An integrative approach to identify sand fly vectors of leishmaniasis in Ethiopia by morphological and molecular techniques

Myrthe Pareyn1*, Vit Dvorak2, Petr Halada3, Natalie Van Houtte1, Nigatu Girma4, Wim de Kesel1, Behailu Merdekios5, Fekadu Massebo4, Herwig Leirs1 and Petr Volf2*

Abstract

Background: Ethiopia is affected by human leishmaniasis caused by several *Leishmania* species and transmitted by a variety of sand fly vectors of the genus *Phlebotomus*. The sand fly fauna in Ethiopia is highly diverse and some species are closely related and similar in morphology, resulting in difficulties with species identification that requires deployment of molecular techniques. DNA barcoding entails high costs, requires time and lacks reference sequences for many Ethiopian species. Yet, proper species identification is pivotal for epidemiological surveillance as species differ in their actual involvement in transmission cycles. Recently, protein profiling using MALDI-TOF mass spectrometry has been introduced as a promising technique for sand fly identification.

Methods: In our study, we used an integrative taxonomic approach to identify most of the important sand fly vectors of leishmaniasis in Ethiopia, applying three complementary methods: morphological assessment, sequencing analysis of two genetic markers, and MALDI-TOF MS protein profiling.

Results: Although morphological assessment resulted in some inconclusive identifications, both DNA- and protein-based techniques performed well, providing a similar hierarchical clustering pattern for the analyzed species. Both methods generated species-specific sequences or protein patterns for all species except for *Phlebotomus pedifer* and *P. longipes*, the two presumed vectors of *Leishmania aethiopica*, suggesting that they may represent a single species, *P. longipes* Parrot & Martin. All three approaches also revealed that the collected specimens of *Adlerius* sp. differ from *P. (Adlerius) arabicus*, the only species of *Adlerius* currently reported in Ethiopia, and molecular comparisons indicate that it may represent a yet undescribed new species.

Conclusions: Our study uses three complementary taxonomical methods for species identification of taxonomically challenging and yet medically import Ethiopian sand flies. The generated MALDI-TOF MS protein profiles resulted in unambiguous identifications, hence showing suitability of this technique for sand fly species identification. Furthermore, our results contribute to the still inadequate knowledge of the sand fly fauna of Ethiopia, a country severely burdened with human leishmaniasis.

Keywords: Sand flies, *Phlebotomus*, Morphology, DNA barcoding, MALDI-TOF mass spectrometry, Protein profiling, Ethiopia
Background

Phlebotomine sand flies (Diptera, Phlebotominae) are hematophagous insects of great medical importance as the females of some species are the vectors of the protozoans *Leishmania* spp., which are transmitted during blood-feeding on a vertebrate host. Human leishmaniasis manifests in three major clinical forms: visceral (VL), cutaneous (CL), and mucocutaneous (MCL) leishmaniasis [1, 2].

All three forms affect people in Ethiopia, particularly the poorest part of the population living in rural areas [3, 4]. VL is generally endemic in the lowlands widespread in the country and is caused by *Leishmania donovani*. Proven vectors of VL in different regions in Ethiopia are *Phlebotomus orientalis*, *P. martini* and to a lesser extent *P. celiae* [5–9]. Localized and diffuse forms of CL, and MCL occur mainly at mid-highland altitudes, on the mountain ridges of the Ethiopian Rift Valley. The main causative parasite species is *L. aethiopica*, which is transmitted by *P. longipes* in northern and central Ethiopia and *P. pedifer* in southwestern Ethiopia [10–16].

It is pivotal for entomological and eco-epidemiological research to accurately identify the sand fly species that act as vectors in a particular area and assess their ecology and behavior, as this information is a prerequisite for implementation of efficient, targeted control programmes [17].

Morphological identification is performed by examination of the mounted head and abdomen of the sand flies which bear the main distinctive characteristics for classification (cibarial and pharengial armature, genitalia). However, this taxonomic approach is labor-intensive, demands high proficiency, and morphological keys are often obsolete and incomprehensive, leading to incorrect identifications [18–20].

To overcome these drawbacks, studies are frequently shifting to molecular techniques, mostly DNA sequencing analyses which allow simultaneous processing of samples, provide reliable identifications and have good reference sequences available for many species. Yet, this approach is technically demanding, considered costly for large-scale studies and its validity depends on the genetic variability of the target locus [21, 22].

Recently, an alternative molecular method, protein profiling by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), has been introduced for species identification in different arthropod families, including sand flies [20, 23–28]. This approach provides unique protein profiles that allow unambiguous species identification and sample processing is rapid, simple and cost-effective. However, it still needs validation on field specimens and requires a centralized database to approve its use for routine species identification of sand flies [20, 29]. As the method utilizes only certain body parts, in sand flies particularly the thorax in standardized protocols [20], a coordinated sample preparation allows simultaneous or later application of other techniques, including morphological analysis of the mounted head and genitalia and DNA-based techniques using template DNA isolated from the sand fly abdomen.

