Phlebotomus (*Adlerius*) *simici* NITZULESCU, 1931: first record in Austria and phylogenetic relationship with other *Adlerius* species

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Research

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Abstract

Background: Phlebotomine sand flies are the principal vectors of *Leishmania* spp. (Kinetoplastida: Trypanosomatidae). Sand fly findings in Central Europe are scarce and in Austria, to date only *Phlebotomus mascittii* has been recorded. In 2018 and 2019, entomological surveys were conducted in Austria with the aim to further clarify sand fly distribution and species composition.

Results: In 2019, a *Ph. simici* specimen was trapped in Austria for the first time. Analyses of two commonly used marker genes, *coxI* and *cytb*, revealed high sequence identity with *Ph. simici* specimens from North Macedonia and Greece. Phylogenetic analyses showed high intraspecific distances within *Ph. simici*, thereby dividing this species into three lineages, from Europe, Turkey and Israel, respectively. Low interspecific distances between *Ph. simici*, *Ph. brevis* and a yet unidentified *Adlerius* sp. from Turkey and Armenia highlights that molecular identification can be challenging within the *Adlerius* complex, even when applying standard marker genes.

Conclusion: This study provides the first finding of *Ph. simici* in Austria and the northernmost record so far. Moreover, it reveals valuable insights into the phylogenetic relationships of species within the *Adlerius* subgenus. *Ph. simici* is a suspected vector of *Leishmania infantum* and therefore of medical and veterinary importance. Potential sand fly expansion in Central Europe due to climatic change and the increasing import of *Leishmania*-infected dogs from endemic areas, urge the need for further studies on sand fly distribution in Austria and Central Europe in general.

Introduction

Phlebotomine sand flies (Diptera: Psychodidae: Phlebotominae) are small hematophagous insects and the vectors of the protozoan parasites *Leishmania* spp., the causative agents of leishmaniasis. In Europe, sand flies were primarily considered to be present in the Mediterranean basin, where both visceral (VL) and cutaneous leishmaniasis (CL) are endemic [1]. Sand fly occurrence north of the Alps was overlooked until *Phlebotomus mascittii* GRASSI, 1908, and *Phlebotomus perniciosus* NEWSTEAD, 1911, were found in Germany in 1999 and 2001, respectively [2,3]. Shortly after, *Ph. mascittii* was reported from northern France and Belgium [4]. Further surveys also revealed stable *Ph. mascittii* populations in four federal states of eastern Austria in 2010 to 2013 [5–7] and a singular specimen was trapped in western Slovakia in 2016 [8]. In addition to records of *Ph. mascittii*, stable populations of *Phlebotomus neglectus* TONNOIR, 1921, were reported from Hungary [9,10].

Sand flies in Central Europe are assumed to be remnants of post-glacial recolonization events from Mediterranean refugial areas, which have survived in small, microclimatic areas [11]. This hypothesis is further supported by a model of a potential distribution of Mediterranean sand fly species up to northern European countries, including the British islands, during the Holocene optimum approximately 6,000 years ago [11]. *Ph. mascittii*, an unproven but suspected vector for *Leishmania* spp., has been assumed to be the only sand fly species in Austria; however, considering the absence of a

geographic barrier between Hungary, Slovenia and eastern Austria, the possible occurrence of other species via prospective dispersal to Austria is likely. Reports of suspected autochthonous leishmaniasis cases in Austria highlight the necessity for further research [12,13].

To update our knowledge on species composition and distribution of sand flies in Austria, entomological surveys were conducted in 2018 and 2019. Species identification of caught specimens was achieved by a combination of morphological and molecular approaches and their phylogenetic status was evaluated. Here we report findings relating to a newly reported species.

Materials And Methods

Entomological survey

An entomological sand fly survey was conducted in six federal states of Austria, in July and August 2018 and 2019. Trappings were performed with battery-operated CDC miniature light traps using fine gossamer collection bags (model #512, John W. Hock Company, Gainesville, Florida) at appropriate trapping sites close to human dwellings and animal barns. Dry ice was occasionally used as a CO_2 bait.

Geographical and weather data acquisition

Geographical data from trapping sites was recorded by a global-positioning-system (TomTom, Amsterdam, Netherlands). Hourly temperature and relative humidity data of trapping regions were retrospectively obtained from the Central Institute for Meteorology and Geodynamics (ZAMG). Together with sand fly findings of this study, published trapping sites were georeferenced into a distribution map using QGIS 3.4.11 [14].

Morphological identification

Head and terminal segments of the abdomen of all caught sand fly specimens were dissected and slide-mounted in CMCP-10 high viscosity mountant (Polysciences, Germany). Identification was based on morphological parameters of male genitalia, female spermatheca and the pharyngeal armature [15]. Additionally, fluorescence microscopy was performed using a NIKON Eclipse E 800 (Nikon Instruments, Amstelveen, Netherlands) to detect and identify hardly visible female spermatheca which can be illuminated by autofluorescence under UV light at 330–380 nm wave length.

Molecular identification

DNA was isolated from the remaining bodies with a QIAamp® DNA Mini Kit 250 (QIAGEN, Hilden, Germany). For species identification, PCR amplification of a 658 bp fragment of the cytochrome oxidase subunit I (*coxI*) gene was performed following the protocol of Folmer et al. [16] using the primers LCO-1490 and a newly designed reverse primer CoxUniEr (5'-AAA CTT CAG GGT GAC CAA AAA ATC-3'), as initially used reverse primers [16] did not deliver satisfying PCR results in this case. Confirmation was obtained by amplifying a 652 bp segment of the cytochrome *b* (*cytb*) gene and the neighboring tRNA-Ser

gene using the newly designed primers CytbEf1 (5'-CAA TGA ATT TGA GGA GGA TTT GT-3') and CytbEr2 (5'-CTA TCT AAT GTT TTC AAA ACA ATT G-3'). Oligo Calc was used to calculate GC-contents, melting temperatures, optimal primer lengths and to exclude self-complementarity (http://biotools.nubic.northwestern.edu/OligoCalc.html). Amplification by PCR was conducted containing 10 x Reaction Buffer B, 2.5 mM $MgCl_2$, 1.6 mM dNTPs, 1mM primers, 1.25 units DNA polymerase and 1-5 ml DNA. Sterile H_2O was added to a final volume of 50 ml. The gene fragment was amplified using the following conditions: 95°C for 15 min, followed by 35 cycles of 95°C for 1 min (denaturation), 52°C for 1:30 min (annealing) and 72°C for 2 min (elongation), followed by a final extension of 72°C for 10 min.

