

Distribution, Fauna and Seasonal Variation of Sandflies, Simultaneous Detection of Nuclear Internal Transcribed Spacer Ribosomal DNA Gene of *Leishmania major* in *Rhombomys opimus* and *Phlebotomus papatasi*, in Natanz District in Central Part of Iran

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ABSTRACT

Background: Zoonotic cutaneous leishmaniasis (ZCL) due to *Leishmania major* is increasing in many parts of Iran. This disease originally is a disease found in gerbils. *Leishmania* parasites are transmitted by sandflies that live and breed in gerbil burrows. Nested PCR amplified *Leishmania ITS1-5.8S rRNA* gene in both main reservoir host "*Rhombomys opimus*" and in the "*Phlebotomus papatasi*" main vector of ZCL, in Iran. Population differentiation and seasonal variation of sandflies were analyzed at a microgeographical level in order to identify any isolation by distance, habitat or seasons. **Methods:** Populations of sandflies were sampled from the edges of villages in Natanz, Isfahan province, Iran, using the Centers for Disease Control traps and sticky papers. Individual sandflies were identified based on external and internal morphological characters. Nested PCR protocols were used to amplify *Leishmania ITS1-5.8S rRNA* gene, which were shown to be species-specific via DNA sequence. **Results:** A total of 4500 sandflies were collected and identified. *P. papatasi*, *Phlebotomus sergenti* and *Phlebotomus jacusieli* from genus *Phlebotomus* and *Sergentomyia sintoni* and *Sergentomyia clydei* from genus *Sergentomyia* were identified in this region. *P. papatasi* was the most abundant sandfly in the collections. Ten out of 549 female *P. papatasi* and four out of 19 *R. opimus* were found to be infected with *L. major*. **Conclusion:** Seasonal activity of sandflies starts in June and ends in November. Abundance of *P. papatasi* was in September. Finding and molecular typing of *L. major* in *P. papatasi* and *R. opimus* confirmed the main vector and reservoir in this region. *Iran. Biomed. J.* 16 (2): 113-120, 2012

Keywords: *Leishmania major*, Sandflies, Leishmaniasis, Iran

INTRODUCTION

Leishmaniasis is caused by human infection with parasites of the genus *Leishmania*, which are transmitted by phlebotomine sandflies [1, 2]. *Rhombomys opimus* is believed to be the principal reservoirs and the phlebotomine sandfly *Phlebotomus (Phlebotomus) papatasi* (Scopoli) (Diptera: Psychodidae) is the proven vector of the parasitic protozoan *Leishmania major* Yakimoff and Schokhor (Kinetoplastida: Trypanosomatidae), the causative agent of zoonotic cutaneous leishmaniasis (ZCL) in Iran [3] the ex-USSR (Uzbekistan, Turkmenistan and

Azerbaijan) [4], eastern Saudi Arabia [5], the Jordan Valley [6], central Tunisia [7], and southern Morocco [8]. In Iran, ZCL has great public health importance in rural regions of 15 out of 32 provinces [9]. This disease is generally restricted to the areas where are heavily infested by the sandflies, as the vector of this disease.

More than 46 species of sandflies have been reported in Iran [10], but only a few of them are common in ZCL foci and gerbils' bite and/or people. Many sandflies from the foci of rural ZCL have been dissected or screened by PCR and found to be infected with leptomonads [3, 11-13].