The sand fly fauna in Ethiopia is very diverse, comprising remarkable numbers of species in both genera *Sergentomyia* and *Phlebotomus*, within which species of at least six subgenera were recorded, many of them proven or suspected vectors of several *Leishmania* species and thus of great medical significance. Among these, some closely related sand fly species are remarkably similar in morphological characteristics, resulting in many difficulties for conclusive species identification [30–33]. Especially females of some species are challenging to distinguish from each other as species-specific morphological features are poorly described or undefined. These species, however, often do not play the same role in *Leishmania* transmission which is hindering sand fly ecology studies and implementation of control measures accordingly, indicating that more sophisticated techniques are necessary for species differentiation [30, 31]. DNA barcoding could be an appropriate alternative, although very few reference sequences of the Ethiopian sand fly species are currently available in genetic databases and analyses are rather too costly to process large sample sizes.

Our study presents for the first time an integrative taxonomic approach to identify most of the important sand fly vectors of leishmaniasis in Ethiopia, applying three complementary methods: morphological assessment, sequencing analysis of two genetic markers, and MALDI-TOF MS protein profiling. We aimed to demonstrate that MALDI-TOF MS protein profiling could be a suitable taxonomical technique, providing unambiguous sand fly species identification in Ethiopia. Furthermore, our results contribute to the currently still inadequate knowledge of the sand fly fauna in Ethiopia, a region among those most affected by the burden of human leishmaniasis.

Methods

Sand flies

Field specimens were captured in May and September 2019 from different CL (Hagere Selam in the north, Saris in the center and Ochollo in the southwest) and VL (Aba Roba, Dimeka and Turmi in the south) foci in Ethiopia (Fig. 1). Sand flies were captured with CDC miniature light traps (John W. Hock Company, Gainesville, Florida, USA), which were set at 18:00 h and collected at 7:00 h the next morning. In order to capture CL vectors in the
mid-highlands, traps were placed in caves or around rocky areas, whereas to capture VL vectors in the lowlands, traps were set nearby termite hills and human dwellings [11, 34, 35]. Collected sand flies were preserved in 70% ethanol and stored at −20 °C [36].

**Morphological identification of sand fly species**
The head and terminal segments of the abdomen of the sand fly specimens were mounted using CMCP-10 high viscosity mounting medium (Polysciences, Hirschberg, Germany) and species identification was done according to relevant morphological keys [30–32, 37–42]. Slide-mounted specimens were observed 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).

**Molecular identification of sand fly species**
After mounting, the remaining part of the abdomen was used for molecular identification of the sand fly species. DNA was isolated using the QiAamp DNA Mini Kit (Qiagen, Hilden, Germany). Samples were subsequently subjected to two PCRs targeting (i) a fragment of the cytochrome c oxidase subunit 1 (cox1) with primers LCO 1490 (5′-GGT CAA ATC ATA AAG ATA TTG G-3′) and HCO 2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) in a total reaction volume of 25 µl as described by Folmer et al. [43]; and (ii) the nad4 gene with primers ND4ar (5′-AAR GCT CAT GTT GAA GC-3′) and ND4c (5′-ATT TAA AGG YAA TCA ATG TAA-3′) in a reaction volume of 50 µl based on Soto et al. [44]. Amplicons were visualized on a 1% agarose gel and obtained products were purified with the QiAquick PCR Purification Kit (Qiagen). These were sent to Vlaams Instituut voor Biotechnologie (VIB; University of Antwerp, Wilrijk, Belgium) or BIOCEV OMICS genetika (Charles University, Vestec, Czech Republic) for Sanger sequencing in two directions with the primers used for DNA amplification.

**Phylogenetic analyses**
The obtained chromatograms were edited and consensus sequences were generated for each specimen, which were compared with reference sequences in GenBank using BLAST. Multiple sequence alignment of the cox1 and nad4 sequences was performed using the Clustal W tool implemented in MEGA X 10.1 and primers were trimmed to have sequences with an equal length of 658 bp and 597 bp, respectively [45, 46]. The nucleotide composition and sequence divergence were calculated with the Kimura 2-parameter model (K2P) and a distance matrix was generated [47, 48]. A neighbor-joining (NJ) tree of the K2P distances was created using a bootstrapping method with 1000 replicates for a graphical presentation of the clustering pattern of the sand fly species [46, 49]. To generate a NJ tree for our specimens of the subgenus *Anthelepis* in combination with other species of the same subgenus, cox1 sequences were retrieved from GenBank. Based on the NJ trees, groups of species were indicated to assess the inter- and intra-species distances.