All PCR amplifications were performed with an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany). Bands were analyzed with a Gel Doc™ XR+ Imager (Bio-Rad Laboratories, Inc., California, USA) and cut out from the gel and purified with an Illustra™ GFX™ PCR DNA and Gel Purification Kit (GE Healthcare, Buckinghamshire, UK). Sanger sequencing was performed with a Thermo Fisher Scientific SeqStudio (Thermo Fisher Scientific, Massachusetts, USA). Sequences were obtained from both strands and a consensus sequence was generated in GeneDoc 2.7.0. Sequence identities were revealed by comparing obtained sequences to sequences available in the GenBank.

Screening for *Leishmania* spp.

Female specimens were screened by PCR and amplifications were performed as described above. The primers LITSR/L5.8S targeting the internal transcribed spacer 1 (ITS1) gene were used, following the PCR protocol of El Tai et al. [17].

DNA sequence analyses

Available sequences for comparison were downloaded from GenBank and aligned with the obtained sequences using ClustalX 2.1 for multiple alignment and GeneDoc 2.7.0. for manual editing and data analysis. DnaSP v.5 [18] was used to identify unique haplotypes. To assess genetic structure among groups, among populations, and within populations we calculated and visualized median joining networks [19] and analysis of molecular variance (AMOVA) with Popart v.1.7 [20]. For further clarification of species boundaries, pairwise distances and Maximum Likelihood (ML) analyses using unique haplotypes were calculated in MEGA X [21]. Based on best fit evolutionary model selection, Tamura 3-parameter and Tamura-Nei-parameter with 1000 replications bootstrap support were applied for *coxI* and *cytb*, respectively.

Results were compared to calculations of Automatic Barcode Gap Discovery (ABGD) web-interface program (https://bioinfo.mnhn.fr/abi/public/abgd/), which generates Kimura-2-parameter (K2P) distances and assigns sequences to hypothetical species. Default settings of intraspecific divergence (*P*) of 0.001–0.1 were applied [22].

All sequence data was submitted to GenBank and barcodes, collection details and voucher material were deposited with ABOL and BOLD.

Results

Entomological survey

Inspection of caught insects revealed, as in previous studies, *Phlebotomus mascittii*, in very low numbers, but also a single female specimen of *Phlebotomus simici* NITZULESCU, 1931, namely from Orth an der Donau (48.14462411 latitude, 16.69736534 longitude) in the night of July 8th to July 9th at a local farm (Fig. 1). The CDC light trap baited with dry ice had been put up at the property in a barn with natural floor used for hay storage. Several animals including a dog, cats, chicken, geese, goats, pigs and rabbits were kept at the property. The mean night temperature was 15.6°C and the mean relative humidity (RH) was 62.3% in the respective trap night of July 9th. On July 10th and 11th when no sand flies were in the traps, the mean night temperature was 15.5°C and 13.2°C, respectively and the mean RH was 53.4% and 71.4%, respectively. The village is located in the federal state of Lower Austria in the eastern part of Austria directly along the River Danube, approximately 15 km west of Vienna. The annual mean temperature in Orth an der Donau is 9.9°C and the annual mean precipitation is 627 mm.

Species identification

The specimen was morphologically identified by characters of the pharynx and spermatheca as belonging to the subgenus *Adlerius* NITZULESCU (Fig. 2). The obtained *coxI* sequence (GenBank: MN812831.1) was queried against available sequences in GenBank by BLAST and identified as *Phlebotomus simici* NITZULESCU, 1931 [23]. Sequence identity ranged from 95.99% to 99.85% compared to sequences of *Ph. simici* originating from Turkey (MN086700.1) and Greece (KU519497.1), respectively. BLAST analysis of the obtained *cytb* sequence (GenBank: MN812836.1) confirmed species identification and showed 95.0% to 100% sequence identity with sequences of specimens from Crete, Greece (GenBank: MT452061.1) and North Macedonia (GenBank: MT452053.1), respectively. No *Leishmania* spp. DNA was detected in any of the sand flies by PCR.

Haplotype analysis of *Ph. simici* based on *coxI*

Sequences of *Ph. simici* available in GenBank were edited to compile a dataset of 51 *coxI* sequences with a length of 551 bp without gaps and stop codons for haplotype analysis (Table 1). 40 haplotypes were identified, defined by 53 variable sites, of which 36 were parsimony informative with an overall haplotype diversity (Hd) of 0.985 and an overall nucleotide diversity (p) of 0.229.

The haplotype of the Austrian *Ph. simici* specimen (Hap_1) clustered within a conserved European group including haplotypes of specimens from North Macedonia (Hap_2, Hap_3), Thessaloniki, Greece (Hap_3–Hap_7) and Peloponnese, Greece (Hap_8, Hap_9). The haplotype from a specimen originating from Crete, Greece (Hap_10) clustered within the haplotypes of specimens originating from Turkey (Hap_11–Hap_38). A small third group was observed, consisting of both haplotypes of specimens from Israel (Hap_39, Hap_40) (Fig. 3). Analysis of molecular variance revealed 85.6% genetic variation between the

three groups and the comparably large genetic distance between the groups was supported by a high F_{ST} value (Table 2).

Haplotype analysis of *Ph. simici* based on *cytb*

Altogether, seven sequences with a length of 609 bp were included in the analysis (Table 1). Six haplotypes were identified, defined by 34 variable sites of which six were parsimony informative with an overall haplotype diversity (Hd) of 0.952 and an overall nucleotide diversity (p) of 0.322.

The sequence of *Ph. simici* from Austria was of the same haplotype (Hap_1) as a *Ph. simici* specimen from North Macedonia, both clustering with the haplotypes of other *Ph. simici* specimens from North Macedonia (Hap_2) and Peloponnese, Greece (Hap_3–Hap_5). The haplotype from a specimen from Crete, Greece (Hap_6) was clearly separated from all other haplotypes (Fig. 4). As availability of *cytb* sequences was limited, AMOVA calculation was redundant.

Pairwise sequence comparisons of *Adlerius* species

Altogether, 82 *coxI* sequences of *Ph. simici* and 8 other species of the *Adlerius* subgenus with a length of 551 bp were included in the analysis (Additional file 1: Table S1). Pairwise distances ranged from 0–18.1%. Hap_1 (Austria) showed the smallest distance (Pd: 0.18%) to Hap_3, which is shared by specimens from North Macedonia and Thessaloniki, Greece, which further corroborated the clustering of the Austrian specimen within the European group in the haplotype network (Additional file 2: Table S2).

