Phlebotomine Sand Fly Survey in the Republic of Moldova: Species Composition, Distribution and Host Preferences

Tatiana Sulesco (tatiana_sulesco@yahoo.com)
Laboratory of Entomology, Institute of Zoology, Chisinau, Republic of Moldova https://orcid.org/0000-0002-8287-1461
Ozge Erisoz Kasap
Hacettepe University: Hacettepe Universitesi
Petr Halada
BioCeV, Institute of Microbiology of the Czech Academy of Sciences
Gizem Oguz
Hacettepe University: Hacettepe Universitesi
Dimian Rusnac
Institute of Zoology
Gresova Marketa
Charles University Faculty of Science: Univerzita Karlova Prirodovedecka fakulta
Bulent Alten
Hacettepe University: Hacettepe Universitesi
Petr Volf
Charles University Faculty of Science: Univerzita Karlova Prirodovedecka fakulta
Vit Dvorak
Charles University Faculty of Science: Univerzita Karlova Prirodovedecka fakulta

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Abstract

Background: Phlebotomine sand flies (Diptera: Psychodiae) in the Republic of Moldova have been understudied for decades. Our study provides a first update on their occurrence, species composition and bloodmeal sources after fifty years.

Methods: During 5 seasons (2013-2017), 58 localities from 20 regions were surveyed for presence of sand flies using CDC light traps and manual aspirators. Species identification was done by a combination of morphological and molecular approaches (DNA barcoding, MALDI-TOF MS protein profiling). In engorged females, host blood was identified by three molecular techniques (RFLP, *cytb* sequencing and MALDI-TOF peptide mass mapping). Population structure of most abundant species was studied by *cox*1 haplotyping, phylogenetic analyses of ITS2 and *cox*1 genetic markers were used to resolve relationships of other detected species.

Results: In total, 780 sand flies were collected at 30 (51.7%) localities from 12 regions of Moldova. Three species were identified by an integrative morphological and molecular approach: *Phlebotomus papatasi*, *P. perfiliewi* and *Adlerius* sp., the first being the most abundant and widespread, markedly anthropophilic based of bloodmeal analyses and occurring also indoor, showing low structuring of population with only 5 haplotypes of *cox*1 detected. Distinct morphological and molecular characters of *Adlerius* sp. specimens suggest presence of yet undescribed species.

Conclusions: Our study revealed a presence of stable sand fly population of three species in Moldova that pose a nuisance by biting as well as potential thread of pathogen transmission and shall be further studied.

Background

Phlebotomine sand flies (Diptera: Psychodidae) are vectors of several infectious pathogens, including parasitic protozoans of the genus *Leishmania* and phleboviruses, and thus of great importance in human and veterinary medicine (Maroli et al., 2013). In Europe, they are incriminated in *Leishmania* transmission that occurs mostly in the Mediterranean countries where *L. infantum* transmitted by several species of the subgenus *Larroussius* is well established and two other *Leishmania* species have recently re-emerged (Antoniou et al., 2013). However, expansion of sand flies due to climatic and environmental changes into regions where they were not previously established is expected (Medlock et al., 2014), posing a risk of *Leishmania* introduction into non-endemic areas (Tánczos et al., 2012). Recently, permanent sand fly populations were recorded in several countries north of their traditional distribution (Poeppl et al., 2013, Oerther et al., 2020) and knowledge of sand fly fauna in long-time understudied regions of Balkan countries (Dvorak et al., 2020b) and Romania (Cazan et al., 2019) was significantly updated, further demonstrating the importance of entomological field research at the edge of their occurrence.

Moldova is considered a country non-endemic for leishmaniasis, there are no records of autochthonous human or canine cases (Mihalca et al., 2019). In the past, presence of three *Phlebotomus* species was

reported in Moldova: *P. papatasi* (Scopoli), *P. perfiliewi* Parrot and *P. chinensis* Newstead (Petrishcheva, 1962, Petrishcheva, 1967). However, the historical data provided no information about their geographical distribution within the country and there is no recent knowledge regarding sand fly presence, species composition, spatial distribution and bloodmeal preferences. This study presents first data in the last 50 years on these aspects of sand fly occurrence in Moldova.

Methods

Sand fly sampling

A countrywide field survey was conducted to detect sand flies at 58 localities from 20 regions between 2013 and 2017. First surveys in Moldova sampled one rural locality in southern Moldova (July 2013, August 2014 and June - September 2015) and one urban area in central Moldova (July-September 2013) by miniature Centre for Disease Control (CDC) light traps (John W. Hock Company, model 512, Gainesville, Florida, U.S.A.) and manual aspirators (Additional file 1: Table S1). In 2015, two CDC light traps were operated on the same sites close to the animal shelter between June 22 and September 26, 2015. Cross-sectional entomological surveys were conducted between 2016 and 2017. A total of 55 localities from 16 regions were surveyed in 2016 and 10 localities from 9 regions in 2017 (Fig. 1a). Sampling was performed using CDC light traps baited with CO_2 (dry ice) and placed inside or outside of the animal shelters (livestock sheds, hen houses, dog kennels) (Fig. 1b). They were used overnight in the places protected from wind exposure. Entomological collections were complemented by mouth aspirators inside the houses and animal shelters. Each collected site was numbered and mapped using global positioning system (GPS). One rural locality in southern Moldova has been selected for systematic sand fly collections in the years 2015 and 2017 by CDC traps and manual aspirators (Ceadir-Lunga, WGS84 coordinates: 46.06549 N, 28.84219E).

