ur kandi	1551	Stone crevices	09.07.06	SP	Cyt b	GQ169332
P.major	MSH-619	Male	Mueel	2780	Stone crevices	10.07.06	SP	Cyt b	GQ169333
P.major	MSH-861	Male	Niaz suee	1080	Fox burrows	22.07.06	SP	Cyt b	GQ169334
P.major	MSH-007	Female	Alni	1267	Fox burrows	16.06.06	SP	Cyt b	GQ169335
P.major	MSH-052	Male	Ghurt tappeh	1547	Rodent burrow	17.06.06	SP	Cyt b	GQ169336
P.major	MSH-1397	Male	Mueel	2780	Stone crevices	07.08.06	SP	Cyt b	GQ169337
P. neglectus (GenBank)								Cyt b	AF161191
P. neglectus (GenBank)								Cyt b	AF161193
P.perfiliewi	MSH-721	Male	Dushan lu	1209	Rodent burrow	22.07.06	SP	Cyt b	GQ169338
P.perfiliewi	MSH-738	Male	Dushan lu	1209	Rodent burrow	22.07.06	SP	Cyt b	GQ169339
P.perfiliewi	MSH-756	Female	Dushan lu	1209	Yard	22.07.06	CDC	Cyt b	GQ169340
P.perfiliewi	MSH-795	Female	Niaz suee	1080	Yard	22.07.06	CDC	Cyt b	GQ169341
P.perfiliewi	MSH-812	Female	Niaz suee	1080	Yard	22.07.06	CDC	Cyt b	GQ169342
P.perfiliewi	MSH-836	Male	Niaz suee	1080	Stone crevices	22.07.06	SP	Cyt b	GQ169343
P.perfiliewi (GenBank)								Cyt b	AF161201
P.tobbi	MSH-480	Male	Ghurt tappeh	1547	Rodent burrow	09.07.06	SP	Cyt b	GQ169344
P.tobbi	MSH-551	Male	Ghurt tappeh	1547	Stone crevices	09.07.06	SP	Cyt b	GQ169345
P.tobbi	MSH-903	Male	Ghurt tappeh	1547	Stone crevices	23.07.06	SP	Cyt b	GQ169346
P.tobbi	MSH-1292	Male	Ghurt tappeh	1547	Stone crevices	06.08.06	SP	Cyt b	GQ169347
P.tobbi	MSH-1481	Male	Ghurt tappeh	1547	Stone crevices	20.08.06	SP	Cyt b	GQ169348
P.tobbi	MSH-1657	Male	Ghurt tappeh	1547	Stone crevices	03.09.06	SP	Cyt b	GQ169349
P.tobbi	MSH-899	Female	Ghurt tappeh	1547	Stone crevices	23.07.06	SP	Cyt b	GQ169350
P,tobbi (GenBank)								Cyt b	AF161212
P.tobbi (GenBank)								Cyt b	AF161210
P.caucasicus (GenBank/Out group)								Cyt b	FJ217390
P.sergenti (GenBank/Out group)								Cyt b	EU980367
P.major	MSH-297	Male	Ur kandi	1551	Stone crevices	09.07.06	SP	EF-1α	GQ169351
P.major	MSH-312	Male	Ur kandi	1551	Bed room	09.07.06	ASP	EF-1α	GQ169352
P.major	MSH-317	Male	Ur kandi	1551	Hen nest	09.07.06	SP	EF-1α	GQ169353
P.major	MSH-522	Male	Ghurt tappeh	1547	Rodent burrow	09.07.06	SP	EF-1α	GQ169354
P.major (GenBank)								EF-1α	EF416834
*P.*neglectus (GenBank)								EF-1α	AF160802
P.neglectus (GenBank)								EF-1α	AF160801
P.perfiliewi	MSH-721	Male	Dushan lu	1209	Rodent burrow	22.07.06	SP	EF-1α	GQ169355
P.perfiliewi	MSH-738	Male	Dushan lu	1209	Rodent burrow	22.07.06	SP	EF-1α	GQ169356
P.perfiliewi	MSH-836	Male	Niaz suee	1080	Stone crevices	22.07.06	SP	EF-1α	GQ169357
P.perfiliewi (GenBank)								EF-1α	AF160805
P.tobbi	MSH-903	Male	Ghurt tappeh	1547	Stone crevices	23.07.06	SP	EF-1α	GQ169358
P.tobbi	MSH-1292	Male	Ghurt tappeh	1547	Stone crevices	06.08.06	SP	EF-1α	GQ169359
P.tobbi	MSH-1481	Male	Ghurt tappeh	1547	Stone crevices	20.08.06	SP	EF-1α	GQ169360
P.tobbi (GenBank)								EF-1α	AF160810
P.caucasicus (GenBank/Out group)								EF-1α	EF416836