When grouping sequences by species, calculated intraspecific mean distances ranged from 0.1–2.4%, the highest being calculated for *Ph. simici* (Table 3). After a further division into three lineages, namely Europe, Turkey and Israel, mean intraspecific distances were 0.4%, 1.5% and 0.7%, respectively.

Interspecific mean distances between species ranged from 0.8–17.3%. While interspecific distances were low between *Ph. simici* and *Ph. brevis* as well as with an unknown *Adlerius* species from Turkey and Armenia (5.3–6.1%), interspecific distances were high between *Ph. simici* and other *Adlerius* species (14.2–17.3%) included. Mean distances between *Ph. simici* lineages ranged from 2.5–3.9% and 4.5–6.4% between *Ph. simici* groups, *Ph. brevis* and *Adlerius* spp. from Turkey and Armenia (Table 3). The lowest interspecific mean distance of 0.8% was observed between *Adlerius* specimens from Turkey and Armenia, clearly indicating that these two belong to the same unidentified species.

Nineteen *cytb* sequences with a length of 609 bp of specimens belonging to the subgenus *Adlerius* were included in the analysis (Additional file 3: Table S3). Pairwise distances ranged from 0–17.7%. The sequence of *Ph. simici* from Austria was 100% identical to a *Ph. simici* specimen from North Macedonia. Pairwise distances to other *Ph. simici* sequences ranged from 0.5–4.9%, of which the highest was observed to *Ph. simici* from Crete, Greece (Additional file 4: Table S4).

Intraspecific mean distances were calculated for *Ph. simici* (1.9%), *Ph. halepensis* (1.0%) and *Ph. chinensis* (2.7%), as only one sequence of *Ph. brevis* was available (Table 4). After splitting *Ph. simici* into

a European lineage and a Turkish lineage including the specimen from Crete, the intraspecific mean distances within the European *Ph. simici* lineage was 0.6%.

Interspecific mean distances ranged from 9.0% between *Ph. simici* and *Ph. brevis* to 15.5% between *Ph. halepensis* and *Ph. chinensis* (Table 4). After splitting *Ph. simici* into a European and a Turkish lineage (including the specimen from Crete), interspecific mean distances were 5.1% between the two groups, 9.0% between *Ph. simici* European lineage and *Ph. brevis* and 9.3% between *Ph. simici* Turkey lineage and *Ph. brevis*.

Maximum likelihood analysis of *coxI*

The 82 sequences used for pairwise distance calculations showed 74 unique haplotypes, which were used for ML analysis. *Phlebotomus (Transphlebotomus) mascittii* GRASSI, 1908 and *Phlebotomus* (*Transphlebotomus*) *anatolicus* KASAP, DEPAQUIT & ALTEN, 2015, as well as *Phlebotomus neglectus* and *Phlebotomus perfiliewi* PARROT, 1930, were used as outgroups in two different approaches, respectively. In both approaches, two well-supported major clades were observed, clade 1 comprised *Ph. simici*, *Ph. brevis* THEODOR & MESGHALI, 1964, and an unidentified *Adlerius* species from Turkey and Armenia. Clade 2 comprised all other *Adlerius* species, namely *Ph. chinensis* NITZULESCU, 1931, *Ph. longiductus* PARROT, 1928, *Ph. balcanicus* THEODOR, 1948, *Ph. arabicus* THEODOR, 1953, *Ph. kyreniae* THEODOR, 1958, and *Ph. halepensis* THEODOR, 1948, (Fig. 5, Fig S1). Calculations resulted in three well-supported lineages of *Ph. simici* that matched the clustering of the median-joining network. An intraspecific threshold value of 0.7% was used for ABGD analysis, which partitioned the sequences into 11 groups. Calculations were in concordance with ML, with one exception, *Ph. simici* was split into two hypothetical species, namely Turkey + Israel and Europe. The unknown *Adlerius* sp. specimens from Turkey and Armenia were shown to belong to one single species and were identified as a sister species of *Ph. brevis* and together forming the sister group of *Ph. simici* (Fig. 5).

Maximum likelihood analysis of *cytb*

The 19 sequences used for pairwise distance calculations showed 15 unique haplotypes, which were used for ML analysis. *Phlebotomus mascittii* and *Phlebotomus anatolicus* as well as *Phlebotomus neglectus* and *Phlebotomus perfiliewi* were used as outgroups in two different approaches. In both approaches, two well supported major clades were observed, clade 1 comprised *Ph. simici*, *Ph. brevis* and *Ph. halepensis*. *Ph. simici* and *Ph. brevis* which further corroborated that they are sister species. Clade 2 comprised *Ph. chinensis*, which was split into two lineages. An intraspecific threshold value of 1.29% was used for ABGD analysis, which partitioned the sequences into six groups. ABGD grouped all four species as different groups with two exceptions. *Ph. chinensis* was split into two lineages and *Ph. simici* from Crete, Greece was computed as a unique *Ph. simici* lineage, which was in concordance with the ML analysis (Fig. 6, Fig. S2).

Discussion

This study reports the first finding of *Phlebotomus simici* in Austria which represents the northern- and westernmost record of this species to date and further highlights the necessity of more detailed sand fly research in Austria and in Central Europe in general. Apart from prior *Ph. mascittii* findings in eastern parts of Austria, the sand fly fauna has remained unexplored and probably underreported in this country [5,6].

The observation of *Ph. simici* in Austria is rather unexpected, as this species has never been reported in any of the bordering countries (Table 5). Prior to this study, only a single species, namely *Ph. mascittii*, had been recorded in Austria, as also in neighboring Slovakia. In those neighboring countries of Austria that are known to harbor more than one species, other species are found (e.g. *Ph. mascittii* and *Ph. perniciosus* in Germany or *Ph. mascittii*, *Ph. perfiliewi*, *Ph. neglectus* and *Ph. papatasi* in Hungary) but never *Ph. simici*. Even in the southern neighboring countries (Italy and Slovenia) that both provide relatively diverse sand fly fauna comprised by several different species, *Ph. simici* was never recorded. Geographically closest recent records of *Ph. simici* are from Serbia, which is neighboring Hungary, a direct neighbor to Austria, in the south.

Ph. simici belongs to the *Adlerius* NITZULESCU subgenus, which includes about 20 described as well as several undescribed species with predominantly Eurasian distribution and an assumed origin in Central Asia [24]. *Ph. simici* is frequently reported in Balkan [25–28] and Middle Eastern countries [29,30]. Recent reports from North Macedonia (Dvořák p.o.), Kosovo [31] and Serbia [32] point towards a northward European distribution, which is further corroborated by an older mention from Croatia [33]. *Ph. simici* is also mentioned in an ex-Yugoslavian study, but it is not entirely clear whether it was indeed recorded in areas which belong to Croatia today [34].