**MALDI-TOF MS analysis of sand fly species**
The MALDI matrix was prepared fresh as an aqueous 60% acetonitrile/0.3% TFA solution of sinapinic acid (30 mg/ml; Bruker Daltonics, Bremen, Germany). Specimens stored in 70% ethanol were air-dried, dissected and thoraces were manually ground in 10 µl of 25% formic acid by a BioVortexer homogenizer (BioSpec, Bartlesville, USA) with sterile disposable pestles. After a short centrifugation of the homogenate (10,000×g for 15 s), 2 µl was mixed with 2 µl of MALDI matrix and 1 µl was deposited and air-dried on a steel target plate (Bruker Daltonics) in duplicate. Protein mass spectra were measured in a mass range of 4–25 kDa by an Autoflex Speed MALDI-TOF spectrometer (Bruker Daltonics) and calibrated externally with the Bruker Protein Calibration Standard I. Each spectrum was acquired as a sum of 4000 manually adjusted laser shots (20 × 200 shots from different positions of the target spot) and visualized by FlexAnalysis 3.4 software (Bruker Daltonics).

For cluster analysis, the protein profiles were processed (normalization, smoothing, baseline subtraction and peak picking) using MALDI Biotyper 3.1. (Bruker Daltonics). The peak picking parameters for generation of a main spectrum (MSP) were to include maximum 100 peaks, which had a signal-to-noise ratio greater than 3 and a relative intensity of minimum 1% of the most intense peak. The desired peak frequency for MSP reference spectra was set to 60%. For MSP dendrogram creation, a correlation distance measure and average linkage parameters were applied. The dendrograms were generated using the individual MSPs or MSP references created for each sand fly species. In addition to six field-caught Ethiopian species, MSP references from our sand fly database were included (country of origin is given in parentheses): *P. ariasi* (France); *P. kandelakii* (Georgia); *P. longipes* (Morocco); *P. neglectus* (Croatia); *P. orientalis* (Ethiopia); *P. perfiliewi* (Macedonia); *P. persici* (Spain); *P. tobbi* (Northern Macedonia); *P. arabicus* (Israel); *P. balcanicus* (Georgia); *P. creticus* (Crete); *P. halepensis* (Georgia); *P. simici* (Macedonia). *Phlebotomus arabicus* and *P. orientalis* originated from colonies maintained in Prague, the others were field-collected.
Results

Morphological species identification

Morphological analysis identified six sand fly species of four subgenera. Three species, *P. (Phlebotomus) duboscqi*, *P. (Synphlebotomus) celiae* and *P. (Sy.) martini*, were identified in VL endemic sites in the south (Dimeka, Turmi and Aba Roba). *Phlebotomus (Larroussius) pedifer* was collected in Ochollo and *P. (L.a.) longipes* in Saris and Hagere Selam. A main feature to differentiate the two species of the subgenus *Larroussius* is based on the bending of the tip of the male aedeagus [31, 41]. However, our specimens showed a varying range of tip endings within a single species (Fig. 2) that did not allow clear differentiation at the species level based on morphological features [30, 41, 50, 51].

Seven males and one female of an additional species were captured from a single cave nearby Hagere Selam (13° 40′ 14″ N, 39° 07′ 29″ E) and identified as a species of the subgenus *Adlerius* (further referred to as ‘*Adlerius* sp.’). Morphometric analysis of four male specimens of *Adlerius* sp. (Table 1) indicates that our sand flies show high similarity in the number and position of coxite hairs and the aedeagus tip to tooth distance as described for *P. davidi* [37]. The style, coxite and aedeagus length of our specimens, however, are longer than reported for *P. davidi*, but shorter than measured and described for *P. arabicus* and fit more to *P. naqbenius*. Accordingly, no conclusive species identification could be reached for *Adlerius* sp.

Sand fly species identification by molecular techniques

Most specimens tested gave reproducible MALDI-TOF MS spectra with a high number of intense signals (Fig. 3a). Except for the protein profiles of *P. pedifer* and *P. longipes*, each species generated a heterogeneous spectrum with species-specific peaks allowing
unambiguous species identification. The dendrogram of the specimen’s protein profiles (Fig. 3b) indicates that all subgenera cluster on distinct branches. The closely related *P. martini* and *P. celiae* were clearly distinguished from each other, whereas the specimens of the subgenus *Larroussius* from all three sites grouped in a single cluster. The *Adlerius* sp. formed its own separate branch.