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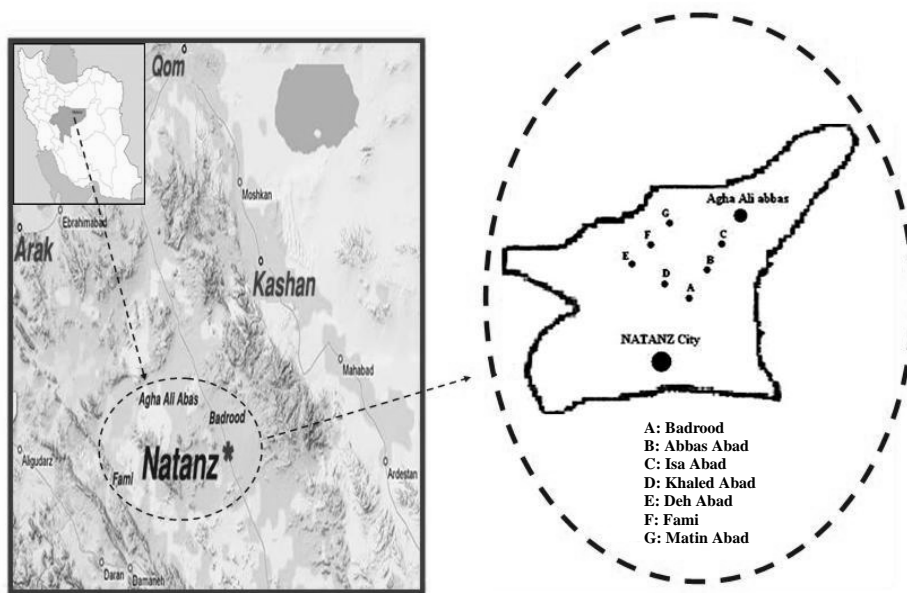


Fig. 1. Location of villages, Natanz, Isfahan province of Iran, where sandflies and *R. opimus* were sampled and screened for *Leishmania* infections.

In the present report, the population differentiation and seasonal variation of sandflies have been analyzed at a microgeographical level to identify any isolation by distance (among villages) or by habitat (between villages and colonies of the gerbil reservoir hosts of *L. major*) or by seasons (among different months of activity). Most of our collections were from sites at the edge of rural villages on a high-altitude cultivated plain (Fig. 1) within an important ZCL focus to the north of Natanz city, Isfahan province, in central part of Iran [11, 10].

Following the objectives of this report, we attempted to identify and type molecularly *L. major* in *P. papatasi* as the main vector and in *R. opimus* as the reservoir host of ZCL in Natanz district, in central part of Iran.

MATERIALS AND METHODS

The collections were carried out from the first half of June through the second half of November 2009 (during the season of activity of adult sandflies) in seven villages near Natanz city (Isfahan province, Iran) (Fig. 1). These areas are 3,397 km² with an altitude of 1600 meter above the sea level (33°40' N, 51°25' E). Also, the climate condition is very hot (up to +41°C) in the summer and cold (about -9°C) in the winter. The total annual rainfall is 270 mm.

Sand flies were collected on sticky papers (A4 sheets of white papers soaked in castor oil) placed overnight in ruined outhouses and at the entrances to gerbil burrows (1 paper per burrow; papers: 2-10 m apart, 30-

40 per site, over a range of 120-200 m at each site). The Centers for Disease Control miniature light traps [14] (with the white-light bulb 1-2 m above ground level) were set overnight to sample sand flies in domestic animal shelters (1-2 traps per site), and a manual aspirator was sometimes used by a single collector to capture sand flies resting inside houses in the morning [15].

Sand flies captured in light traps and aspirators were narcotized with cigarette smoke, and those caught on sticky papers were removed with needles or fine brushes dipped in 70% ethanol. All specimens were then stored in analytical grade 80% ethanol, firstly at 4°C (in villages) and later at -20°C (in Tehran).

All sand flies were identified based on external and internal morphological characters of the head and abdominal Terminalia [4, 16], which were slide-mounted in Berlese's fluid [17], following dissection with sterilized forceps and micro-needles [18].

Rodents were caught with live traps by baiting cucumbers and dates. All rodents and sand flies from these collections were screened for infections of *Leishmania* species using nested PCR of *ITS-rDNA* gene [10, 19].

PCR products were directly sequenced to identify *Leishmania* haplotypes infecting individual rodent and sandfly, and all haplotypes were identified by species using phylogenetic analysis. DNA sequences were edited and aligned using Sequencher™ 3.1.1 software (Gene Codes Corporation), and the multiple alignments of new DNA haplotypes and homologous GenBank sequences were exported into PAUP* software [20] for phylogenetic analysis.