Morphological identification of sand flies

The collected insects were killed by freezing in dry ice and preserved in 70 or 96% molecular grade ethanol. For morphological identification, head and genitalia of each specimen were dissected and mounted on slides using CMCP-10 high viscosity mounting medium (Polysciences, Hirschberg, Germany) or Berlese mounting medium and the rest of the body was stored in ethanol for molecular analyses. Species identification was based on decisive morphological characters using published keys and descriptions (Perfiliev 1968, Lewis 1982). For specimens belonging to the subgenus *Adlerius*, morphometric measurements of decisive characters on head and genitalia were done using a light microscope Olympus BX51 (Olympus Life Science, Waltham, USA) with a camera system Olympus D70. Morphological characters were measured using the QuickPHOTO MICRO 3.0 software (Promicra, Prague, Czech Republic) and compared with previously published values (Artemiev 1980, Dvorak et al., 2020a).

Molecular taxonomy of sand flies

The remaining body parts of sand flies were stored in 96% ethanol for DNA extraction that was done using High Pure PCR Template Preparation Kit (Roche Life Science, Penzberg, Germany). Species identity of sand fly specimens was further assessed by amplification of cytochrome oxidase I (*cox*1) of mtDNA region using LCO/HCO primer pair or amplification of the second internal transcribed spacer 2 (ITS2) using the primer pair JTS3/C1a following the protocols reported in Folmer et al., 1994 and Depaquit et al., 2002, respectively. 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. Sequences were edited and aligned using the BioEdit (v.7.0.9) (Hall, 1999) alignment editor.

*cox*1 sequences obtained for *P. papatasi* were compared with those available in GenBank using BLAST algorithm. Haplotype network for 36 *P. papatasi* specimens collected at 20 different localities (Additional file 2: Table S2) was constructed by PopArt (Leigh 2015) using TCS method. The position of collected *P. perfiliewi* specimens within the species complex *Phlebotomus perfiliewi* sensu lato was assessed by comparing their ITS2 sequences with those deposited in GenBank. c*ox*1 sequences obtained for *Adlerius* sp. from Moldova and the sequences available for the other members of the subgenus in GenBank were analyzed together to construct a Neighbor Joining tree based on the Kimura's two parameter (K2P) substitution model in MEGA v.6.0 (Tamura et al., 2013). Relationship between the members of *Adlerius* subgenus was further evaluated by constructing parsimony networks using TCS method in PopArt.

Identification of sand flies using MALDI-TOF mass spectrometry

Sample preparation and analysis by MALDI-TOF MS protein profiling followed protocol optimized for sand flies (Dvorak et al., 2014): thoraxes of dissected specimens were homogenized by a manual BioVortexer homogenizer (BioSpec, Bartlesville, USA) with sterile disposable pestles in 10 µl of 25% formic acid, briefly centrifuged at 10000 g for 15 s. Two µl of the homogenate were mixed with 2 µl of freshly prepared MALDI matrix which was an aqueous 60% acetonitrile/0.3% TFA solution of sinapinic acid (30 mg/ml; Bruker Daltonics, Bremen, Germany). One µl of the mixture was then spotted on a steel MALDI plate in duplicate. Protein mass spectra were measured in a mass range of 4-25 kDa on an Ultraflex III MALDI-TOF spectrometer (Bruker Daltonics) as a sum of 2000 laser shots (20x100 shots from different positions of the sample spot) and analyzed by FlexAnalysis 3.4 software. For species identification and cluster analysis, the protein profiles were processed using MALDI Biotyper 3.1 and compared with reference spectra of an in-house database constructed using protein spectra of 25 different sand fly species. For MSP dendrogram creation, an individual main spectrum (MSP) was generated from each analyzed spectrum.

Bloodmealanalysis

Bloodmeals of engorged females, all identified as *P. papatasi* and captured in 11 different villages mainly by manual aspirators, were analyzed by three molecular approaches, using two DNA-based techniques and a mass spectrometry approach. Specimens were dissected and slide-mounted for morphological species identification, abdomens containing host blood were used for molecular techniques. Species identity of specimens where morphological identification was not conclusive was further confirmed by

MALDI-TOF MS protein profiling using their remaining thoraxes. Additional file 3: Table S3 summarizes details about all engorged females analyzed for identification of blood origin and indicates which methods were applied.

