Sand fly and *Leishmania* spp. survey in Vojvodina (Serbia): first detection of *Leishmania infantum* DNA in sand flies and the first record of *Phlebotomus (Transphlebotomus) mascittii* Grassi, 1908

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**Abstract**

**Background:** Leishmaniasis in Serbia was an endemic disease, and is considered to be eradicated for more than 40 years. In the past decade sporadic cases of canine leishmaniasis started to emerge for the first time in Vojvodina Province (previously non-endemic region of Serbia). Reports of introduced, and later on autochthonous cases of leishmaniasis alerted the possibility of disease emergence. The aim of this study was to bridge more than a half a century wide gap in entomological surveillance of sand fly vectors in Vojvodina, as well as to verify the presence of the vector species that could support *Leishmania* spp. circulation.

**Results:** During the period 2013–2015, a total of 136 sand flies were collected from 48 of 80 surveyed locations. Four sand fly species of the genus *Phlebotomus* were detected: *P. papatasi*, *P. perfiliewi*, *P. mascittii* and *P. neglectus*. Detection of *P. mascittii* represents the first record of this species for the sand fly fauna in Vojvodina and in Serbia. All female specimens (*n* = 80) were tested for *Leishmania* spp. DNA, and three blood-fed *P. papatasi* specimens were positive (4%). One positive DNA sample was successfully amplified by ITS1 nPCR. The RFLP analysis of the resulting 350 bp fragment showed a typical pattern of *L. infantum*, and the ITS1 partial sequence blasted in GenBank confirmed 100% identity with *L. infantum* and *L. donovani* complex sequences. This result represents the first record of both *Leishmania* spp. and *L. infantum* DNA from sand flies in Vojvodina, and in Serbia.

**Conclusions:** Presence of autochthonous canine leishmaniasis cases, records of *Phlebotomus (Larroussius)* species proven vectors of *L. infantum* (*P. perfiliewi* and *P. neglectus*) and detection of *L. infantum* DNA from wild caught (non-competent) vectors, prove that *L. infantum* is present in Vojvodina and indicates a probable circulation in the region.

**Keywords:** Sand fly, *Phlebotomus*, Leishmaniasis, *Leishmania infantum*, Serbia
**Background**

The leishmaniasis are major vector-borne diseases caused by protozoan parasites belonging to the genus *Leishmania*. The parasites are transmitted to humans and other vertebrates by the bite of infected female sand flies (Psychodidae, Phlebotominae). Around one billion of people are at risk of infection while number of reported cases per year is estimated at 0.7–1.3 million for cutaneous leishmaniasis (CL) and 200,000–400,000 for visceral leishmaniasis (VL), causing over 20,000 deaths annually [1].

In Europe, leishmaniasis is endemic in all southern countries, with ~700 (3950 if Turkey is included) autochthonous human cases reported every year [1]. The spread of *Leishmania infantum*, causative agent of zoonotic VL and CL in humans and domestic dogs (reservoir host) represents a major threat to Europe. Increasing dog and human travel, as well as the ongoing migrant crisis, pose a significant risk of *L. infantum* introduction into central Europe. Climate and land cover changes could also support northward dispersal of vectors, establishment of seasonal biting rates matching those of southern Europe, hence permitting autochthonous transmission [2].

In Serbia, leishmaniasis started to emerge after the Second World War. Due to the composition and abundance of sand fly species at that time, poor hygienic and health conditions in the post-war period, the disease rapidly assumed an epidemic character [3]. The first autochthonous case of VL was reported in 1945 [4]. From 1945 to 1955, leishmaniasis spread in epidemic waves from the southern parts of the country northwards, reaching its northernmost limit in central Serbia. After the last major epidemic (1953), the number of new cases started to diminish and, in the following years, the disease appeared only sporadically. The last case of VL was reported in 1968 [5], thereafter the disease was considered eradicated.

Sand fly research in Serbia was initiated in 1947, soon after first cases of autochthonous VL emerged, and was terminated in 1990. During this period, the presence of seven species of the genus *Phlebotomus* was recorded: *Phlebotomus papatasi*, *P. perfiliewi*, *P. tobbi*, *P. neglectus*, *P. simici*, *P. sergenti* and *P. balcanicus* [6]. The most intensive sand fly investigations were conducted during the VL epidemics, mainly in the infested areas. Following the disease spread, sand fly research was mainly focused on the south-east, east and central Serbia, leaving all other areas of country unexplored or partially explored. One of these less investigated and leishmaniasis-free region of Serbia includes Vojvodina Province, located in the north of the country.