RESULTS

Approximately, 4500 sandflies were collected from various habitats in different regions from Natanz. A number of 1646 sandflies were dissected from outdoors (ruined outhouses, yards and at the entrances to gerbil burrows), and indoors (domestic animal shelters, resting houses) by sticky paper and the Centers for Disease Control light traps.

Overall, *P. papatasi* was the most abundant sandfly in the collections (34.9%), followed by *P. sergenti* (26.9%), *Sergentomyia (Sergentomyia) sintoni* Pringle (22.4%), *S. clydei* (8.1%) and *P. jacusieli* (7.7%) (Tables 1 and 2).

Ten out of 549 female *P. papatasi* were found to be infected with *L. major* using nested primers ITS1F and ITS2R4. Fifty one (9.2%) out of 549 sandflies, collected from gerbil burrow, were screened for *Leishmania* infection and only one (0.18%) was found to be infected with *L. major*. In addition, 498 (90.7%) out of 549 sandflies, collected from animal shelters inside houses and yards, were screened for *Leishmania* infection and nine were found to be infected with *L. major*. *L. major* was also detected in 4 out of 19 *R. opimus* captured in two villages.

The analyses of *L. major ITS-rDNA* gene were based on the 211 bp of the *ITS1* gene and 168 bp of 5.8S gene, followed by 15 bp of the *ITS2* gene (total 394 bp).

At least 14 males and 14 females of each sandfly species were taken from each collection sample sites for identification, seasonal variation, fauna and analysis of population. Morphological identification was based on external and internal morphological characters of the head and abdominal Terminalia. Only undamaged sandflies were used, but selection was otherwise made at random.

The proportion of male and female of sandfly

population in different locations of Natanz has been shown in Table 1. In our previous report [11] after female's gonotrophic stage analyzing, we showed that total *Leishmania* infection rates were higher for females with large eggs (semi-gravid and gravid). Also in *P. papatasi* with or without blood meal remains, we found low *Leishmania* infections (Table 1).

Prevalence of sandfly species in different habitats of Natanz villages has been shown in Table 2. Also, the numbers, frequency rate and seasonal activity of sandfly species collected from various villages of Natanz have been presented in Figure 2.

Among dissected sandflies, *P. papatasi* with high density (34.9%) was collected during activity season of sandflies from outdoors (ruined outhouses, yard and at the entrances to gerbil burrows) and indoors (domestic animal shelters, resting inside houses).

DISCUSSION

Sequences and homologous of *ITS1-5.8S rRNA* gene of *L. major* fragment from *P. papatasi* and *R. opimus* were searched in GenBank and 3 haplotypes were identified (Fig. 3). After phylogenetic analysis, the sequences from Iranian sandflies and rodents were found to be diagnostic for *L. major*. Three haplotypes of *L. major* were identified. The common haplotype of *L. major* was found to be identical to that of isolates of *L. major* from Iran and Sudan (GenBank accession no. EF413075 and AJ300481, respectively), and it predominated Iranian sandflies and rodents infected with this species (10/12 infections). Also, this common haplotype (GenBank accession no. EF413075) differs pair wise by only one nucleotide position from a haplotype of *L. major* from Central Asia and Sudan (GenBank accession no. AJ000310 and AJ272383, respectively); also by two nucleotide