Taxonomic clustering of the cox1 and nad4 sequences in the NJ tree (Fig. 4a, b, respectively) substantiates the hierarchical clustering of the protein profiles (Fig. 3b), separating all species except for the two *Larroussius* species. The mean intra-group K2P distances within the *P. longipes* and *P. pedifer* cluster was only 0.3% (SD 0.1) for cox1 and 0.1% (SD 0.1) for nad4 genes (Additional file 1: Table S1), indicating that there is no genetic difference between the two presumable species. The K2P distance between *P. martini* and *P. celiae* was only 1.6% (SD 0.4) for cox1 and 1.2% (SD 0.3) for nad4 genes, yet the species clearly clustered in distinct clades (Additional file 2: Table S2).

The dendrogram including the MSP reference spectra of *P. pedifer*, *P. longipes*, *Adlerius* sp. and other available species of the subgenera *Larroussius* and *Adlerius* from our library (Fig. 5) demonstrates that only the two Ethiopian CL vectors cannot be distinguished from each other, while the protein profiling could clearly differentiate all other analyzed species, including the closely related *P. tobbi* and *P. perfiliewi* that provide highly similar, yet species-specific protein spectra. The dendrogram depicts that *Adlerius* sp. is closely related with *P. arabicus* from Israel but some distinct peaks were found in their protein profiles (Additional file 3: Figure S1). Moreover, there is a large relative distance between the MSP reference spectra of the two species (Fig. 5), demonstrating that *Adlerius* sp. is definitely not *P. arabicus*.

Furthermore, the cox1 sequence profile of the *Adlerius* sp. was compared with available GenBank sequences of other species of the *Adlerius* subgenus (Fig. 6), which supports the MSP dendrogram (Fig. 5), indicating that the species is closely related to *P. arabicus* but constitutes a distinct branch with a K2P distance of 6.5% (SD 1.2, Additional file 4: Table S3).

**Discussion**

Since East Africa accounts worldwide among the regions most affected by both cutaneous and visceral leishmaniasis, phlebotomine sand flies occurring in the Horn of Africa have been extensively studied due to their exclusive

---

**Fig. 2** Aedeagi of male *Phlebotomus longipes* (left two columns 1 and 2) and *P. pedifer* (right column 3). *Phlebotomus longipes* was acquired for Hagere Selam and Saris (northern and central Ethiopia) and *P. pedifer* from Ochollo (southern Ethiopia)
role as vectors in the transmission cycles. Unfortunately, whereas considerable amount of knowledge regarding the diverse sand fly fauna of Ethiopia has been gradually gathered, the last comprehensive morphological key for species identification has not been updated for more than five decades [42], leaving the attempt to identify the specimens collected in field surveys very tedious and challenging. This further urges the need to deploy alternative molecular techniques that have recently emerged and were successfully adopted for species identification of various medically significant arthropods. In this study, we applied an integrative taxonomic approach to identify most CL and VL vectors in Ethiopia using a combination of morphological assessment, sequencing analysis of two genetic markers and MALDI-TOF MS protein profiling, and demonstrate some novelties in the complex sand fly fauna of Ethiopia.

The three vectors of *L. donovani* in Ethiopia are *P. orientalis*, *P. martini* and *P. ceiae*. The former is a species of the subgenus *Larroussius*, whereas the latter two belong to the subgenus *Symphebotomus* and occur sympatriically in Aba Roba focus in southwestern Ethiopia [32, 35]. While to our knowledge this is the only place where *P. ceiae* has been identified in Ethiopia, *P. martini* is more widespread, serving as a vector also in the northwestern and southeastern parts of the country, where it sometimes cohabitates with *P. orientalis* [8, 52, 53].

### Table 1: Morphometric analysis of the *Adlerius* sp. from Ethiopia, in comparison with other *Adlerius* species

| Species (country, reference) | Ascoid formula | Style length | Coxite length | No. of coxite hairs | Position coxite hairs | Aedeagus length | Aedeagus tip to tooth |
|------------------------------|----------------|--------------|---------------|---------------------|------------------------|-----------------|----------------------|
| *Adlerius* sp.* (Ethiopia, Pareyn) | 2/3-7, 1/8-15 | 173 (158–188) | 366 (355–387) | 45 (36–52) | 0.56 (0.51–0.57) | 186 (181–195) | 13 (11–15) |
| *P. davidi* (Yemen & Ethiopia, Artemiev [37]) | 2/3-7, 1/8-15 | 157 (156–160) | 315 (300–328) | 46 (38–59) | 0.59 (0.55–0.64) | 162 (152–172) | 12 (10–14) |
| *P. arabicus* (Israel, PV, unpublished) | 2/3-7 | 189 (176–206) | 386 (370–428) | 64 (55–76) | – | 183 (176–206) | 20 (17–25) |
| *P. arabicus* TYPE (Saudi Arabia, Lewis & Büttiker [64]) | 2/7 (8) | 190 | 360 | 57 | 0.59 | 190 | – |
| *P. arabicus* HES L A (Saudi Arabia, Lewis & Büttiker [64]) | 2/7 (8) | – | – | 54 (42–69) | (0.50–0.59) | – | (12–22) |
| *P. Naqben* species (Saudi Arabia, Lewis & Büttiker [64]) | 2/7 | – | – | 54–98 | 0.58 | – | (15–22) |
| *P. naqbenius* (Saudi Arabia, Lewis & Büttiker, [65]) | 2/7 | 185 | 357 | 60 | – | 172 | 16 |
| *P. naqbenius* holotype (PV, unpublished) | 2/7 | 185 | 357 | 60 | – | 172 | 16 |
| *P. naqbenius* syntype (PV, unpublished) | 2/7 | 196 | 381 | 80 | 0.6 | 185 | 21 |