Vojvodina was surveyed only briefly between 1949 and 1951. Research was conducted irregularly (through the years and seasons) and only in a small number of locations. Studies revealed the presence of only three species: *P. papatasi*, *P. perfiliewi* and *P. neglectus* [7]. The number of specimens caught was rather low, with several samples collected from various villages or from various houses in the same village. Low sand fly diversity and abundance, as well as the absence of human cases, resulted in the neglect of sand fly studies in this area for more than 60 years.

In the past decade, since 2006, cases of canine leishmaniasis have started to emerge for the first time in Vojvodina. Clinical symptoms and positive serological findings were first diagnosed in dogs that were imported or had travelled abroad to some of the Mediterranean countries with endemic leishmaniasis [8]. Subsequent findings (2010–2013) involved dogs that had never travelled from their home in Vojvodina [9]. This information suggested that both parasite and vector species are present in the region and implicated the possibility of autochthonous transmission.

The aim of this study was to bridge more than a half a century wide gap in entomological surveillance of sand fly vectors in Vojvodina, a very important transition region of Europe, as well as to verify the presence of the vector species that could support *Leishmania* spp. circulation.

**Methods**

**Sand fly sampling**

Cross-sectional entomological surveys were conducted between 2013 and 2015 in selected sample sites of the Vojvodina Province (North Serbia). A total of 80 villages were surveyed: 17 in 2013, 24 in 2014 and 39 in 2015. Surveys were conducted from the middle of May until the middle of September. Due to the restricted funding, sites sampled positive at first collection were not resampled, whereas negative locations were sampled again. Sampling locations were partly chosen according to the available data about sand fly presence obtained during previous investigations (1948–1951) and data regarding reported and/or suspected cases of canine leishmaniasis (2006–2013). The remaining locations are situated in areas without any data of sand fly and/or leishmaniasis presence.

Multiple sampling techniques were used to increase the number of specimens sampled, as low abundance was expected according to historical data. Indoor and outdoor populations of sand flies were collected using a miniature Centre for Disease Control (CDC) light traps (John W. Hock Company, model 512, Gainesville, Florida, U.S.A.), dry-ice baited traps without light (NS2 type), dry-ice baited traps with light, sticky papers and mouth aspirators. Suction traps were operating overnight, set at 16:00 h and collected at 08:00 h the next day. Traps were placed ~1.5 m above the ground inside (CDC) and outside (traps with dry ice) of the houses and animal shelters. Sticky traps (20 × 30 cm papers coated with commercial castor oil) were placed in holes surrounding walls of animal shelters and houses, and all...
other suitable sand fly resting places, for a period of four days. Indoor collections were performed during the daylight using mouth aspirators.

Morphological identification of sand flies
All specimens collected were immediately transferred to 96% ethanol. Specimens were dissected; head and terminal segments of the abdomen were removed, cleared in Marc Andre solution and mounted in Berlese medium. The head and the tip of the abdomen were used for morphological identification, while the rest of the body was transferred to a separate tube with 70% ethanol and stored for DNA analyses. Morphological identification was based on characters of male genitalia, female spermaticae and pharyngeal armature [10, 11].

Molecular identification of sand flies
In order to validate the morphological identification of sand fly specimens, sequence analysis of cytochrome c oxidase subunit 1 (cox1) mtDNA region was performed. PCR amplification was performed using the LCOI490/HCO2198 primer pairs according to the procedure of Folmer et al. [12]. The amplification products were electrophoresed trough 2% agarose gel and visualised under UV light. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and directly sequenced in both directions, using the same primers used for DNA amplification. Next-generation sequencing (NGS) was performed with BioRobot EZ1-XL Advanced (Qiagen). Sequences obtained were edited and aligned using BioEdit (7.0.9.0) [13] and compared with those available in GenBank using Neighbor Joining algorithm under the assumption of Kimura’s two parameter model in MEGA 6.06 [14].

Detection of *Leishmania* spp.
Presence of leishmanial DNA was assayed individually in all females [15]. Small-subunit (SSU) ribosomal DNA was amplified by a sensitive nested (n) PCR technique using the Kinetoplastida and *Leishmania* genus-specific primers in the first and second PCR round, respectively [16]. Negative (no DNA) and positive (DNA from cultured *L. infantum* promastigotes) controls were used in all experiments. PCR products were electrophoresed through 1.5% agarose gel and visualised under UV light. Positive samples yielded a predicted nPCR product of 358 bp. *Leishmania*-positive DNA samples were then examined individually by internal transcribed spacer (ITS) 1 nPCR using LITSR/L5.8S primers, and the amplified fragment analyzed by both restriction fragment length polymorphism (RFLP) and sequencing analysis for *Leishmania* species identification [17].