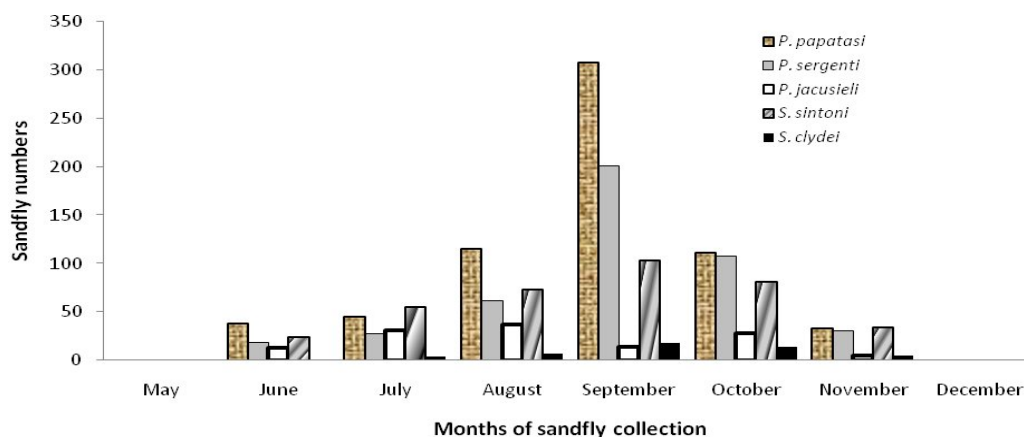


Fig. 2. Seasonal activities of different adult sandflies species in villages of Natanz district, Isfahan province, Iran from May to December 2009.

Table 1. Proportion of male and female of sandfly population in different locations of Natanz.

| Villages | No. | Species | | | | | | | | | | | | | | | | | | | | | | | | |
|----------------|-------------|--------------------|------------|------------|------------|------------|--------------------|-------------|------------|------------|------------|---------------------|------------|-----------|-----------|------------|-------------------|-------------|------------|------------|-------------|------------------|----------|-------------|------------|-------------|
| | | <i>P. papatasi</i> | | | | | <i>P. sergenti</i> | | | | | <i>P. jacusieli</i> | | | | | <i>S. sintoni</i> | | | | | <i>S. clydei</i> | | | | |
| | | M | F | | | | M | F | | | | M | F | | | | M | F | | | | M | F | | | |
| | | | FF | SG | G | UF | | FF | SG | G | UF | | FF | SG | G | UF | | FF | SG | G | UF | | FF | SG | G | UF |
| Seyed Abad | 87 | 9 | 5 | 7 | 9 | 15 | 4 | 1 | 2 | 4 | 6 | 1 | _ | _ | _ | 1 | 4 | 2 | 4 | 5 | 7 | _ | _ | _ | _ | 1 |
| Matin Abad | 178 | 21 | 15 | 19 | 14 | 31 | 11 | 5 | 9 | 7 | 15 | 1 | _ | _ | 1 | 1 | 8 | 1 | 4 | 7 | 5 | 1 | _ | 1 | _ | 1 |
| Khaled Abad | 262 | 27 | 11 | 15 | 6 | 41 | 15 | 5 | 8 | 14 | 25 | 5 | 1 | 3 | 5 | 9 | 14 | 7 | 4 | 13 | 24 | 2 | _ | 1 | 3 | 4 |
| Isa Abad | 237 | 15 | 14 | 14 | 11 | 43 | 11 | 4 | 6 | 8 | 18 | 4 | 2 | 6 | 5 | 11 | 19 | 10 | 8 | 9 | 13 | 2 | _ | 1 | 1 | 2 |
| Fami | 157 | 10 | 7 | 5 | 8 | 14 | 13 | 4 | 3 | 8 | 11 | 7 | _ | 3 | 5 | 13 | 8 | 2 | 5 | 9 | 15 | 2 | _ | 2 | 1 | 2 |
| Deh Abad | 167 | 21 | 6 | 14 | 11 | 18 | 15 | 5 | 7 | 8 | 10 | 5 | 2 | 3 | 1 | 8 | 5 | 9 | 4 | 1 | 6 | 2 | _ | 1 | 2 | 3 |
| Chane Abad | 83 | 7 | 3 | 5 | 3 | 9 | 6 | 2 | 7 | 5 | 6 | 1 | _ | _ | _ | 2 | 4 | 2 | 1 | 3 | 14 | 1 | _ | _ | 1 | 1 |
| Chahe Arabha | 124 | 9 | 5 | 11 | 7 | 12 | 9 | 3 | 5 | 14 | 18 | 2 | _ | _ | 1 | 1 | 9 | 1 | 5 | 3 | 8 | _ | _ | _ | _ | 1 |
| Badrood | 77 | 5 | _ | 2 | 4 | 6 | 4 | 1 | 2 | 5 | 11 | 3 | _ | 1 | 1 | 3 | 9 | _ | 2 | 5 | 10 | 1 | _ | _ | _ | 2 |
| Agha Ali Abas | 150 | 20 | 3 | 6 | 11 | 16 | 11 | 2 | 7 | 5 | 21 | 2 | _ | _ | 1 | 2 | 10 | 3 | 9 | 4 | 15 | 1 | _ | _ | _ | 1 |
| Abas Abad | 124 | 18 | 5 | 8 | 3 | 16 | 7 | 4 | 8 | 7 | 12 | 2 | _ | _ | 3 | _ | 11 | 2 | 7 | 2 | 4 | 2 | _ | 1 | 2 | _ |
| Total | 1646 | 162 | 74 | 106 | 87 | 221 | 106 | 36 | 64 | 85 | 153 | 33 | 5 | 16 | 23 | 51 | 101 | 39 | 53 | 61 | 121 | 14 | 0 | 7 | 10 | 18 |
| Percent | 100 | 9.8 | 4.5 | 6.4 | 5.3 | 13 | 6.4 | 2.19 | 3.9 | 5.2 | 9.3 | 2 | 0.3 | 1 | 1 | 3.1 | 6.1 | 2.37 | 3.2 | 3.7 | 7.35 | 0.9 | 0 | 0.43 | 0.6 | 1.09 |