Bloodmeals of engorged females collected in 2016, all identified as *P. papatasi* were analyzed by combination of RFLP assay by HaeIII and HinfI restriction enzymes and sequencing of cytochrome B (*cyt b*). In total, 100 bloodfed females were analyzed, originating from 10 of the surveyed localities: Andrusul de Jos (n=6), Balanesti (n=11), Boscana (n=23), Ciopleni (n=10), Colibasi (n=8), Corjova (n=2), Rosu (n=11), Slobozia Mare (n=14), Malaesti (n=10), Malaesti Noi (n=5). A fragment of 359 bp of vertebrate *cyt b* gene was amplified using the modified vertebrate-universal specific primers cyt bb1/cyt bb2 (Kocher *et al.*, 1983; Malmqvist *et al.*, 2004). PCR amplification was performed as described by González *et al.*, 2015. The PCR product was purified by QIAquick PCR Purification Kit (QIAGEN) and 15 µl were digested by 1 µl of the enzyme HaeIII or HinfI (New England Biolabs) at 37◦C for 20 minutes in 50 µl of total solution according to a protocol of the producer. Digested product was separated on 2% agarose gel and observed under UV light. For chosen specimens, the purified amplicons were sequenced in both directions using same primers cyt bb1/cyt bb2 to confirm the bloodmeal identification by RFLP.

Bloodmeals of chosen engorged females collected in 2016 and 2017, all identified by morphology as *P. papatasi*, were analyzed by peptide mass mapping of host-specific haemoglobin peptides using MALDI-TOF mass spectrometry. In total, 25 females originating from Ceadir-Lunga (n=5), Corjova (n=2) Malaesti Noi (n=4) and Slobozia Mare (n=14) were prepared as described by Hlavackova et al. (2019). From dissected specimens, thoraxes were stored for confirmation of species identification by MALDI-TOF MS protein profiling and abdomens were homogenized in 50 µl of distilled water (Merck KGaA, Darmstadt, Germany) by BioVortexer homogenizer (BioSpec), 10 µl of the homogenate was then incubated with 10 µl of 50 mM N-ethylmorpholine acetate buffer (pH 8.1; Sigma-Aldrich) and 100 ng of trypsin (Promega) at 37˚C for 30 minutes. After the digestion, 0.5 µl of the mixture was deposited on a MALDI plate in duplicate, air-dried and overlaid with 0.5 µl of MALDI matrix (aqueous 50% acetonitrile/0.1% TFA solution of α-cyano-4-hydroxycinnamic acid; 5 mg/ml; Bruker Daltonics). Peptide mass mapping spectra were acquired on an Ultraflex III MALDI-TOF instrument in the mass range of 700–4000 Da and calibrated externally using a peptide standard PepMix II (Bruker Daltonics). At least two peptides per sample were selected for MS/MS sequencing using LIFT technology. MS/MS data were searched against the SwissProt database subset of vertebrate proteins using in-house MASCOT 2.1 search engine (Matrix Science).

Results

Entomological survey

In total, 151 sites from 58 localities were sampled by CDC traps and manual aspirators, of which 65 (43.0%) sites were positive for sand flies (Additional file 1: Table S1). A total of 780 sand flies (34.7%

males) were collected and identified between 2013 and 2017 from 30 (51.7%) localities belonged to 12 regions of Moldova (Fig. 1a).

Overall, 66.9% (522/780) of specimens were collected by manual aspirators from 31 sites belonged to 21 villages, inside the houses (381 specimens, 26 sites) and animal shelters (141 specimens, 5 sites) (Fig. 1b). Three sand fly species of the genus *Phlebotomus* were identified based on their morphological characters as described later: *P. papatasi*, *P. perfiliewi* and *Adlerius* sp. The most abundant species was *P. papatasi* (n = 741; 265 males, 476 females), followed by *Adlerius* sp. (n = 20; 3 males, 17 females) and *P. perfiliewi* (n = 19; 4 males, 15 females). *Phlebotomus papatasi* was the most distributed species, present in 48.3% (n = 28) of all sampled localities and 41.7% (n = 65) of all collected sites from ten regions in Moldova. During the entomological surveys, the species was present in high numbers inside the hen houses and human dwellings located in central and southern Moldova. Overall, 71.5 % (n = 530) of *P. papatasi* specimens were collected by manual aspirators inside the buildings. *Phlebotomus perfiliewi* was present in 13.8% (n = 8) of all sampled localities and 6.6% (n = 10) of all sites, located in six regions of the country. This species was sampled only by CDC light traps with or without dry ice, placed inside or outside the animal shelters with the potential animal hosts represented mainly by goats, poultry and dogs. *Adlerius sp.* specimens were collected from 10.3% (n = 6) of all inspected localities belonged to four regions. This taxon was sampled mainly by CDC light traps, only two females were captured by manual aspirator inside the hen house and one male was collected inside the house.

A total of 225 and 47 sand flies were sampled in 2015 and 2017, respectively, in the locality Ceadir-Lunga selected for permanent sand fly collections to study population dynamics during the summer season. Two trapping methods were used.