Results
During 2013–2015, a total of 136 sand flies were collected from 48 of 80 locations (Fig. 1). Four sand fly species of the genus *Phlebotomus* were identified: *P. papatasi*, *P. perfiliewi*, *P. mascittii* and *P. neglectus* (Table 1). The majority of specimens (*n* = 130) belonged to *P. papatasi* (75 females, 54 males, 1 undetermined) (GenBank: KY848828), three females were identified as *P. perfiliewi* (GenBank: KY848829), two females as *P. mascittii* and one female as *P. neglectus* (GenBank: KY848830). *Phlebotomus papatasi* was the predominant species in Vojvodina, found in 55% of surveyed locations (91.67% of positive locations).

Presence of *P. mascittii*, a member of the *Phlebotomus* (*Transphlebotomus*) subgenus had not previously been reported from Vojvodina (or Serbia). During 2015, two female specimens of *P. mascittii* were collected in urban environment of Ležimir and Parage villages (geographical position given in Table 1). The first specimen was collected in Ležimir (18/06/2015) in a CDC/CO₂ trap placed under a concrete roof that connects a brick storage shed and pig stain, creating a approximately 7 m tunnel like passage between the front (human) and back yard (animal dwelling). Mean monthly temperature for June in Ležimir were 18.6 °C at 07:00 h, 25.9 °C at 14:00 h and 19.1 °C at 21:00 h (source: national network of synoptic stations, station at Sremska Mitrovica, ~35 km from Ležimir). The second specimen was collected in Parage 01/08/2015 with a CO₂ trap placed next to the brick wall of the house approximately 50 m from animals. Mean monthly temperature for August in Parage were 20.5 °C at 07:00 h, 30.6 °C at 14:00 h and 23.4 °C at 21:00 h (source: national network of synoptic stations, station at Rimski Sančevi, ~35 km from Parage). Both specimens were identified morphologically, and specimen identification was confirmed by sequence analysis of the cox1 mitochondrial gene region. Sequences obtained (GenBank: KY848831) were blasted against the database from GenBank and were identified as *P. mascittii* (Fig. 2). This report of *P. mascittii* represents the first record of this species in sand fly fauna of Vojvodina and in Serbia.

All female specimens (*n* = 80) were tested for *Leishmania* DNA; three blood-fed *P. papatasi* specimens were positive (4%). All positive females were collected in 2013 (3/37) from different settlements (Golubinci, Indija and Opovo), thus resulting in 8.1% infection prevalence in that year. Only one positive DNA sample was successfully amplified by ITS1 nPCR. The RFLP analysis of the resulting 358 bp fragment showed a typical pattern of *L. infantum*, and the ITS1 partial sequence (GenBank: KY646445) was compared to those in GenBank, which confirmed the identity as *L. infantum* and *L. donovani* complex sequences (100% match). These results represent the first record of both *Leishmania* and *L. infantum* DNA from sand flies in Vojvodina and in Serbia.
Due to the low number of specimens sampled, we were not able to statistically compare different sampling techniques. However, most of the specimens were sampled by CDC (n = 80), then CDC/CO₂ (n = 31) and the least by only CO₂ (n = 25). No sand flies were sampled by sticky traps and mouth aspirators.

**Discussion**

Sand fly research in Vojvodina was neglected for more than 60 years. Previous investigations conducted in the period 1949–1951 indicated a low diversity of sand flies, with only three species reported (P. papatasi, P. perfiliewi and P. neglectus) [18]. According to the old data P. papatasi was predominant species, being found in the majority of investigated locations in low numbers (1–2 specimens from various locations of the same village), while P. neglectus [19] and P. perfiliewi [20] were present in both, a limited number of locations and in the number of individuals (1–2 specimens from various villages).

Our results coincide partially with these data, confirming the presence of three previously reported species and indicating that, even in low numbers, P. papatasi remained the predominant species in Vojvodina, being found in 55% of all surveyed locations (91.67% of positive locations) (Table 1). Low densities of P. perfiliewi and P. neglectus were also confirmed; three specimens of P. perfiliewi were found (1 in 2013, 2 in 2015) and just one specimen of P. neglectus (2015).