SP = Sticky paper ; CDC = CDC Light trap ; Asp = Aspirator
Cyt b = Cytochrome b ; EF-1α = Elongation Factor 1-α

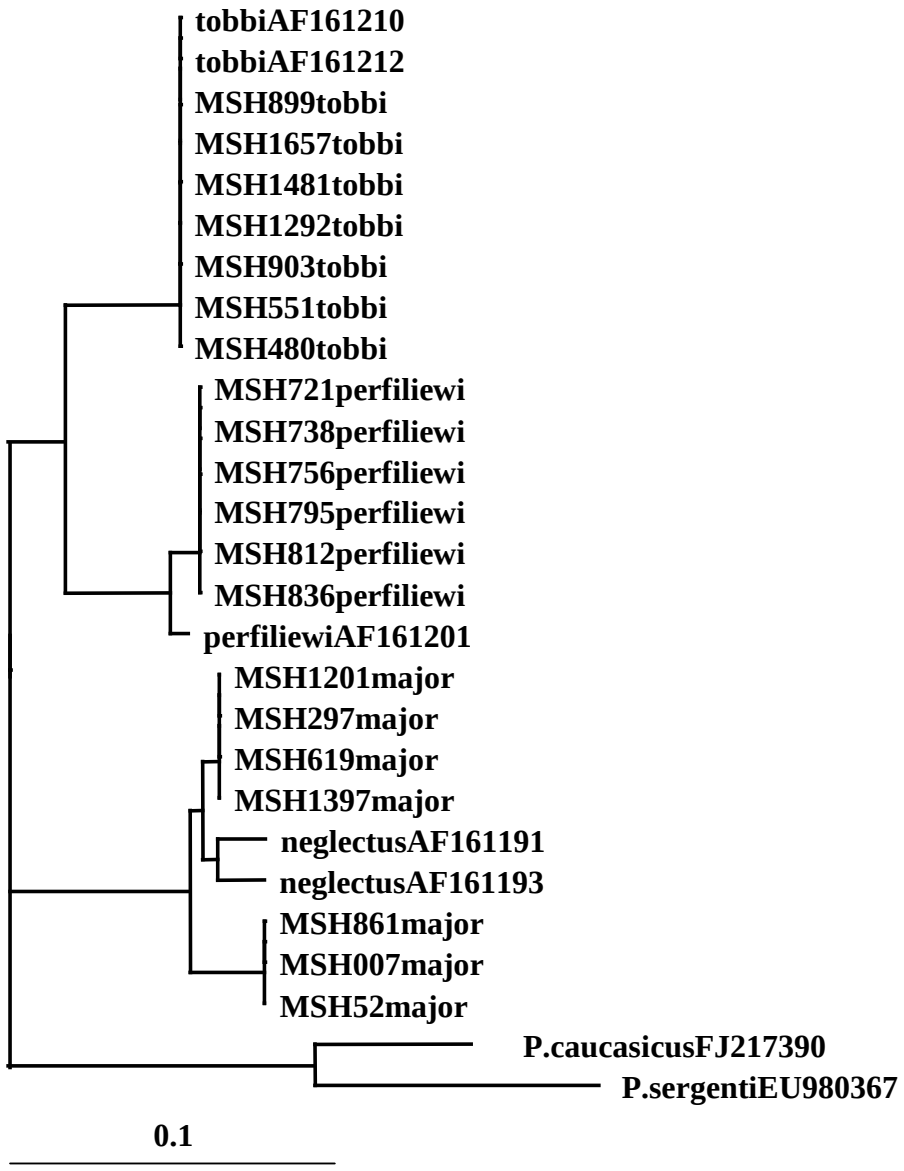

Fig. 1. Neighbor-joining phylogenetic tree for DNA haplotypes of Cyt b (mtDNA) of the subgenus *Larroussius* sand flies species