The periurban village where the *Ph. simici* specimen was caught in Austria is located in the Danube valley in the very eastern part of the country, belonging to the warmest parts of Austria. Microclimatic conditions in river valleys support the establishment and prevalence of local populations of sand flies north of the core area of European distributions shown by occurrence of *Ph. mascittii* in the Rhine valley [35]. The Danube valley has been assumed to be particularly suitable for sand fly occurrence [36]. The sampling location exhibits perfect breeding site requirements for sand flies, having several buildings with natural floors and various animal hosts, including a dog, poultry, swine, rabbits and goats, close to human dwellings. Typically, also *Ph. mascittii* is found at similar locations in Central Europe [2,5,6,8], which raises the question if possibly these two species overlap also in other regions and more *Ph. simici* populations are already established and have been overlooked in the past.

The fact that only a single specimen was detected may be attributed to several factors. Firstly, even though July is usually the warmest month in Austria, abnormal weather conditions were observed in 2019, with great temperature fluctuations. The night temperature was only 15.6°C in the trapping night and decreased in the consecutive nights, probably temporarily suspending sand fly activity. In Romania, *Phlebotomus perfiliewi* was observed to be active at 15°C minimum night temperature, but no activity was observed below this temperature [37]. Secondly, this finding supposedly represents the northern

distribution limit of this species and thus, low population densities and consequently small trapping numbers are to be expected. In Austria, trapping rates are generally extremely low, also for *Ph. mascittii,* with typical trapping numbers of less than five specimens per night [5,6,38]. In Slovakia only a single specimen of *Ph. mascittii* has been trapped, namely in 2016 [6,8]. A comparative study by Obwaller et al. [7] reported huge differences in numbers of trapped *Ph. mascittii* specimens in consecutive years at two locations in Austria. These observations suggest that sand fly activity, and thus trapping success might not only depend on temperature but other factors may also play a role.

Identification of the female specimen was challenging and morphological identification was only possible to the subgenus level. Both, pharynx and spermatheca, showed typical *Adlerius* structures, however, spermatheca were hardly visible by light microscopy. An additional assessment of the spermatheca under UV light illuminated structures confirming the *Adlerius* subgenus. To our knowledge, the use of autoimmunofluorescence for sand fly identification has never been reported before. The application of this technique might add a valuable tool for the morphological examination of spermatheca. While its suitability for identification to the species level has to be further evaluated, it clearly contributes to the visualization of the otherwise often hardly visible spermatheca. The impossibility to morphologically identify the female specimen to the species level is not surprising, *Adlerius* females are often unidentifiable by morphology. This is particularly known for *Ph. simici* and *Ph. brevis*, two species that overlap in all morphological characters used to distinguish females of the subgenus *Adlerius* [24]. For example, Perrotey et al. [30] reported that females of sympatrically occurring *Ph. simici* and *Ph. brevis* in Lebanon were undistinguishable by morphological characters.

To clarify conflicting morphological identifications, molecular approaches using suitable marker genes are needed. In our study, species identification was possible by sequencing the *coxI* gene, a classical DNA barcoding marker. Interestingly, sequence identity ranged from 95.99% to 99.85% with sequences of *Ph. simici* from Turkey and Greece, respectively. Further sequence analyses revealed a monophyletic group of three distinct lineages of *Ph. simici*, however, mean pairwise distances between the three lineages were unexpectedly high for within one species. In addition, interspecific distances to *Ph. brevis* and the unidentified *Adlerius* species from Turkey and Armenia were rather low (<10%) compared to distances to the other *Adlerius* species (>10%) included. This finding indicates that *Ph. simici*, *Ph. brevis* and other *Adlerius* spp. are genetically very close and *coxI* might not be an ideal genetic marker for such closely related species.

CoxI has been a commonly used genetic marker for species identification since its introduction as “the barcoding gene” by Hebert et al. [39] and thus, sequence availability in GenBank is high and *coxI* is frequently used for sand fly identification and interspecific comparisons [40]. Although there is no common cut-off value for species delimitation, Hebert et al. [39] observed a mean divergence value of 11.3% between species and only a small fraction showed 2% or less divergence. However, in this study we observed pairwise distances between *Ph. simici*, *Ph. brevis* and another *Adlerius* sp. far below 10%. In particular, the mean interspecific distance between *Ph. simici* and *Ph. brevis* was only marginally higher than the mean interspecific distances between the three observed *Ph. simici* lineages. This clearly

indicates that *Ph. simici*, *Ph. brevis* and the yet unidentified *Adlerius* species have a short history of divergence and are thus challenging to differentiate by *coxI*. In contrast, interspecific distances of *Ph. simici* to further *Adlerius* specimens included in the analysis were far above 10% and thus species easy to separate.

To corroborate our results, *cytb* was used as a second genetic marker, albeit sequence availability is rather poor for *Adlerius* species. *Cytb* is the most used genetic marker in sand fly systematics [40]. Further confirmation of species delimitation was achieved by comparing the obtained *Ph. simici* sequences with reference sequences of *Ph. simici* and *Ph. brevis* from Iran. Although the intraspecific distance within *Ph. simici* was similarly high as observed for *coxI*, the calculated interspecific distance almost doubled the mean interspecific distance of *coxI* between *Ph. simici* and *Ph. brevis* (9.1%) and clearly separated these two species.

Ph. simici is an assumed but unproven vector species for *Leishmania infantum* [24]. Even though the specimen found was tested negative for *Leishmania* DNA, this species has been shown to be highly anthropophilic [26], which is important for its potential relevance in *Leishmania* transmission to humans.