Notes: Measurements were collected from: *four males from Ethiopia; the holotype and syntype of *P. naqbenius* were loaned from The National History Museum, London, UK; *examination of the type-series; *examination of own samples from Hesua; *We speculate that the authors attributed the numbers of the coxite hairs incorrectly; All measurements are in µm.
Fig. 3  MALDI-TOF mass spectra (a) and the corresponding dendrogram of the protein profiles (b) of Ethiopian sand fly species. Zoomed mass range of 4 to 15 kDa is shown in a and distances in b are displayed in relative units.
Fig. 4 Neighbor-joining tree based on K2P distances of *cox1* (a) and *nad4* (b) sequences of Ethiopian sand flies. Abbreviations: K2P, Kimura 2-parameter model.

Fig. 5 Dendrogram of MSP reference spectra of *Larroussius* and *Adlerius* sand flies from Ethiopia and our library. The origin of the species is given in the methods section. Specifically, *P. arabicus* was derived from the sand fly facility at Charles University, Czech Republic, which originated from northern Israel. Distances are displayed in relative units. Abbreviations: MSP, main spectrum.
measurements can presumably not be extrapolated to a larger area without leading to misidentifications.

*Phlebotomus martini* and *P. celaiae* have a different distribution, infection prevalence and abundance, and thus their contribution to disease transmission varies [35]. Therefore, proper species identification is pivotal and requires novel determination techniques. Both genetic and MALDI-TOF MS analyses in this study pointed out that DNA marker sequences and protein profiles were similar, and enable rapid and conclusive species identification, indicating that this is a suitable approach for species identification.

Sand flies were also captured from different CL endemic sites in Ethiopia, where we found two species of the subgenus *Larroussius*, identified morphologically as *P. pedifer* and *P. longipes*. The latter species was first described by Parrot et al. [56] in 1939 in Ethiopia, but the species complex was re-examined by Lewis et al. [41] in 1972, who compared presumed *P. longipes* specimens from Kenya and South Sudan with *P. longipes* from different places in northern, western and central Ethiopia. The species from Kenya appeared smaller than the ones from Ethiopia, although this was probably a geographical difference. Moreover, the authors describe that male specimens from Kenya had an up-turned foot-like aedeagus, whereas it was only slightly up-turned in the Ethiopian species. Therefore, the Kenyan specimens were described as a new related species, *P. pedifer*, which was later also found as the CL vector in southwestern Ethiopia (Ochollo) [41]. Killick-Kendrick et al. [31] later confirmed that males of *P. pedifer* and *P. longipes* can be distinguished based on this slight difference in the aedeagus shape and additionally also the number of inner surface coxite hairs, being 50–60 for *P. pedifer* and 35–50 for *P. longipes*. In contrast, females of the two species are considered indistinguishable [30, 41], except for a slight difference in the base of the spermathecal ducts, which often still results in inconclusive identifications [31].

Results of the present study show that the tips of the aedeagi of the Ethiopian CL vectors varied among all collected specimens, being partially dependent on the orientation of the specimen on the slide. Also, the number of coxite hairs overlapped significantly between *P. pedifer* and *P. longipes* and seems more geographically dependent than species-specific. Accordingly, we conclude that these morphological characters are not appropriate for species identification. Moreover, sequences of two genetic markers and protein profiles of *P. pedifer* and *P. longipes* were found identical, whereas all other species of the subgenus *Larroussius* in our database could be easily distinguished using MALDI-TOF MS. This includes also *P. orientalis* protein profiles generated by colony specimens originating from an Ethiopian VL endemic site, Melka Werer. Even though *P. orientalis* is morphologically closely related to *P. pedifer* and *P. longipes* [51], our results show that this species can easily be distinguished based on MALDI-TOF MS protein profiling. Among other analyzed *Larroussius* species, the method also clearly differentiated between *P. perfiliewi* and *P. tobbi* which are very similar morphologically and have a similar geographical distribution [57–59], demonstrating the discriminatory power of the approach. Furthermore, the ecology of *P. pedifer* and *P. longipes* is comparable, as both occur in close association with hyraxes, inhabiting caves, basalt cliffs, cracks in boulders and gorges at mid-highland altitudes [10, 11, 34]. Collectively, our results suggest that the two species incriminated in the transmission cycle of *L. aethiopica* may actually represent a single species, *P. longipes*, which was described first.