Two CDC light traps operated on two same sites in Ceadir-Lunga close to the animal shelter between June 22 and September 26, 2015, in total, trapped 18 sand flies with all three species present: *P. papatasi* (4 males, 5 females), *P. perfiliewi* (4 females) and *Adlerius* sp. (5 females). First specimens were trapped in mid-July and last in mid-August. Collection by manual aspirators inside houses and animals shelters between June 26 and September 14, 2015 yielded 207 sand flies. The highest number of sand flies (n = 160) of two species was collected in July in hen houses: *P. papatasi* (49 males, 108 females) and *Adlerius* sp. (1 male, 2 females). Indoor sand fly activity was markedly longer, first specimens being collected at first aspiration in June 24 and last in September 14 which was the last day of collections by aspirators.

In 2017, a total of 18 sand flies were collected by two CDC traps, operated on the permanent sites between July 6 and September 25, 2017. The majority of specimens (n = 10) belonged to *P. papatasi* (3 males, 3 females), *Adlerius* sp. (3 females) and *P. perfiliewi* (1 female) was collected in July. Seven sand fly specimens of two species (4 males of *P. papatasi* and 3 females of *Adlerius* sp.) were sampled in August and one specimen (*P. papatasi* male) in September 2017.

Morphological species identification

Specimens of the subgenera *Phlebotomus* and *Larroussius* were all identified based on morphological characters on their head (pharyngeal armature) and genitalia (spermathecas of females, morphology of external terminalia of males) as *Phlebotomus papatasi* and *P. perfiliewi*, respectively. For *Adlerius* sp. specimens, decisive morphological characters were compared with previously published values of described species within the subgenus, especially four species previously reported from Europe: *Phlebotomus balcanicus*, *P. creticus*, *P. longiductus*, and *P. simici* (Table 1). Three analyzed males had markedly longer flagellomere 3 (A3) and labrum than the European species with *P. chinensis* and *P. longiductus* having the closest values of the remaining *Adlerius* species. Length of appendices on the external genitalia (coxite, style, parameral sheath) varied but did not match any of the *Adlerius* species completely, the number of coxite hairs was lower than for

Table 1 Mean values obtained for the descriptive morphological characters for three males (A) and 15 females (B) of *Adlerius* sp. in Moldova compared with other *Adlerius* species reviewed in Artemiev (1980) and newly described *P. creticus* (Dvorak et al., 2020a). Minimum and maximum values of each measurement and count were given in parenthesis. * Measurements in µm.

(A)	A3*	A3/Labrum*	Style length*	Coxite length*	Parameral sheath length*	Coxite /Parameral sheath*	No. of coxite hairs
Adlerius sp.	461.67	1.82	215. 95	436.75	183.67	2.38	60
	(439. 44 - 493.20)	(1.69 - 1.86)	(207.10 - 223.00)	(402.10 - 461.95)	(176.47 - 191.60)	(2.20 - 2.61)	(57 - 64)
P. balcanicus	372.00	1.39	200.00	424.00	188.00	2.28	106
	(320.00 - 408.00)	(1.17-1.52)	(184.00 - 216.00)	(388.00 - 464.00)	(164.00 - 208.00)	(2.08 - 2.52)	(92 - 130)
P. chinensis	419.00	1.59	202.00	364.00	175.00	2.06	24
	(396.00 - 454.00)	(1.54 - 1.64)	(188.00 - 215.00)	(344.00 - 380.00)	(164.00 - 182.00)	(1.95 - 2.09)	(20 - 27)
P. creticus	340.00	1.29	x	405.20	195.30	x	69
	(285.00 - 406)	(1.14 - 1.53)	x	(345.00 - 451.00)	(178.00 - 219.00)	x	(54 - 85)
P. longiductus	417.00	1.52	204.00	386.00	195.00	1.98	64
	(340.00- 500.00)	(1.31 - 1.83)	(180.00 - 236.00)	(340.00 - 428.00)	(172.00- 240.00)	(1.64 - 2.23)	(51 - 81)
P. simici	275.00	1.18	148.00	296.00	189.00	1.57	20
	(240.00 - 290.00)	(1.10- 1.20)	(140.00 - 156.00)	(270.00 - 320.00)	(170.00 - 200.00)	(1.55 - 1.59)	(19- 22)

(B)	A3*	A3/Labrum*
Adlerius sp.	397.80	1.26
	(269.90 - 458)	(0.98 - 1.52)
P. balcanicus	304.00	0.90
	(244.00 - 360.00)	(0.69-0.99)
P. chinensis	325.00	1.27
	(280.00 - 368.00)	(1.25 - 1.31)
P. creticus	298.00	0.88
	(268.00 - 326.00)	(0.80 -0.94)
P. longiductus	388.00	1.06
	(332.00-440.00)	(0.93 - 1.30)
P. simici	244.00	0.80
	(200.00 - 270.00)	(0.75- 0.86)

P. balcanicus and higher than *P. simici* and *P. chinensis*. In 15 analyzed females, the length of A3 exceeded all *Adlerius* species except for *P. longiductus* which also has similar morphology of pharyngeal armature (Fig. 2)

Species identification by molecular techniques

DNA barcoding by *cox*1 were performed for 36 randomly selected sand fly specimens morphologically identified as *P. papatasi*. The final alignment was 602 bp long and five unique haplotypes were found to be distributed in the sampling localities. Obtained sequences of all specimens morphologically identified as *P. papatasi* confirmed the species identification when compared with sequences from GenBank using BLAST algorithm (99.46% - 100% identity). Constructed haplotype network placed five haplotypes into a single network. Two haplotypes (hap1 and hap2) are dominant, and present at most of surveyed localities while other three (hap3, hap4, and hap5) have a restricted occurrence, two of them unique at a single locality, Malaesti and Crihana Veche, respectively. Among these five haplotypes, only five polymorphic positions were found (Fig. 3).