M, male; F, female; FF, fresh fed; SG, semi-gravid; G, gravid; UF, un-fed

Table 2. Prevalence of sandflies in different habitats of Natanz's villages.

| Habitats | Species | | | | | | | | | | | | | | | | | | | | |
|----------------|--------------------|--------------|--------------|-------------|--------------------|-------------|-------------|-------------|---------------------|-------------|-------------|-------------|-------------------|-------------|-------------|-------------|------------------|-------------|-------------|-------------|---|
| | <i>P. papatasi</i> | | | | <i>P. sergenti</i> | | | | <i>P. jacusieli</i> | | | | <i>S. sintoni</i> | | | | <i>S. clydei</i> | | | | |
| | Home | A.Sh | R.B | Yard | Home | A.Sh | R.B | Yard | Home | A.Sh | R.B | Yard | Home | A.Sh | R.B | Yard | Home | A.Sh | R.B | Yard | |
| Villages | Seyed Abad | 11 | 18 | 9 | 7 | 7 | 5 | 4 | 1 | _ | 2 | _ | _ | 7 | 4 | 6 | 5 | _ | 1 | _ | _ |
| | Matin Abad | 27 | 37 | 21 | 15 | 17 | 12 | 11 | 7 | _ | _ | 2 | 1 | 8 | 10 | 7 | _ | _ | 1 | 2 | _ |
| | Khaled Abad | 18 | 38 | 27 | 17 | 16 | 23 | 15 | 13 | 4 | 6 | 8 | 5 | 15 | 18 | 18 | 11 | _ | 4 | 6 | _ |
| | Isa abad | 21 | 34 | 28 | 14 | 13 | 14 | 9 | 11 | 6 | 11 | 9 | 2 | 18 | 14 | 19 | 8 | 1 | 3 | 2 | _ |
| | Fami | 11 | 10 | 13 | 10 | 8 | 11 | 13 | 7 | 2 | 9 | 13 | 4 | 4 | 12 | 10 | 8 | 1 | 2 | 4 | _ |
| | Deh Abad | 10 | 26 | 23 | 11 | 6 | 16 | 14 | 9 | _ | 2 | 14 | 3 | 3 | 9 | 7 | 6 | 2 | 2 | 3 | 1 |
| | Chane Abad | 7 | 9 | 6 | 5 | 5 | 10 | 7 | 4 | _ | 2 | 1 | _ | 1 | 11 | 9 | 3 | 1 | _ | 2 | _ |
| | Chahe Arabha | 9 | 16 | 13 | 6 | 9 | 19 | 14 | 7 | _ | 1 | 3 | _ | 3 | 10 | 11 | 2 | _ | 1 | _ | _ |
| | Badrood | 3 | 11 | 3 | _ | 2 | 9 | 7 | 5 | 2 | 3 | 2 | 1 | 4 | 9 | 6 | 7 | _ | 2 | 1 | _ |
| | Agha Ali Abas | 17 | 15 | 17 | 7 | 8 | 18 | 15 | 5 | 1 | 2 | 2 | _ | 8 | 16 | 11 | 6 | _ | 1 | 1 | _ |
| Abas Abad | 8 | 16 | 15 | 11 | 17 | 10 | 7 | 4 | 1 | 3 | 1 | _ | 3 | 8 | 11 | 4 | 1 | 2 | 1 | 1 | |
| Total | 142 | 230 | 175 | 103 | 108 | 147 | 116 | 73 | 16 | 41 | 55 | 16 | 74 | 121 | 115 | 60 | 6 | 19 | 22 | 2 | |
| Percent | 8.63 | 13.97 | 10.63 | 6.26 | 6.56 | 8.93 | 7.05 | 4.43 | 0.97 | 2.49 | 3.34 | 0.97 | 4.5 | 7.35 | 6.99 | 3.65 | 0.36 | 1.15 | 1.34 | 0.12 | |