*Phlebotomus duboscqi* was found in the Aba Roba focus and could easily be identified by MALDI-TOF MS as it matched with *P. duboscqi* from Senegal in our reference database. Two other species of the subgenus *Phlebotomus* were recorded elsewhere in Ethiopia, namely *P. papatasi* and *P. bergeroti*, which are sympatric with *P. duboscqi* in some areas [60, 61]. In these locations, taxonomic tools like MALDI-TOF MS protein profiling can be very useful for entomological studies to differentiate similar species and to thoroughly investigate their potential roles in *Leishmania* transmission.

Another *Phlebotomus* species was found in Hagere Selam, which belonged to the subgenus *Adleriuss* according to the morphology of the collected specimens. The only other currently reported *Adleriuss* species in Ethiopia is *P. arabicus*, which has been described in the upper Awash Valley, where it was captured from rocky valleys and found infected with *Leishmania* parasites, although the species could not be identified due to contamination
In Israel, *P. arabicus* is known as an efficient vector of *L. tropica* in rocky places colonized by hyraxes [63]. Our specimens were also captured in caves together with *P. longipes*. Morphometric and molecular analyses in our study, however, show that the captured species is definitely not *P. arabicus*.

In 1980, Artemiev [37] described four males and one female sand fly from Yemen and one male and one female sand fly from Ethiopia as *P. davidii*. The Ethiopian specimens were derived from the area around Langano Lake, approximately 200 km south of Addis Ababa. Our measurements of several morphological parameters, namely number of coxite hairs and the distance from the aedeagus tip to tooth, fit best with the description of *P. davidii* [37]. However, the coxite, style and aedeagus length were slightly larger than described by Artemiev [37], which can be due to small sample sizes and geographical variation. Moreover, this author described that the antennae of the male specimen from Ethiopia were missing and that the female specimen from Ethiopia had a different pharynx than the one from Yemen, requiring more specimens from both countries to determine the true taxonomy [37]. This suggests that the species from Ethiopia was potentially different from the holotype of *P. davidii* collected in Yemen.

Lewis & Büttiker [64] questioned the validity of *P. davidii* as a species based on doubts about the value of the ascoid formula that also distinguished it from *P. arabicus*. While not addressing apparently different numbers of coxite hairs and emphasizing the overlapping values of wing lengths and possible same or varying ascoid formulae, they concluded that specimens from Ethiopia previously identified as *P. davidii* are a geographical variant of *P. arabicus* and regarded *P. davidii* as its synonym. Interestingly, in the same study, the authors collected specimens of another *Adlerius* species in several localities in Saudi Arabia, provisionally named “*P. Naqben*”, with a remarkably wide range in number of coxite hairs (54–98) [64]. In a later study, Lewis & Büttiker [65] formally described it as *P. naqbenius*. However, for this new species they do not provide the morphological characters regarded as important for the subgenus *Adlerius* by Artemiev [37] (ascoid formula, length of style, coxite and aedeagus tip to tooth) and they base its description by Artemiev [37]. This suggests that the species from Ethiopia was potentially different from the holotype of *P. davidii* collected in Yemen.

In conclusion, MALDI-TOF MS protein profiling and DNA barcoding provided similar results in this study, demonstrating that methods of molecular taxonomy provide a viable alternative to often poorly characterized and minuscule morphological characters required for traditional species identification. An integrated approach is especially useful in regions where the sand fly fauna is highly diverse. Because DNA barcoding is much more expensive, time consuming and sequences of many Ethiopian species are not available in online genetic databases, we suggest to develop a centralized MALDI-TOF MS protein profile database and use this approach for routine identification of sand fly specimens from field surveys.

## Conclusions

To the best of our knowledge, this study demonstrates for the first time that MALDI-TOF MS protein profiling is a suitable taxonomical approach for cost-effective, unambiguous species identification of Ethiopian sand flies. DNA- and protein-based molecular techniques as well as morphometric analysis suggest that the vectors of *L. aethiopica, P. pedifer* and *P. longipes*, may represent a single species. We also report that the *Adlerius* species we found, differs from *P. arabicus*, the only *Adlerius* species reported in Ethiopia to date. This *Adlerius* species probably represents a new species. Collectively, our results contribute to the understanding of the complex sand fly fauna in Ethiopia, a region heavily burdened with human leishmaniasis.

## Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13071-020-04450-2.

### Additional file 1: Table S1

Intraspecies K2P distances of cox1 and Nad4 genes of Ethiopian sand flies. Abbreviations: K2P, Kimura 2-parameter model; SD, standard deviation.

### Additional file 2: Table S2

Interspecies K2P distances of cox1 and Nad4 genes of Ethiopian sand flies. The lower left quadrant presents the K2P distances (SD) of the cox1 gene, the upper right quadrant of the nad4 gene. The nad4 gene of *P. duboscqi* specimens was not included in the analysis.
Additional file 3: Figure S1. Comparison of MALDI-TOF MS protein profiles of Ethiopian Adelius sp. with five other species of the subgenus Adelius. Zoomed mass range of 4 to 15 kDa is depicted.

Additional file 4: Table S3 Interspecies K2P distances of crx1 gene of Phlebotomus sand flies of the subgenus Adelius.

Abbreviations
BLAST: Basic Local Alignment Search Tool; crx1: Cytochrome c oxidase subunit 1; CL: Cutaneous leishmaniasis; K2P: Kimura two-parameter model; MALDI-TOF MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MCL: Mucocutaneous leishmaniasis; MEGA: Molecular Evolutionary Genetics Analysis; MSP: Main spectrum; NJ: Neighbor joining; SD: Standard deviation; SNRPR Southern Nations, Nationalities, and Peoples Region; VL: Visceral leishmaniasis.

Acknowledgements
We are very grateful to Arba Minch University and Mekelle University and all staff of the regional, zonal and woreda (district) offices for their cooperation and coordination of the field work. Furthermore, we would like to thank Dr Mweshepa Belkew for his advice on trapping localities and sand fly identifications.

Authors’ contributions
MP, VD and PV designed the study. MP, NG, WD, BM and FM coordinated and carried out the field work. MP, VD, PH and NVH carried out the laboratory analyses. MP, VD, PH and PV analyzed and interpreted the data and wrote the initial manuscript. Resources were arranged by HL, FM and PV. HL, BM, FM, HL and PV participated in the coordination of the project and revised the manuscript. All authors read and approved the final manuscript.

Funding
MP is a PhD-fellow in the VLADOC program of the Flemish Interuniversity Council VlIR-UOS (NDOC2016PR083) and her research stay at Charles University was supported by an Infravec2 Grant (Agreement No. 731060). HL is part of the University of Antwerp Center of Excellence VAX-IDEA. This research was supported by the projects BIOCEV CZ.1.05/1.1.00/02.0109 from the European Regional Development Fund. Additional support from the Czech Infrastructure for Integrative Structural Biology (LM2015043 funded by MEYS CR) is gratefully acknowledged.

Availability of data and materials
The datasets analyzed during the present study are available from the corresponding authors on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
1 Evolutionary Ecology Group, Biology Department, University of Antwerp, Antwerp, Belgium. 2 Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic. 3 BioCEv-Institute of Microbiology, The Czech Academy of Sciences, Vestec, Czech Republic. 4 Biology Department, Arba Minch University, Arba Minch, Ethiopia. 5 Department of Public Health, Arba Minch University, Arba Minch, Ethiopia.

Received: 15 July 2020 Accepted: 30 October 2020
Published online: 17 November 2020