The two specimens of P. mascittii represent first record for Vojvodina and the whole of Serbia, and may indicate a change in the composition of sand fly fauna in the region. *Phlebotomus mascittii* is a species found in Mediterranean region [21], as well as in countries with colder climate like Austria [22], Belgium [23] and Germany [24] with a northernmost border in Slovakia [25]. Considering its usually low abundance [26], and fact that species is present in countries bordering with our research area (e.g. Croatia [27] and Hungary [28]), there is a possibility that *P. mascittii* was already present in Serbia for several years, but remained undetected before 2015.

Patchy distribution and low density of sand flies is more prominent in Vojvodina than in other parts of Serbia, since Vojvodina is predominantly an agricultural region with a heavy use of insecticides. Large cultivated fields greatly influence sand fly dispersal potential and limit their distribution to small isolated clusters. Favourable weather condition, suitable topography and plentiful of food sources enable sand flies to maintain small, but steady, populations. Our data show that fragmented populations of sand flies are present across all of Vojvodina (Fig. 1).
| Locality       | Latitude   | Longitude   | No. of specimens | Date       | Species and gender (male/female) | Positive trap type |
|---------------|------------|-------------|------------------|------------|----------------------------------|--------------------|
| Opovo         | 45.0501220 | 20.4341260  | 8                | 21/07/2013 | P. papatasi (1 m/5f)             | ×                  |
| Indija        | 45.0353556 | 20.0790806  | 7                | 11/06/2014 | P. papatasi (1 m/1f)             | ×                  |
| Golubinci     | 44.9836306 | 20.0715722  | 3                | 24/08/2013 | P. papatasi (2 m/1f)             | ×                  |
| Putinci       | 44.9952167 | 19.9623000  | 10               | 24/08/2013 | P. papatasi (2 m/8f)             | ×                  |
| Ljukovo       | 45.0295639 | 20.0227056  | 1                | 24/08/2013 | P. papatasi (1 m)                | ×                  |
| Vladimirovci  | 45.0322167 | 20.8762667  | 20               | 21/07/2013 | P. papatasi (7 m/13f)            | ×                  |
| Kačarevo      | 44.9623333 | 20.7128667  | 1                | 21/07/2013 | P. perfiliewi (1f)               | ×                  |
| Bečej         | 45.6151970 | 20.0268460  | 2                | 24/07/2013 | P. papatasi (1f)                 | ×                  |
| Kać           | 45.3033710 | 19.9292900  | 4                | 25/07/2013 | P. papatasi (1f)                 | ×                  |
| Begeča        | 45.2380420 | 19.6228380  | 1                | 31/07/2013 | P. papatasi (1 m)                | ×                  |
| Čenej         | 45.3692500 | 19.8042833  | 1                | 30/07/2013 | P. papatasi (1 m/1f)             | ×                  |
| Stara Pazova  | 44.9839278 | 20.1787111  | 2                | 02/09/2015 | P. papatasi (1 m/1f)             | ×                  |
| Budisava      | 45.2803167 | 19.9908333  | 1                | 23/07/2013 | P. papatasi (1f)                 | ×                  |
| Šajkaš        | 45.2664833 | 20.9258333  | 5                | 17/07/2013 | P. papatasi (1 m/2f)             | ×                  |
| Žabalj        | 45.3755500 | 20.0761333  | 5                | 17/07/2014 | P. papatasi (1 m)                | ×                  |
| Mošorin       | 45.2967333 | 20.1547333  | 2                | 26/07/2014 | P. papatasi (1 m/1f)             | ×                  |
| Vilovo        | 45.2463500 | 20.1535500  | 1                | 26/07/2014 | P. papatasi (1 m)                | ×                  |
| Perlez        | 45.2098500 | 20.3902333  | 2                | 26/07/2014 | P. papatasi (2f)                 | ×                  |
| Knićanin      | 45.183167  | 20.164167   | 2                | 26/07/2014 | P. papatasi (1 m/1f)             | ×                  |
| Čačar         | 45.1568333 | 19.391167   | 1                | 19/08/2014 | P. papatasi (1f)                 | ×                  |
| Kuzmin        | 45.3040833 | 19.9285889  | 1                | 30/07/2014 | P. papatasi (1f)                 | ×                  |
| Vrbas         | 45.5809000 | 19.6322000  | 1                | 14/08/2014 | P. papatasi (1f)                 | ×                  |
| Kula          | 45.6178500 | 19.5127333  | 1                | 14/08/2014 | P. papatasi (1f)                 | ×                  |
| Lok           | 45.2183000 | 20.2113333  | 4                | 26/07/2014 | P. papatasi (4f)                 | ×                  |
| Lug           | 45.1876667 | 19.5429667  | 1                | 21/07/2015 | P. papatasi (1 m)                | ×                  |
| Divolš         | 45.1115167 | 19.5093333  | 5                | 02/09/2015 | P. papatasi (2f)                 | ×                  |
| Martinči      | 45.0132167 | 19.4452000  | 1                | 02/09/2015 | P. papatasi (1m)                 | ×                  |
| Ležmir         | 45.1138333 | 19.5672500  | 3                | 02/09/2015 | P. papatasi (1m)                 | ×                  |
| Bašaid         | 45.6356500 | 20.4080500  | 2                | 01/06/2015 | P. papatasi (1 m)                | ×                  |
| Nova Crnja     | 45.6698167 | 20.6086833  | 2                | 16/07/2015 | P. papatasi (1 f)                | ×                  |
| Banatsko Veliko Selo | 45.8157167 | 20.6091500  | 1                | 16/07/2015 | P. papatasi (1 f)                | ×                  |
| Banatski Monostor | 45.9633000 | 20.2824167  | 1                | 16/07/2015 | P. perfiliewi (1f)               | ×                  |
| Svetozar Miletić | 45.8497833 | 19.1695667  | 1                | 20/08/2015 | P. neglectus (1f)               | ×                  |
Table 1 Positive sand fly localities (coordinates, trapping dates, trap type, number of samples, species and gender) (Continued)