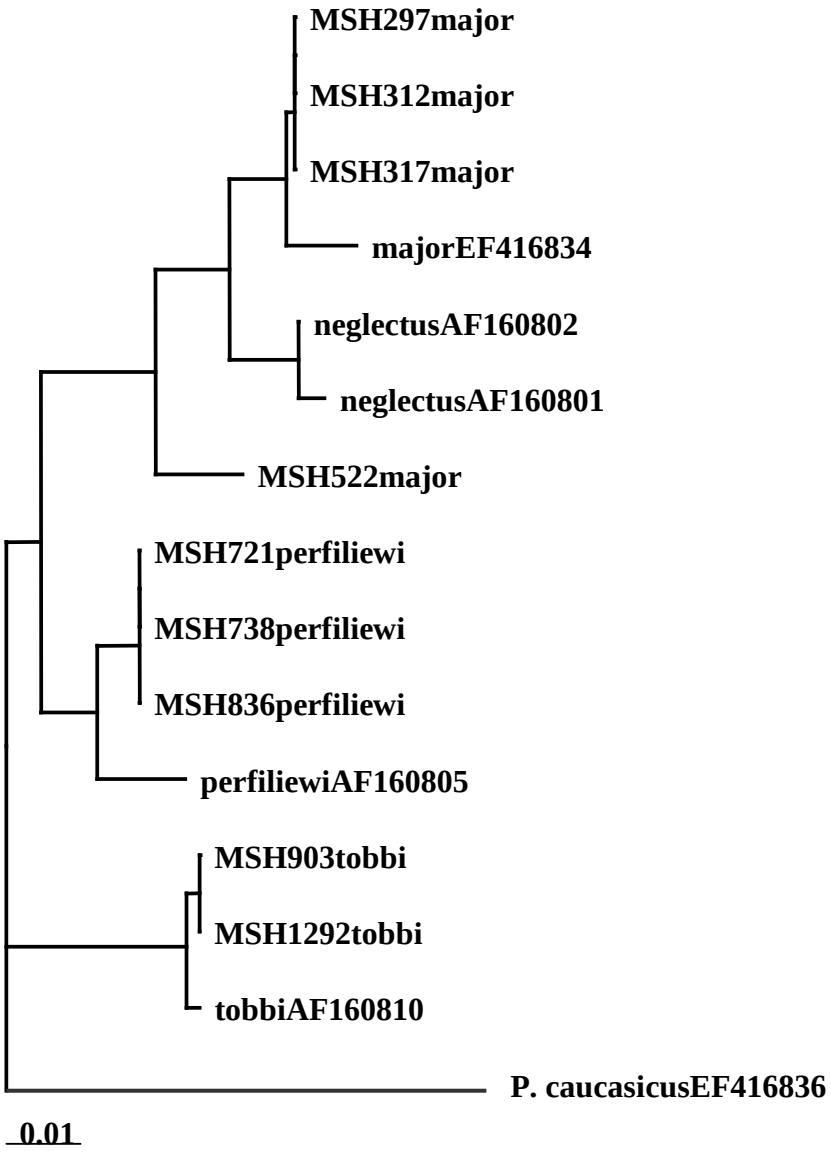

Fig. 2. Neighbor-joining phylogenetic tree for DNA haplotypes of EF-1α (nDNA) of the subgenus *Larroussius* sand flies species