Altogether, the finding of a single *Ph. simici* specimen in Austria does not allow to infer on deeper population genetic structures, however, interesting results at the sequence level were obtained and should be considered in future studies. It is obvious that a single specimen cannot prove the existence of a permanent population and does not give any information on the actual population size. However, particularly eastern parts of Austria have been shown to be suitable for sand flies, which is underlined by continuous trappings of *Ph. mascittii*, the closest population being found in Rohrau approximately 15 km away from the location reported in this study [5,6]. Yet, the origin and routes of dispersal are still unclear. By finding a unique but genetically very close haplotype and a shared haplotype of *coxI* and *cytb*, respectively, to a haplotype from North Macedonia, post-glacial northward recolonization from this area seems likely. This is further corroborated by recent findings in Serbia [32]. Temperatures in Central Europe during the Holocene optimum around 6,000 years ago were comparable to today and the presence of Mediterranean species in Central Europe may result from northward recolonization events from different refugial areas at that time [41]. The known distribution of *Ph. simici* and the high interspecific distances between the European, Turkish and Israeli lineages suggest that *Ph. simici* is most certainly a polycentric Balkanopontomediterranean species. The split between the European and the Turkish *Ph. simici* lineages might have taken place during one of several complex paleogeographic events that separated the Aegean region into eastern and western parts as demonstrated for the *Transphlebotomus* subgenus, where separation of the five species, including *Ph. mascittii*, were dated back to major biogeographic events in the Aegean region [42]. Inference on genetic divergence can be tricky and high mutation rates based on molecular clock calibrations of 5.7%/Mya [43] and 19.2%/Mya [44] have been published at population level compared to a commonly applied rate of 2.3%/Mya for mitochondrial DNA [45]. Thus, the clarification of separation events between *Ph. simici* lineages and between other *Adlerius* species should be subject of further studies including a more representative set of populations.

Conclusions

Although the finding of only a single *Ph. simici* specimen is reported, this study presents a unique and important finding for Austria and Central Europe in general. It clearly shows that current knowledge on sand fly distribution and species diversity is still scarce in Austria, but also in the larger area. Further entomological surveys are needed to elucidate the current distribution and species composition as well as to assess their epidemiological significance in Central Europe; especially in climatically favorable areas which may already be inhabited by overlooked populations of known and unknown species. This is of greatest importance, as a warming climate may lead to further growth of established populations and hence further dispersal. However, the increasing absence of traditional farms as commonly observed microhabitats for sand flies might have a limiting effect for future dispersal in Austria. Evaluation and sampling of other potential trapping sites should be attempted in future studies. Moreover, this study corroborates that morphological discrimination of sand fly species can be tricky or even impossible. The newly introduced approach taking advantage of the autofluorescence of chitin might constitute a very valuable tool. Molecular identification techniques have limitations and should always be interpreted with caution, particularly for closely related or cryptic species. The inclusion of at least a second marker gene or technique is advised in these cases. Although precise dispersal routes from refugial areas to Central Europe remain unknown, phylogenetic analyses in this study shed light on the relationships within *Ph. simici* and between *Adlerius* species.

Declarations

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Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated and analysed during this study was included in the article.

Conflict of interest

The authors declare that they have no competing interests.

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Authors' contributions

EK, AGO, WP, GM and JW designed the study. EK, AGO, MA, AC, LP and JS conducted field work. EK and VD performed laboratory work. EK, VD, MM and MK analysed the data. EK, VD, PV and JW wrote the manuscript. All authors reviewed, edited and approved the manuscript.

Abbreviations

ABGD: Automatic Barcode Gap Discovery Program; ABOL: The Austrian Barcode of Life; BOLD: The Barcode of Life Data System; CDC: Centers for Disease Control and Prevention; *coxI*: cytochrome oxidase subunit I; *cytb*: cytochrome b; df: degrees of freedom; ML: Maximum Likelihood; Mya: Million years; ZAMG: Central Institute for Meteorology and Geodynamics

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Tables

Table 1 Data on all *Ph. simici* specimens included in the haplotype network analysis based on *coxI* and *cytb* gene sequences.

region	*coxI*		*cytb*		reference
	GenBank	haplotype	GenBank	haplotype	
Austria	MN812831.1	Hap_1	MN812836.1	Hap_1	present study
North Macedonia	MT452050.1, MT452051.1	Hap_2, Hap_3	MT452052.1, MT452053.1	Hap_1, Hap_2	Stefanovski et al. (GenBank)
Greece, Thessaloniki	KU519497.1–KU519500.1	Hap_3–Hap_7	-	-	Chaskopoulou et al. (2016) [26]
Greece, Peloponnese	MT452054.1–MT452056.1	Hap_8, Hap_9	MT452057.1–MT452059.1	Hap_3–Hap_5	Chaskopolou et al. (GenBank)
Greece, Crete	MT452060.1	Hap_10	MT452061.1	Hap_6	Antoniou et al. (GenBank)
Turkey	MN086690.1–MN086717.1	Hap_11–Hap_38	-	-	Kasap et al. (2019) [28]
Israel	KX822734.1, KX822735.1	Hap_39, Hap_40	-	-	Akad et al. (GenBank)

Table 2 Analysis of molecular variance (AMOVA) of 51 *Ph. simici* individuals based on *coxI*.

variance	df	sum of squares	s^2	% variance	statistics	*P-value*
among groups	2	3618.423	174.418	85.63431	$F_{ST} = 0.87663$	<0.001
among populations	5	237.54	4.131	2.02828	$F_{SC} = 0.14119$	0.003
within populations	43	1080.527	25.129	12.33741	$F_{CT} = 0.85634$	<0.001
total	50	4936.49	203.678	100		

Table 3 Interspecific mean *coxI* genetic distances (%) based on the Tamura-3-parameter-model. Diagonal bold values indicate intraspecific mean distances.

		1	2	3	4	5	6	7	8	9	10
1	*Ph. simici*	**2.4**[b]									
2	*Ph. brevis*	5.3[b]	**0.9**								
3	*Adlerius* sp. Turkey	6.1[b]	3.9[b]	**0.8**							
4	*Adlerius* sp. Armenia	5.7[b]	3.6[b]	0.8[b]	**0.1**						
5	*Ph. balcanicus*	14.2	13.4	14.3	14.1	**3.7**[b]					
6	*Ph. halepensis*	14.5	12.3	13.1	13.0	8.6[b]	**1.4**				
7	*Ph. kyreniae*	14.8	13.5	14.9	14.6	7.1[b]	9.2[b]	**0.5**			
8	*Ph. chinensis*	15.7	15.8	15.5	15.4	13.6	13.1	14.8	**0.5**		
9	*Ph. longiductus*	16.8	15.8	16.1	15.2	13.6	12.4	14.1	14.5	**0.2**	
10	*Ph. arabicus*	17.3	15.4	15.8	15.5	11.2	8.1[b]	12.3	15.1	13.9	-[a]

[a]only one sequence available

[b]indicates small interspecific distance or large intraspecific distance

Table 4 Interspecific mean *cytb* genetic distances (%) based on the Tamura-Nei-model. Diagonal bold values indicate intraspecific mean distances.