A.Sh, animal shelter; R.B, rodent burrow

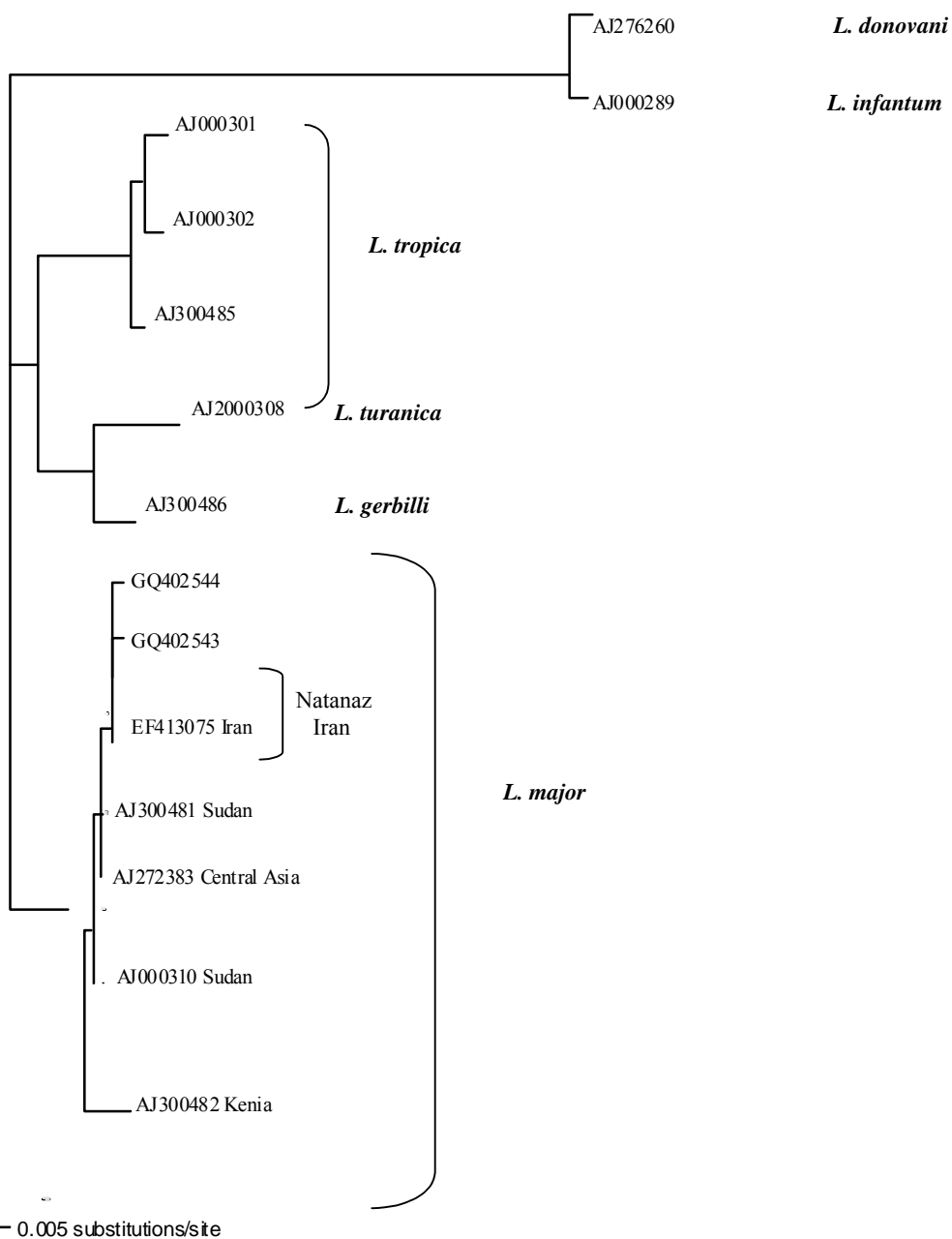


Fig. 3. Unrooted neighbour-joining tree showing the relationships of the haplotypes of the *ITS1-5.8S rDNA* for the isolates of *L. major* as the species infecting sandflies and rodents, mentioned in text using PAUP* software [31], and it relates *ITS1-5.8S rDNA* haplotypes in GenBank.

positions from a haplotype from Kenya (GenBank accession no. AJ300482). Two new haplotypes of *L. major* (GenBank accession no. GQ402543 and GQ402544) were also found in single infections with only one nucleotide position from a common haplotype (GenBank accession no. EF413075) of *L. major* (Fig. 3).

No great differences were found in different species, locations or habitats. Regarding monthly distribution, there are no sandflies in May and before that and also in December 2006. In August, September and October, abundance of sandflies is much higher, especially abundance of *P. papatasi* in September is very high.

Among sandfly vectors of *Leishmania*, *P. papatasi* may acquire *L. major* from rodent reservoir hosts living in the peridomestic habitats. Immigration of vectors from more distant colonies of *R. opimus* was less likely. Because mark-release-recapture experiments had shown that *P. papatasi* rarely disperses more than 1.5 km, with the extent of dispersal depending on the availability of resting and breeding sites (for both sexes) and of blood meal sources (females) [21].