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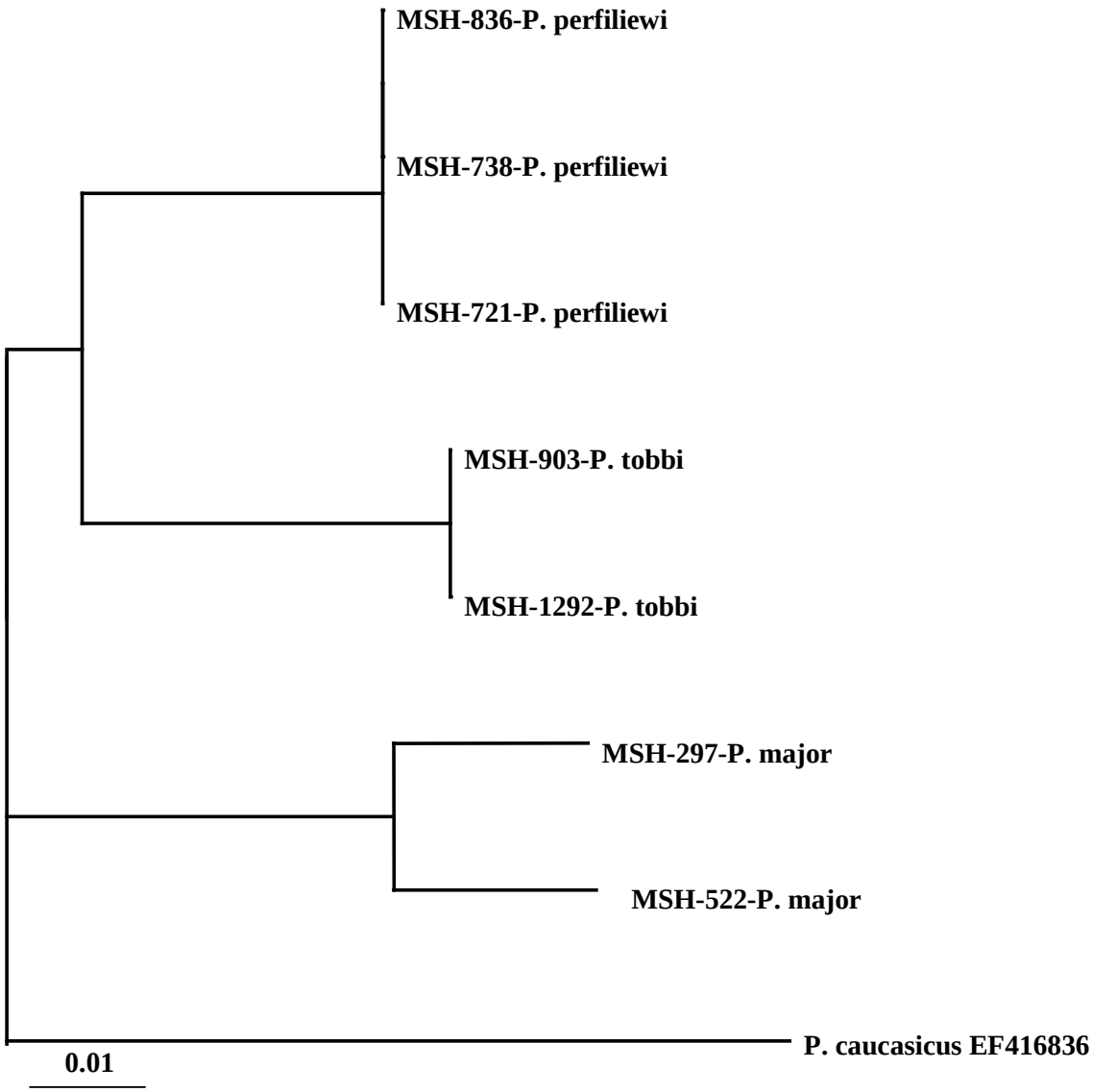

Fig. 3. Neighbor-joining phylogenetic tree for DNA haplotypes of combination of Cyt b and EF-1α of the subgenus *Larroussius* sand flies species.