For collected specimens of *P. perfiliewi*, sequencing of ITS2 was done to assess their position within *P. perfiliewi* species complex. All analyzed specimens provided identical sequence and a Neighbor Joining analysis with sequences of *P. perfiliewi* species complex available from GenBank showed their position in a lineage of *P. perfiliewi* s.s. with populations from Greece and Ukraine.

The final alignment of the 12 *cox*1 sequences obtained for the *Adlerius* sp. specimens collected from Moldova was 547 bp long. All the Moldovan *Adlerius* specimens were revealed to belong to the same species as they clustered together in the NJ tree, and have diverged from the rest of the *Adlerius* species included in the analysis (mean K2P distance: 6.5%- 16.7%). The clade comprised of *P. longiductus* from China, *Adlerius* sp. from Moldova and *P. chinensis* from China was divided into three well supported (100% bootstrap values) lineages (Fig. 4). Concordantly, the mean K2P distances between *Adlerius* sp. from Moldova and *P. chinensis* and *P. longiductus* from China were higher (13.4% and 6.5%, respectively) than those yielded between some species pairs within the subgenus *Adlerius* (Additional file 4: Table S4). Supporting these results, TCS identified three independent networks for these three taxa. The first network included only the five *Adlerius* sp. haplotypes from Moldova, while the second and third networks comprised of *P. longiductus* (n=2) and *P. chinensis* (n=5) haplotypes from China, respectively. With a 95% connection limit, 32 mutational steps were required to connect Moldovan *Adlerius* sp. to *P. longiductus* and 64 mutational steps to connect *Adlerius* sp. from Moldova to *P. chinensis* (Fig. 5).

The NJ tree clearly showed that the recently identified *P. creticus* (Dvorak et al., 2020a) is highly diverged from *Adlerius* sp. from Moldova (mean K2P distance=12.3%), clustering together with *P. balcanicus* sequences from Romania and Serbia. This group was placed as a sister taxon to *P. balcanicus* from Turkey (Fig. 4). The mean K2P distance between *P. creticus* and *P. balcanicus* from Romania and Serbia was lower (1.8%) than the one yielded between *P. creticus* and *P. balcanicus* from Turkey (4.5%) (Additional file 5: Table S4). These results were in concordance with the TCS results, which grouped *P. creticus* and *P. balcanicus* from Romania and Serbia in one network and constructed an independent network for *P. balcanicus* from Turkey (Additional file 5: Fig. S1). *P. simici* specimens originated from Austria and Greece were clustered together, while Turkish and Israeli *P. simici* sequences were placed in a distinct lineage in the NJ tree, with high bootstrap support (>95.0%). Rest of the taxa classified in the subgenus *Adlerius* were each represented by a single lineage (Fig. 4).

MALDI-TOF mass spectrometric protein profiling

Species identification of chosen specimens from collections performed in 2016 and 2017 was confirmed by MALDI-TOF MS protein profiling. In total, 20 specimens were analyzed (10 collected in 2016, 10 collected in 2017). All except two produced intense, species-specific protein profiles of high quality.

Of material collected in 2016, protein profiles of 4 specimens originating from two localities (Balabanesti and Ciopleni) identified by morphology as *Phlebotomus papatasi* indeed matched with protein profiles of this species in the reference database with a log score value (LSV) > 2.0 that is accepted as the unambiguous assignment. Similarly, 4 specimens identified by morphology as *P. perfiliewi* (1 male and 1 female from Braniste, 2 females from Balabanesti) produced spectra similar to *P. perfiliewi* in the reference database with LSV > 2.0 and thus conclusively identified as this species. Protein spectra of 1 male and 1 female *Adlerius* sp. originating from Magdacesti and Balabanesti, respectively, showed differences from spectra of all *Adlerius* species represented in the reference database (*P. arabicus*, *P.*

balcanicus, *P. creticus*, *P. halepensis*, *P. simici*), indicating that Moldovan specimens represent another species within the subgenus *Adlerius*.

Of material collected in 2017, most effort was therefore focused on the further analysis of *Adlerius* sp. Seven of ten analyzed specimens (1 male from Corten, 6 females from Ceadir-Lunga) produced identical protein profiles similar with those of *Adlerius* sp. collected in 2016. In addition, protein profiles of 1 male of *P. papatasi* (locality Ceadir-Lunga) and 2 females of *P. perfiliewi* (locality Ceadir-Lunga and Slobozia Mare) were obtained, all similar with protein spectra of respective species obtained in the previous season.