The same results were reported for *P. papatasi* living in similar habitats in the Jordan Valley [22]. Schlein *et al.* [22] showed that non-gravid female flies were found to travel at least 800 m between colonies of the local reservoir host (*Psammomys obesus*) of *L. major*, and fed of turkeys, which provided many blood meals for female sandflies. Using exit-entrance traps in gerbil burrows [23] and light traps without bulbs in nearby fields [24] males and gravid females were shown to be active mostly between sunset and midnight, when they left burrows to find sugar meals from nearby plants and then sought resting sites in nearby burrows [25]. In contrast, non-gravid females were active throughout the night, sometimes dispersing across fields to seek blood meals and often between midnight and sunrise, entering burrows far from their starting points [23, 25, 26].

Our results demonstrate that there has been no isolation of sandflies within different villages (Table 1) and habitats (Table 2) in Natanz, Isfahan province, Iran.

Within the ZCL focus, *P. papatasi* has been frequently collected in and around houses in villages, where it was shown to be the predominant *Phlebotomus* species and to obtain many of its blood meals from humans, birds and large domestic mammals [27, 28]. It could be concluded that most peridomestic populations of *P. papatasi* are isolated from those in gerbil colonies, with peridomestic female sandflies rarely feeding on gerbils; therefore, rarely becoming infected with *L. major*.

The significance of any phlebotomine species to be as a disease vector and rodent to be as a disease reservoir can be dependent on many factors; therefore, finding *L. major* in *P. papatasi* and in *R. opimus* is not sufficient evidence for considering them as a vector or reservoir [29]. Of the sandflies recorded from Iran, only *P. papatasi* was judged to be a proven vector of *L. major* [30].

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