Fig. 4. Pharyngeal armature of *P. major*

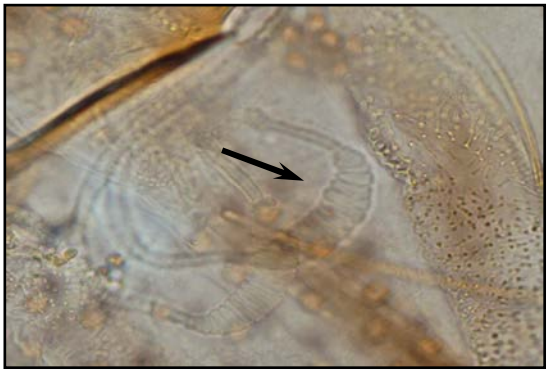

Fig. 5. Spermatheca of *P. major*

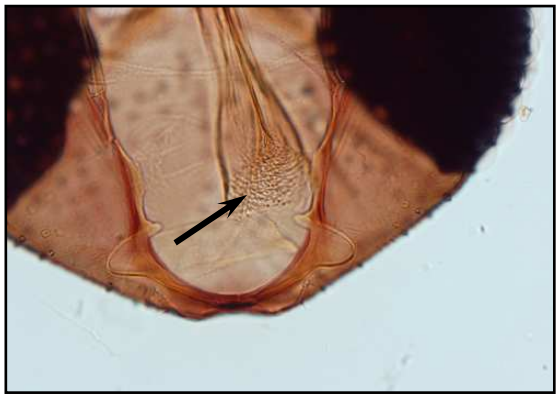

Fig. 6. Pharyngeal armature of *P. tobbi*

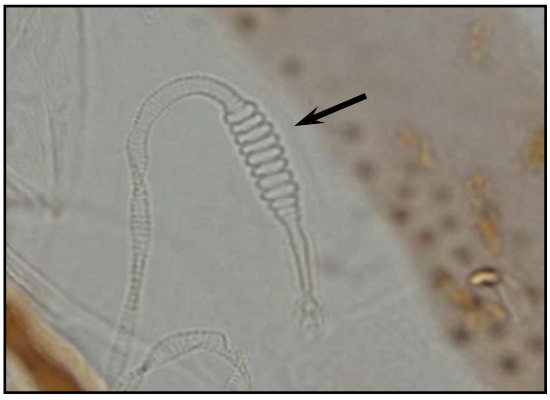

Fig. 7. Spermatheca of *P. tobbi*

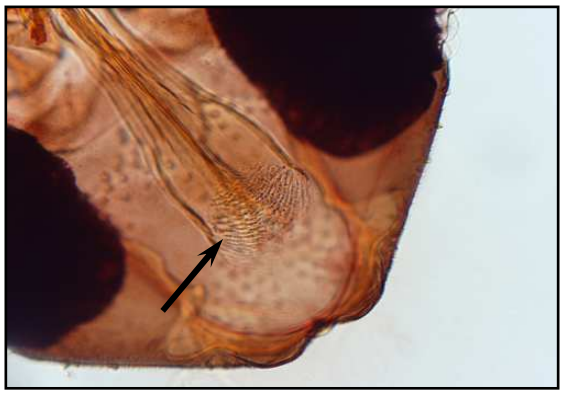

Fig. 8. Pharyngeal armature of *P.perfiliewi*

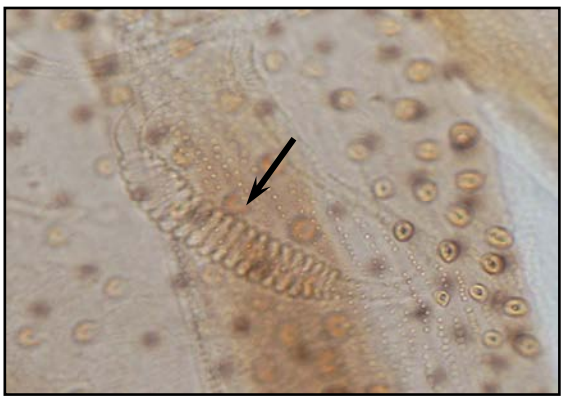

Fig. 9. Spermatheca of *P.perfiliewi*

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Fig. 10. Palpal formula of *P.major*