Specimens of three recorded species that belong to different subgenera of the genus *Phlebotomus* produced reproducible, distinctive and substantially different protein spectra (Fig. 6a), enabling rapid and conclusive species identification. No differences in the protein spectra of specimens from different localities were observed. Dendrogram constructed based on protein spectra of all 20 analyzed specimens shows clear clustering corresponding to these three species (Fig. 6b). It also demonstrates that two specimens with spectra of compromised quality (LCE01, a *P. perfiliewi* female from Ceadir-Lunga and LCU01, an *Adlerius* sp. male from Corten, both collected in 2017) were successfully and correctly identified, albeit positioned on long branches that reflect the lower quality of their spectra.

Protein spectra of collected *Adlerius* sp. specimens were compared with protein profiles of three species of the subgenus *Adlerius* recently known to occur in Europe: *Phlebotomus balcanicus* and *P. simici* from an entomological survey in Bosnia and Herzegovina, Montenegro and Northern Macedonia (Dvorak et al., 2020b) and newly described *P. creticus* from Crete (Dvorak et al., 2020a). Figure 7 shows that specimens of all four species grouped in their own distinct clades, *Adlerius* sp. forming a sister cluster with *P. simici* from Northern Macedonia.

Bloodmeal analysis of engorged females

A total of 100 engorged *P. papatasi* females captured in 10 villages, mainly by manual aspirators, in 2016 were selected for analysis of bloodmeal sources by RFLP assay, digesting the 359 bp part of vertebrate *cyt b* gene using HaeIII and HinfI restriction enzymes. Obtained restriction patterns corresponded with humans in 98 specimens and with chickens (*Gallus gallus*) in 2 specimens. Sequencing analysis of *cyt b* amplicons of 2 specimens tested as feeding on chickens and 20 randomly chosen specimens from different localities confirmed the host identification revealed by RFLP.

MALDI-TOF peptide mass mapping analysis of bloodfed females included specimens collected in 2016 and 2017, providing distinct host peptide maps for 22 of 25 analyzed females. All successfully analyzed specimens displayed 8-16 signals of host-specific alpha- and beta-haemoglobin peptides, except one female with lower number of host peptides which nevertheless still enabled a conclusive identification of the blood source (Additional file 3: Table S3). The sequencing of the peptide fragments by tandem mass spectrometry (MS/MS) and subsequent database searching provided amino acid sequence of these host

peptides and assigned following blood origin: 6 chickens (*Gallus gallus*) in samples from Slobozia Mare, 2 humans from Corjova, 3 humans from Malaesti Noi, 3 humans and 8 chickens from Ceadir-Lunga.

Discussion

This study provides first data on sand fly species composition, spatial distribution and host preferences in Moldova after more than 50 years, substantially updating two short reports that indicated the presence of three species (*P. papatasi*, *P. perfiliewi* and *P. chinensis*) without providing any details about their geographical distribution (Petrishcheva 1962, Petrishcheva 1967).

Our study confirmed low sand fly species diversity reported in Moldova in the past, with three species collected (*P. papatasi, P. perfiliewi* and *Adlerius* sp.). For their species identification, an integrative approach was deployed that enables in parallel morphological as well as molecular identification, utilizing both DNA barcoding as a standard method of molecular typing and MALDI-TOF MS protein profiling, a rapid and cost-effective tool recently emerging as an alternative method for species identification of various organisms including medically important arthropods. It provided 100% correct species determination with most protein profiles of a high quality and stable for specimens collected at different localities and during two consecutive seasons, thus representing a sufficiently robust identification tool. Moreover, correct species determination of few specimens, that provided protein spectra of lower quality, was nevertheless also achieved. These results support the use of such combined taxonomical approach as suitable for processing sand flies collected through field entomological surveys.

Presence of two of three previously detected species in Moldova was confirmed by our sampling, *P. papatasi* being the most abundant and most widely distributed species and found in 93.3% of all positive localities in Moldova. Detailed historical data on *P. papatasi* density and distribution in the country are not available. High densities of *P. papatasi* were reported in the 1950s during the sand fly fever virus outbreak in Reni and Bolgrad regions in southern Ukraine located close to the border with Moldova. The Ukrainian sand fly control efforts focused on treatment with dichlorodiphenyltrichloroethane (DDT) and hexachlorocyclohexane the buildings in the villages between 1951 and 1954 gave a rapid reduction of sand fly fever morbidity and significantly decreased the sand fly densities (Gritsai et al., 1957). Between years 1948 – 1956, the national malaria eradication programs successfully used the same insecticides to combat the malaria vector *Anopheles maculipennis* s.l. in Moldova. It can be assumed that the widespread use of insecticides led to a decrease of the sand fly densities in Moldovan villages as well. Before the 1990s, breeding the livestock in the households was common in the countryside. Deterioration of the economic situation led to a reduction of livestock which was substituted by a high number of backyard poultry in the villages. Perhaps both socioeconomical and environmental changes promoted the increase of *P. papatasi* densities in the villages.