		1	2	3	4
1	*Ph. simici*	**1.9**			
2	*Ph. brevis*	9.0	-[a]		
3	*Ph. halepensis*	13.5	13.7	**1.0**	
4	*Ph. chinensis*	15.4	15.0	15.5	**2.7**[b]

[a]only one sequence available

[b]indicates small interspecific distance or large intraspecific distance

Table 5 Checklist of reported sand fly species in Austria and its neighboring countries. Subgenus, author and year of description are provided at first mention of the respective species. Countries are presented in alphabetical order.

country	species	reference	GenBank *coxI*
Austria	*Phlebotomus* (*Adlerius*) *simici* NITZULESCU, 1931	present study	yes
	Phlebotomus (*Transphlebotomus*) *mascittii* GRASSI, 1908	Naucke et al. 2011 [5], Poeppl et al. 2013 [6]	yes
Czech Republic	none observed	-	-
Germany	*Phlebotomus mascittii*	Oerther et al. 2020 [38]	no
	Phlebotomus (*Laroussius*) *perniciosus* NEWSTEAD, 1911	Naucke et al. 2004 [3]	no
Hungary	*Phlebotomus mascittii*	Trájer et al. 2017 [10]	no
	Phlebotomus papatasi		no
	Phlebotomus neglectus		no
	Phlebotomus perfiliewi		no
Italy	*Phlebotomus mascittii*	Dantas-Torres et al. 2014 [46]	no
	Phlebotomus perniciosus		no
	Phlebotomus (*Phlebotomus*) *papatasi* SCOPOLI, 1786		no
	Phlebotomus (*Laroussius*) *neglectus* TONNOIR, 1921		no
	Phlebotomus (*Laroussius*) *perfiliewi* PARROT, 1930		yes
	Phlebotomus (*Laroussius*) *ariasi* TONNOIR, 1921		no
	Phlebotomus (*Paraphlebotomus*) *sergenti* PARROT, 1917		no
	Sergentomyia minuta		no
Liechtenstein	none observed	-	-
Slovakia	*Phlebotomus mascittii*	Dvořák et al. 2016 [8]	yes
Slovenia	*Phlebotomus mascittii*	Praprotnik 2019 [47]	yes
	Phlebotomus perniciosus	Ivović et al. 2015 [48]	no
	Phlebotomus papatasi		no
	Phlebotomus neglectus		no
	Sergentomyia minuta		no
Switzerland	*Phlebotomus mascittii*	Knechtli & Jenni 1989 [49]	no
	Phlebotomus perniciosus		no
	Sergentomyia (*Sergentomyia*) *minuta* RONDANI, 1843		no

Figures

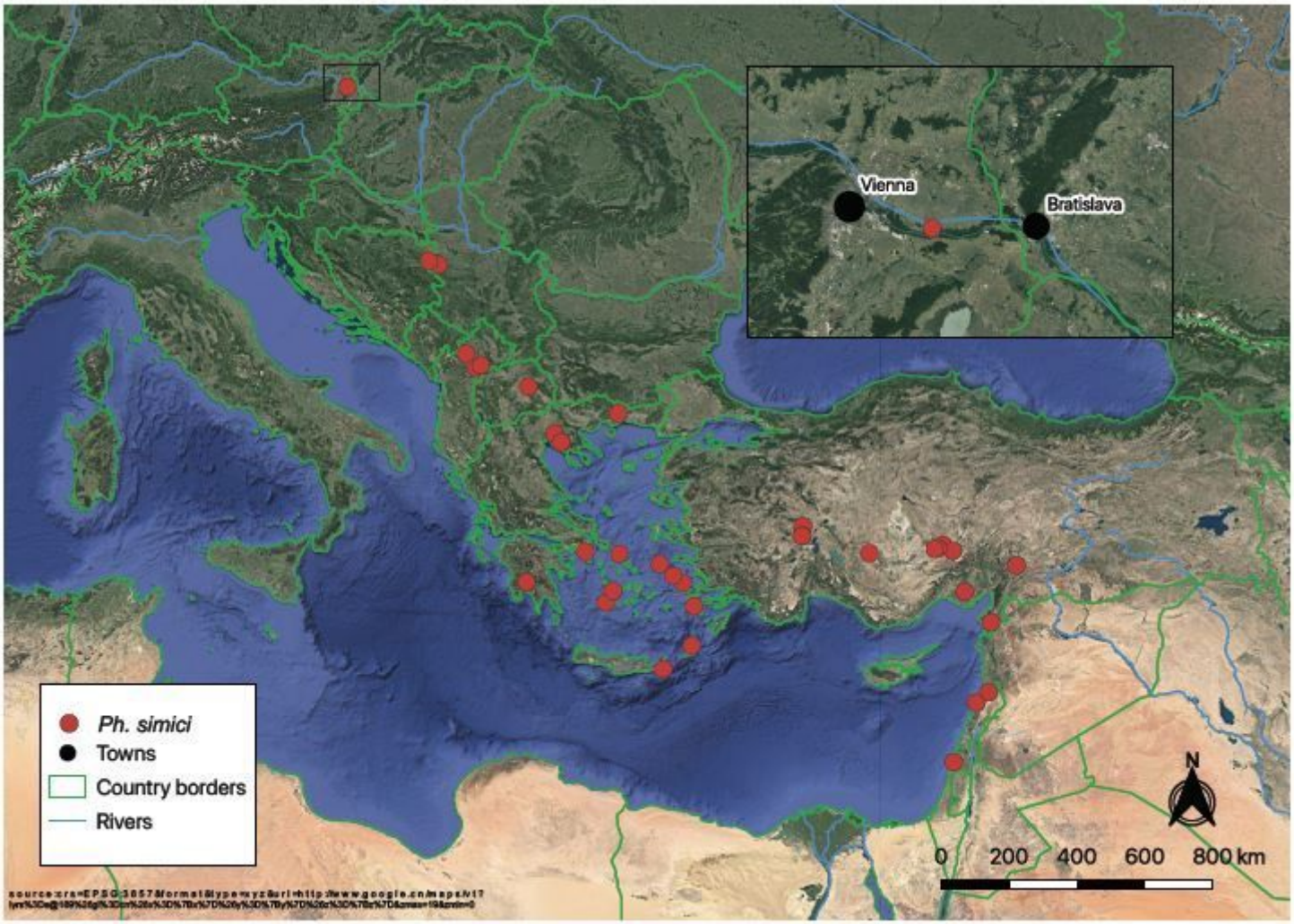

Figure 1

Ph. simici distribution map including the first finding in Austria. All published Ph. simici rec-ords with available coordinates were included. The three Ph. simici lineages suggested by AMOVA, namely Europe, Turkey and Israel, are enclosed in dashed, dash dot and round dot lines, respectively. Note: For some of the georeferenced Ph. simici records no sequence da-ta are available.