| Locality          | Latitude       | Longitude      | No. of specimens | Date       | Species and gender (male/female) | Positive trap type |
|-------------------|----------------|----------------|------------------|------------|----------------------------------|--------------------|
| Markovićevo       | 45.3249833     | 21.0331667     | 1                | 05/09/2015 | P. papatasi (1f)                 | –                  |
| Miletićevo        | 45.3037167     | 21.0601500     | 1                | 22/08/2015 | P. papatasi (1f)                 | –                  |
| Straža            | 44.9722500     | 21.3015000     | 1                | 16/06/2015 | P. papatasi (1m)                 | –                  |
| Vračev Gaj        | 44.8822000     | 21.3702167     | 2                | 22/08/2015 | P. papatasi (1na)                | –                  |
| Banatska Palanka  | 44.8463833     | 21.344667      | 2                | 14/07/2015 | P. papatasi (2m)                 | –                  |
| Melenci           | 45.5282972     | 20.3037944     | 1                | 29/07/2015 | P. perfiliei (1f)                | –                  |
| Paraje            | 45.4154278     | 19.4045500     | 2                | 16/08/2015 | P. papatasi (1f)                 | –                  |
| Staričevo         | 45.4045000     | 19.7076333     | 1                | 28/06/2015 | P. papatasi (1f)                 | –                  |
| Ravno selo        | 45.4525200     | 19.6197600     | 8                | 20/07/2015 | P. papatasi (6m/2f)              | –                  |
| Ratkovo           | 44.4823900     | 19.3300200     | 3                | 19/07/2015 | P. papatasi (3f)                 | –                  |
| Šilbaš             | 45.3800600     | 19.5037600     | 1                | 19/07/2015 | P. papatasi (1f)                 | –                  |
| Kulpm             | 45.3970900     | 19.5907200     | 2                | 20/07/2015 | P. papatasi (2m)                 | –                  |
| Zmajeko           | 45.4445400     | 19.6999000     | 1                | 20/07/2015 | P. papatasi (1m)                 | –                  |
| Tovarišćevo       | 45.3557600     | 19.3191400     | 6                | 19/07/2015 | P. papatasi (2m/4f)              | –                  |
| Total: 48         |                |                | 80               |            |                                  | 31                 |
| Abbreviations: f female, m male, na unknown gender |

*Localities where Leishmania spp. presence in sand flies was recorded

Fig. 2 Sequence analysis based on the cox1 mitochondrial gene region in Phlebotomus species. Neighbor-joining tree based on the partial cox1 sequences used for molecular identification of the sand fly specimens.
Considering the increased number of canine leishmaniasis in Vojvodina in the past decade, and the presence of phlebotomine species competent vectors of *L. infantum*, all collected females were tested for the presence of *Leishmania*. Of 80 samples examined, three *P. papatasi* specimens resulted positive (4%) for *Leishmania* genus by nPCR. All positive females were collected in 2013. Negative results in 2014 and 2015 might be consequence of low number of samples (per locality and year), and low abundance of the vector species in general.