The presence of sand flies inside the human dwellings and animal shelters was first reported by residents from southern Moldova in 2011 when the local population started suffering from sand fly bites during the

summer season. Our entomological surveys revealed the presence of *P. papatasi* in high numbers inside the human dwellings and hen houses in southern and central Moldova. The most northern sites of *P. papatasi* collections were recorded at latitudes 47°35' - 47°47', where the species was present in low numbers only in CDC trap collections.

Bloodmeal analysis by RFLP of engorged *P. papatasi* females collected in 2016 survey mainly by manual aspirators inside the houses demonstrated their strong anthropophily, 98% of successfully analyzed engorged females feeding on humans while domestic chickens were detected as an additional and less frequently utilized host. These findings were confirmed by a sequencing analysis of chosen engorged specimens. Since peptide mass mapping using MALDI-TOF mass spectrometry was recently introduced as a new and effective tool for bloodmeal identification of haematophagous arthropods (Hlavackova et al., 2019), it was also used to identify the blood source of some engorged *P. papatasi* females. Obtained results confirmed both humans and chickens, emphasizing the apparent tight trophic connection of *P. papatasi* with human dwellings.

P. papatasi is a species with markedly wide geographical distribution, however, studies based on several genetic markers showed that various geographical populations are relatively homogeneous and of limited genetic differentiation (Depaquit et al., 2008, Esseghir et al., 1997). More genetic variation was observed mainly among populations geographically separated by prominent natural barriers such as the High Atlas Mountains in Morocco (Guernaoui et al., 2020). In our study, *P. papatasi* collected at various sites in Moldova showed a limited genetic variability in cytochrome oxidase I gene, providing five haplotypes with only five polymorphic sites, suggesting a genetically homogeneous population. With respect to the geography of Moldova and adjacent regions that lack natural barriers of dispersal, we may expect a rather unrestricted gene flow with populations from neighbouring countries.

Two other recorded species, *P. perfiliewi* and *Adlerius* sp. were present sporadically in CDC trap collections inside or outside the animal shelters. *Phlebotomus perfiliewi* is one of the main vectors of *Leishmania infantum* in the Mediterranean basin and in Central Asia (Maroli et al., 2013). It is regarded as a species complex of at least three species, however, recent taxonomic study failed to align their morphological and genetic characters and sequencing analyses of two chosen markers (ITS2 and *cyt* B) provided incongruent phylogenies (Depaquit et al., 2013). ITS2 sequences obtained from specimens collected in Moldova suggest that they belong to *P. perfiliewi* s.s. and are closely related to populations from Greece and Crimea.

The subgenus *Adlerius* harbors over 20 described and formally undescribed species, females of which are usually considered indistinguishable morphologically and their identification is often based on the identification of associated males (Artemiev, 1980, Akhoundi et al., 2012). Previous historical record of *P. chinensis* in Moldova is questionable and shall be considered with respect to later evolvement of taxonomy within the subgenus. *Phlebotomus chinensis* is a species currently understood to occur only in China. In the past, however, such taxonomic designation was applied on sand fly populations from much broader geographical area which were later described as new *Adlerius* species (Artemiev, 1980).

Morphological analysis of males from Moldova, albeit limited to only three trapped specimens, suggests that they differ from *P. chinensis* as well as two of three European *Adlerius* species, *P. balcanicus* and *P. simici* in several characters including number of coxite hairs. This was further confirmed by MALDI-TOF MS protein profiling which added evidence that also a third European *Adlerius* species, only recently described *P. creticus*, provides species-specific protein profiles distinctively different from specimens from Moldova. In a dendrogram derived from their protein spectra (Fig. 7), *Adlerius* sp. from Moldova clustered with *P. simici* while *P. creticus* formed a sister clade with *P. balcanicus*. For the *cox*1 sequences obtained for the *Adlerius* sp. (both male and female specimens) from Moldova, the BLAST search revealed these sequences to be 93.78-94.15% identical to *P. longiductus* from China (GenBank accession numbers: KF137551- KF137553). The NJ analysis placed the Moldovan *Adlerius* sp. and *P. longiductus* in highly diverged lineages (Figure 4), and the mean K2P distance between these two lineages (6.5%) was comparable with those previously reported for different sand fly species classified in the subgenus *Adlerius* (Erisoz Kasap et al., 2019). These results are further corroborated by parsimony network analysis which identified two independent networks for these two lineages, suggesting they may represent two different species.