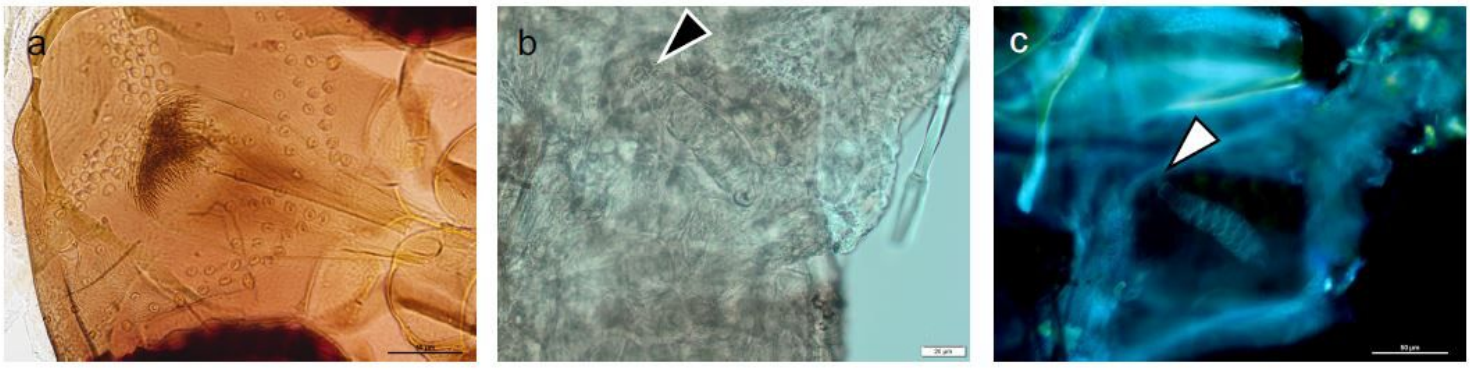

Figure 2

Morphological identification of Ph. simici. Pharynx (a), spermatheca (b), and autofluores-cent spermatheca under UV light (c). Arrow in b and c indicates the tip of the spermatheca and the missing neck, typical for Adlerius.

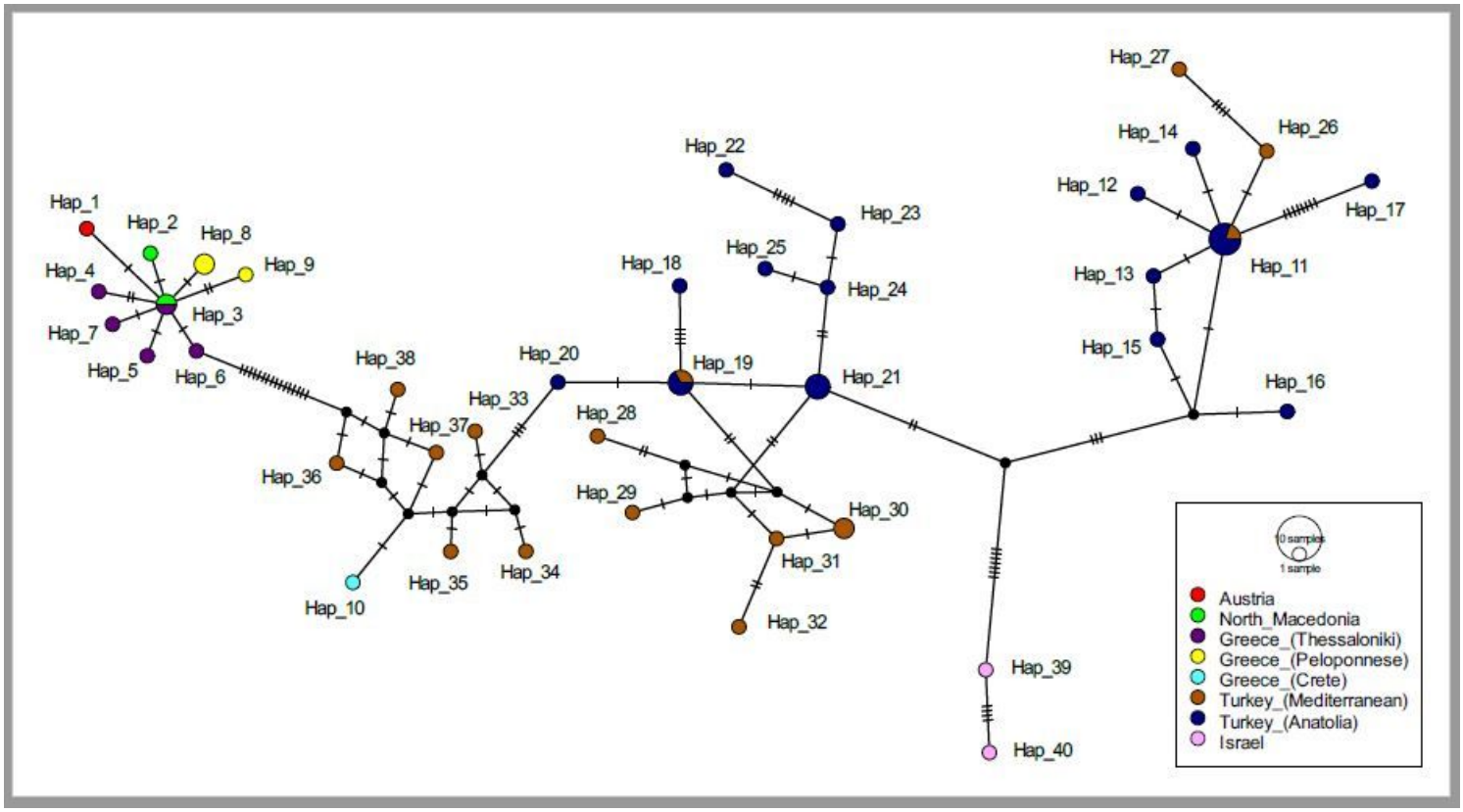

Figure 3

Haplotype network of Ph. simici based on coxI sequences. The three Ph. simici lineages sug-gested by AMOVA, namely Europe, Turkey and Israel, are enclosed in dashed, dash dot and round dot lines, respectively.