Although *P. papatasi* is a highly specific vector of *L. major* [29], presence of *L. infantum* DNA in *P. papatasi* has been previously reported [30]. Detection of leishmanial DNA does not imply that the sand fly species is a vector, as the assay cannot distinguish among developmental phases of promastigotes in the gut of infected specimens. According to Adler & Theodor [31], *P. papatasi* became infected after feeding on dogs with canine leishmaniasis, but the infection rate in the insects diminished continuously from 96% after one day, to 4% after 7 days (i.e. when the blood was digested). Our finding is most likely the direct result of recent blood-feeding, since all positive females had an abdomen full of blood, most likely engorged during the previous night(s).

Even though *L. infantum* DNA was detected from a non-competent vector, the fact that the parasite was identified from wild specimens, and the record of *Phlebotomus* (Larroussius) species proven vectors of *L. infantum* (*P. perfiliewi* and *P. neglectus*) [32, 33], indicates the possibility of *L. infantum* circulation in the surveyed region.

We wish also to highlight the importance of the *P. mascittii* record, since this species has long been suspected as a vector of *L. infantum* [34]. So far, *P. mascittii* has only been assumed to be a putative vector of *Leishmania* spp., however the recent detection of *L. infantum* DNA in specimens of *P. mascittii* from Austria [22] and Italy [21] may support possible competence in transmission (with the limitations exposed above about molecular assays for vector incrimination).

Despite the relatively low number of collected specimens, implication of leishmaniasis circulation in Vojvodina, and Serbia as a whole, seems to be accumulating. New cases of human autochthonous VL were recently registered in both past-endemic [35] and non-endemic areas of south-east Serbia [36]. Along with human and canine leishmaniasis cases, the presence of *Leishmania* spp. was also detected in the spleen of golden jackals (Mammalia, Canidae, *Canis aureus*) in central and east Serbia [37]. The possibility of vertical transmission among canine populations was discussed by Boggiairo et al. [38], and it is not excluded as a probable way of sustaining infection within wild and domestic canid population in Vojvodina/Serbia, since low numbers of sand flies were recorded. Beyond Serbia, reports of the disease are accumulating from all neighbouring countries [39–41]. Constant flow of humans, animals and commodities through Vojvodina increases the risk of parasite introduction and disease emergence, since this region is situated in the main transit route of tourism and trade between the Mediterranean and Middle-eastern countries and Central and northern Europe.

**Conclusions**

Presence of autochthonous canine leishmaniasis cases, detection of *L. infantum* DNA from wild-caught (non-competent) vector, and the record of *Phlebotomus* (Larroussius) species, which are proven vectors of *L. infantum* (*P. perfiliewi* and *P. neglectus*), indicate the possible dynamics of endemic circulation of *L. infantum* in the surveyed region.

**Abbreviations**

CDC: Centre for Disease Control; CL: Cutaneous leishmaniasis; cox1: Cytochrome c oxidase subunit 1; ITS: Internal transcribed spacer; NGS: Next-generation sequencing; nPCR: nested PCR; RFLP: Restriction Fragment Length Polymorphism; SSU: Small subunit; VL: Visceral leishmaniasis

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**Availability of data and materials**

The datasets supporting the conclusions of this article are included within the article. The sequences have been deposited in the GenBank database under the accession numbers: KX848828 (*Phlebotomus papatasi*); KX848829 (*Phlebotomus perfiliewi*); KX848830 (*Phlebotomus neglectus*); KX848831 (*Phlebotomus mascittii*); and KY646445 (*Leishmania*).

**Authors’ contributions**

SV conducted the research planning, field sampling, morphological identification, molecular identification (extraction, PCR, sequencing), *Leishmania* spp. testing, wrote the original manuscript; NA conducted the field sampling, molecular identification of samples (extraction, PCR, sequencing); GO participated in field sampling; SS and YO was involved *Leishmania* spp. sequencing; BA and DP were involved in technical development of Republic of Serbia; and 13981-1 project for Diversity and spatial distribution of sandflies and pathogens they transmit (Leishmania, phleboviruses) in Vojvodina province (Serbia) funded by the Rufford Small Grant Foundation.

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**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.
Competing interests
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