Supporting the sequencing data, both female and male *Adlerius* sp. specimens were found to be different than *P. longiductus* morphologically as described above, although the statistical significance of this difference was not assessed. *P. longiductus* is widely distributed in China, Central and South Asia, and in the Middle East and has been previously reported from Eastern Europe (reviewed in Maroli et al., 2013). Therefore, examination of representative specimens across its range is needed to reveal if these morphological and molecular differences reflect intraspecific geographical variation of local populations or the cryptic speciation within this taxon. Close relationship between *P. balcanicus* sequences from Eastern Europe (Romania and Serbia) and recently identified *P. creticus* needs further evaluation as well as the divergence of two lineages within the widely distributed *P. simici* which has been recently recorded in Austria, suggesting possible northward spread in Europe (Kniha et al., 2021). An integrative approach deploying several molecular methods which study both DNA and proteins to complement traditional morphological “golden standard” was recently successfully applied when describing *Phlebotomus creticus*, a novel species from eastern Mediterranean (Dvorak et al., 2020a), as well as studying sand fly fauna in East Africa (Pareyn et al., 2020). Our findings further advocate its use as a valuable tool that provides better understanding of taxonomy and biogeography of this morphologically challenging yet medically important sand fly subgenus.

Notably, five species of sand flies were recently reported from Romania (Cazan et al., 2019) and six species are known from Ukraine (except Crimea) (Petrishcheva, 1967; Artemiev, 1980; Artemiev and Neronov, 1984). We may speculate that some of the species occurring in these countries may be detected by further entomological surveys also in Moldova, especially when wider surveillance is possible. While this study cannot provide robust data on the seasonality of three detected species, the results obtained in Ceadir-Lunga, a most comprehensively surveyed site located in the south of the country, suggest that sand fly activity indoors may start earlier than outdoors and last until mid-September. Following studies

at different localities with sand fly presence shall provide full understanding of sand fly seasonal dynamics in Moldova.

Moldova is so far regarded as non-endemic country for leishmaniasis (Mihalca et al., 2019). In the past, a single officially published imported human case was reported in a one year old child who travelled with his parents from Rustavi, Georgia to Straseni Region, Moldova in the spring of 2013 (Lungu, 2014). In the 1980s, a total of 6,000 samples of human serum from 28 regions of Moldova were tested for the presence of antibodies to sand fly fever viruses. In Vulcanesti region (southern Moldova), 7.0 % and 10.5 % of humans tested were positive for antibodies to sandfly fever Sicilian virus (SFSV) and Neapolitan sandfly fever viruses, respectively (Scoferta et al., 1984). Precise information on the imported/autochthonous human and canine cases of leishmaniasis in Moldova is not available. However, autochthonous cases of canine leishmaniasis have been recently reported in several countries in proximity of Moldova which are also considered as non-endemic for leishmaniasis, namely Romania (Dumitrache et al., 2016), Hungary (Tánczos et al., 2012) and Ukraine (Gavrilova and Dragushchenko, 2012). The Republic of Moldova continues to preserve the traditional rural lifestyle with domestic pets, poultry and cattle in the backyard animal shelters, but with limited veterinary care. The presence of two proven vectors of leishmaniasis in the country and favourable environmental conditions may have an impact on the emergence and local transmission of leishmaniasis in Moldova. The intensive and continues surveillance should be taken to regularly update the information on sand fly vectors, the presence of *Leishmania* parasites in the potential domestic and wild reservoirs and sand flies.

Conclusions

Three sand fly species of the genus *Phlebotomus* were identified in the Republic of Moldova based on integrated morphological and molecular approaches. *Phlebotomus papatasi* was the most widely distributed and abundant species, feeding indoors and strongly anthropophilic as suggested on bloodmeal analyses. Haplotype network analysis showed low structuring of *P.papatasi* population with only 5 haplotypes of *cox*1 with minor differences detected. Two other recorded species, *P. perfiliewi* and *Adlerius* sp. were present sporadically in CDC trap collections. ITS2 sequences of collected *P. perfiliewi* specimens identified then as *P. perfiliewi* s.s. Distinct morphological and molecular characters of *Adlerius* sp. specimens suggest presence of yet undescribed species that shall be further studied to reveal its relationships with other species within the subgenus and its potential role in pathogen transmission. The presence of two proven vectors of leishmaniasis in the country emphasizes a need for intensive and continuous surveillance to update the information on sand fly populations.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this article and its additional files, available as Table S1, Table S2, Table S3, Table S4, Fig. S1. *cox*1 sequences obtained during this study were deposited in GenBank database under the accession numbers XXXX-XXXX .

Competing interests

All individual authors declare that they have no competing interests.

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Authors' contributions

BA, PV, VD, TȘ - designed the study. TȘ, BA,GO - conducted the field sampling and field data processing. OEK, VD – analyzed DNA sequences. PH, VD, MG – performed MALDI-TOF assays and data analysis. RD - contributed to field data collection and processing. TȘ, VD, OEK - wrote the manuscript. PV, PH, BA - revised the manuscript. All authors read and approved the final manuscript.

Authors' information

[1] Laboratory of Entomology, Institute of Zoology, Chisinau, Republic of Moldova

[2] Department of Biology, Ecology Section, Faculty of Science, VERG Laboratories, Hacettepe University, Ankara, Turkey

[3] BioCeV, Institute of Microbiology of the Czech Academy of Sciences, Vestec, Czech Republic

[4] Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic

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