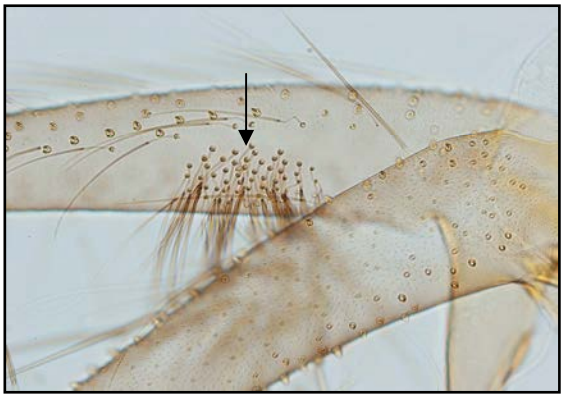

Fig. 11. Coxite hairs of *P.major*

Fig. 12. Palpal formula of *P.neglectus*

Fig. 13. Coxite hairs of *P.neglectus*

According to the finding of the present investigations, the phylogeny of Cyt b and EF-1α haplotypes confirms the morphological relationships among the three species *P. perfiliewi*, *P. major* and *P. tobbi* in the subgenus *Larroussius*. The males of these three species have a several diagnostic morphological characters, whereas the females of these species show very similarities in morphology of spermathecal segment number, pharyngeal armature and other characteristics.

Although morphological characteristics are the most practical methods for species distinguishing, new molecular techniques are very useful to resolve problems of identification in the cases with morphologically similarities.

Access on the genetic diversity and molecular systematic of the *Larroussius* sand flies species not only useful to find the taxonomic status of them, but also indicates the ecological and geographical differences.

In our analysis, all of the Cyt b and EF-1α sequences, the monophyly of the subgenus *Larroussius* was confirmed, in concordance with the morphologically and molecularly based phylogenies of Rispail and Leger (1991, 1998b) and Esseghir et al. (1997). All of the branches of the parsimony and distances trees had strong support and showed identical relationships and indicated the validity of many of characters in inferring evolutionary relationships. The trees were also topologically similar to the parsimony tree of Esseghir et al. (1997).

On morphological characteristics, the present study confirmed the observations of

Perfiliev (1968), Lewis (1982), Leger et al. (1983), and Killick-Kendrick et al. (1991).

Phylogenetic analyses of *P. major* populations showed 2 lineages in different locations such as rodents burrow and stone crevices (Table 3).

According to our molecular and morphological finding, it seems that there are both *P. major* and *P. neglectus* in Meshkinshahr district. This is the first report of *P. neglectus* in this area as well as in Iran (Table 3)

Further studies needs to resolve the taxonomic status of this species. *P. major* has many geographical variants the females of which have conventionally been distinguished by differences in pharyngeal armature. Adler (1933) stressed the tendency of *P. major* to form geographical races. He showed (1946) that they differ mainly in the form of the pharyngeal armature of the females. He also mentioned ecological differences between the races and stresses that some races may have a different importance in the epidemiology of VL.

Our study on phylogenetic analyses of *P. tobbi* populations showed one lineage with single haplotype for either cytB or EF-1α gene. *P. tobbi* was first found in Iran and Israel, and was described as a variety of *P. perniciosus*. Parrot (1934) reported that it is a single species with the name of *P. tobbi*. This was accepted also by Theodor (1948).

Phlebotomus tobbi is mainly found in burrows and is rather common in Transcaucasia and it seems to be identical with the sand flies from Iran and southwest Asia. Therefore, *P. tobbi* is considered a separate species.

Phlebotomus perfiliewi is the probable vector of VL in the north west of Iran. In the present study, this species had one lineage with a single haplotype. Phylogenetic tree retrieved from cytB or combination of cytB and EF-1α showed close relationship of *P. perfiliewi* with *P. tobb*i.

Perfiliev (1966) recognized three subspecies of *P. perfiliewi* (*perfiliewi, galilaeus* and *transcaucasicus*) which were distinguished by minor morphological differences in the aedeagus of the male. Lewis (1982) listed the same three subspecies but commented that the distinction of the male *galilaeus* from *transcaucasicus* uncertain. Artemiev and Neronov (1984) raised *galilaeus* and *transcaucasicus* to level species. The spermatheca of *galilaeus* and *perfiliewi* are indistinguishable, suggesting that these allopatric sand flies are taxonomically very close and are perhaps two subspecies (Lewis, 1982).

To find an exact distinction and identification of the female species of *Larroussius*, needs more investigations in different parts of the world as well as Iran.

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