References
1. Maroli M, Feliciangeli M, Bichaud L, Charrel R, Gradoni L. Phlebotomine sandflies and the spreading of leishmaniasis and other diseases of public health. Med Vet Entomol. 2013;27:123–47.
2. CDC. Center of Disease Control and Prevention. Parasites - Leishmaniasis. Atlanta: Center of Disease Control and Prevention, 2013. https://www.cdc.gov/parasit es/leishmaniasis/biology.html. Accessed 07 Sept 2018.
3. Alvar J, Yactayo S, Bern C. Leishmaniasis and poverty. Trends Parasitol. 2006;22:552–7.
4. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishma- niasis worldwide and global estimates of its incidence. PLoS ONE. 2012;7:e35671.
5. Lemma W, Tekie H, Abassi I, Balkew M, Gebre-michael T, Warburg A, et al. Nocturnal activities and host preferences of Phlebotomus orientalis in extra-domestic habitats of Kafa-Humera lowlands, Kala-azar endemic, Northwest Ethiopia. Parasit Vectors. 2014;7:594.
6. Seblova V, Volfova V, Dvorak V, Puzinova K, Votypka J, Kassahun A, et al. Phlebotomus orientalis sand flies from two geographically distant Ethiopian localities: biology, genetic analyses and susceptibility to Leishmania donovani. PLoS Negl Trop Dis. 2014;7:e2187.
7. Gebreslassie A, Abassi I, Aklilu E, Yared S, Kirstein OD, Moncac A, et al. Host-feeding preference of Phlebotomus orientalis (Diptera: Psychodidae) in an endemic focus of visceral leishmaniasis in northern Ethiopia. Parasit Vectors. 2015;8:270.
8. Gebre-michael T, Malone JB, Balkew M, Ali A, Berhe N, Hallu A, et al. Mapping the potential distribution of Phlebotomus martini and P. orientalis (Diptera: Psychodidae), vectors of kala-azar in Africa by use of geographic information systems. Acta Trop. 2004;90:73–86.
9. Fuller G, Lemma A, Haile T, Gemeda N. Kala-azar in Ethiopia: survey of south-west Ethiopia. The leishmanin skin test and epidemiological stud- ies. Ann Trop Med Parasitol. 1979;73:417–30.
10. Lemma W, Erenso G, Gadisa E, Balkew M, Gebre-michael T, Hallu A. A zoontic focus of cutaneous leishmaniasis in Addis Ababa, Ethiopia. Parasit Vectors. 2009;2:60.
11. Pareyn M, Van Den Bosch E, Girma N, Van Houtte N, Van Dongen S, Van Der Auwerda G, et al. Ecology and seasonality of sandflies and potential reservoirs of cutaneous leishmaniasis in Ochello, a hotspot in southern Ethiopia. PLoS Negl Trop Dis. 2019;13:e0007676.
12. Bray RS, Ashford RW, Bray MA. The parasite causing cutaneous leishma- niasis in Africa. Trans R Soc Trop Med Hyg. 1973;67:345–8.
13. van Henten S, Adriaensen W, Fikke H, Akuffo H, Diero E, Hallu A, et al. Cutaneous leishmaniasis due to Leishmania aethiopica. EClinicalMedicine. 2018;65.69–81.
14. Hallu A, Di T, Abeebe T, Hunegnaw M, Kager PA, Grammicia M, et al. Isola- tion of Leishmania tropica from an Ethiopian cutaneous leishmaniasis patient. Trans R Soc Trop Med Hyg. 2006;100:53–8.
15. Bsrat A, Berhe N, Balkew M, Yohannes M, Tekli T, Gadisa E, et al. Epidemiologi- cal study of cutaneous leishmaniasis in SaeSe Tsade-embta district, eastern Tigray, northern Ethiopia. Parasit Vectors. 2015;8:149.
16. Bugisa G, Hallu A, Demtsu B. The current status of cutaneous leishma- niasis and the pattern of lesions in Ochello primary school students, Ochello, southwestern Ethiopia. Sci J Clin Med. 2014;3.111–6.
17. Pareyn M, Kochora A, Van Rooy L, Eligo N, Vanden Broecke B, Girma N, et al. Feeding behavior and activity of Phlebotomus pedifer and potential reservoir hosts of Leishmania aethiopica in southwestern Ethiopia. PLoS Negl Trop Dis. 2020;14:e0007947.
18. Galati EAB, Galvis-ovallos F, Lawyer P, Léger N, Depaquit J. An illustrated guide for characters and terminology used in descriptions of Phleboto- minae (Diptera, Psychodidae). Parasite. 2017;24:26.
19. Ready PD. Biology of phlebotomine sand flies as vectors of disease agents. Annu Rev Entomol. 2013;58:227–50.
20. Ovraat V, Halada P, Hlavackova K, Dokianakis E, Antoniou M, Volf P. Identification of phlebotomine sand flies (Diptera: Psychodidae) by matrix-assisted laser desorption/ionization time of flight mass spectrometry. Parasit Vectors. 2014;7:21.
21. Depaquit J. Molecular systematics applied to Phlebotomine sandflies: review and perspectives. Infect Genet Evol. 2014;28:744–56.
64. Lewis D, Buttiker W. Insects of Saudi Arabia: the taxonomy and distribution of Saudi Arabian *Phlebotomus* sandflies (Diptera: Psychodidae). Fauna Saudi Arab. 1982;4:353–97.
65. Lewis D, Buttiker W. Some Phlebotomine sandflies (Diptera: Psychodidae) from Saudi Arabia. Fauna Saudi Arab. 1986;8:324–39.
66. OpenAFRICA: Ethiopia shapefiles. 2016. https://africapendata.org/dataset/ethiopia-shapefiles. Accessed 8 Aug 2018.
67. QGIS Development team. QGIS Geographic Information System. Open Source Geospatial Foundation Project; 2019. https://qgis.osgeo.org.

**Publisher’s Note**
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.