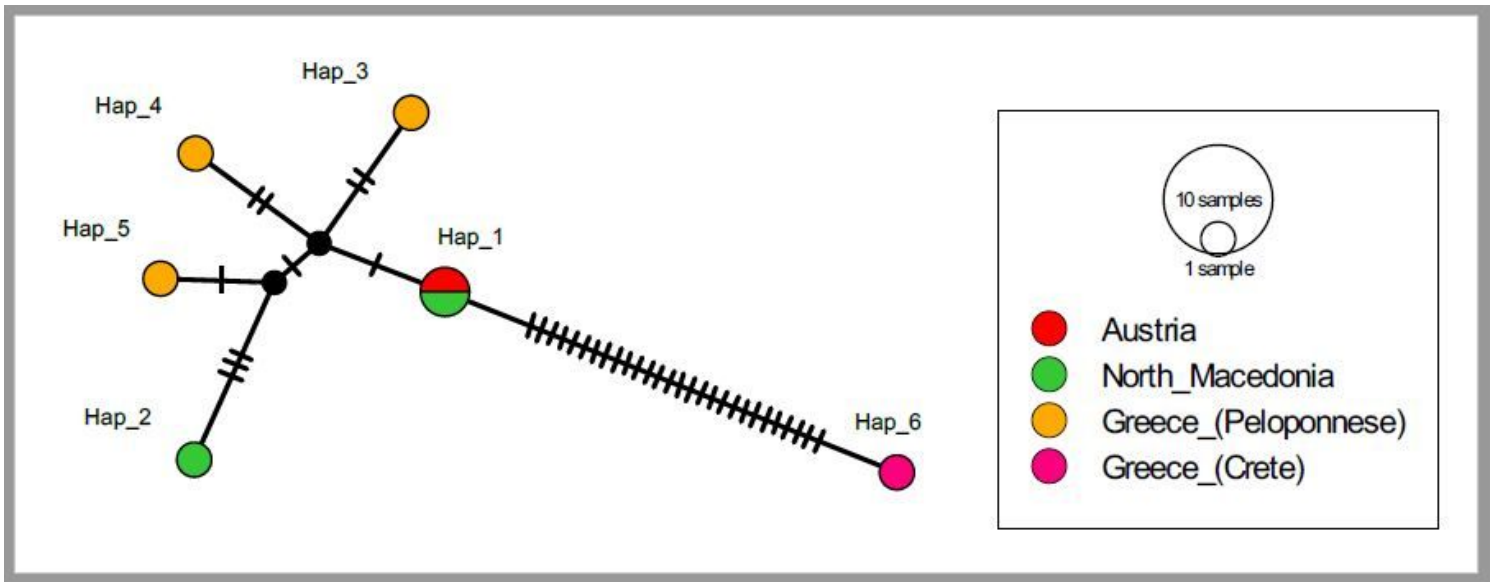

Figure 4

Haplotype network of Ph. simici based on cytb sequences.

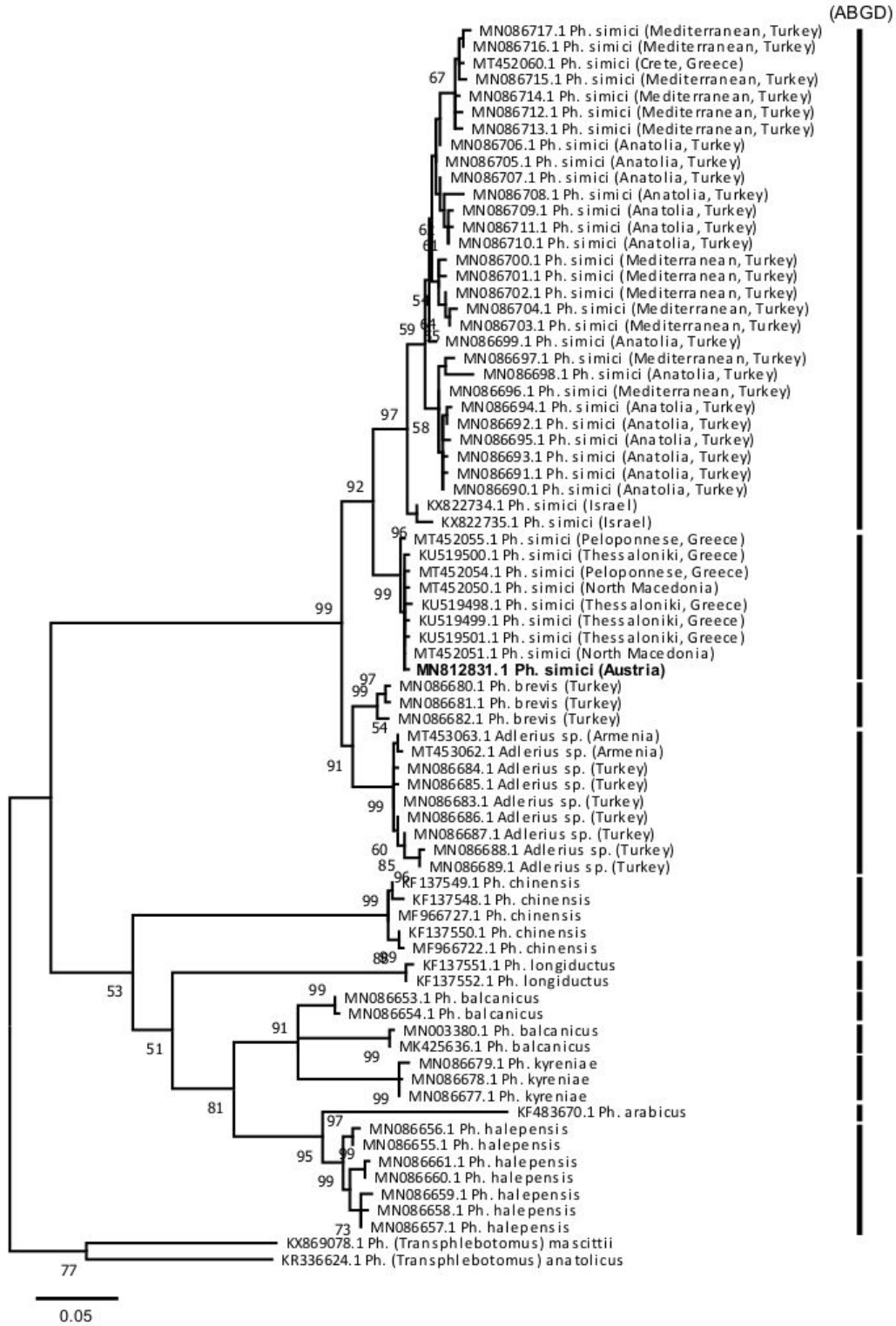

Figure 5

Maximum likelihood (ML) tree calculated based on coxI sequences of Adlerius spp. Ph. (Transphlebotomus) mascittii and Ph. (Transphlebotomus) anatolicus were used as out-group. Vertical bars represent hypothetical species calculated by ABGD. Bootstrap values higher than 50 % are shown.

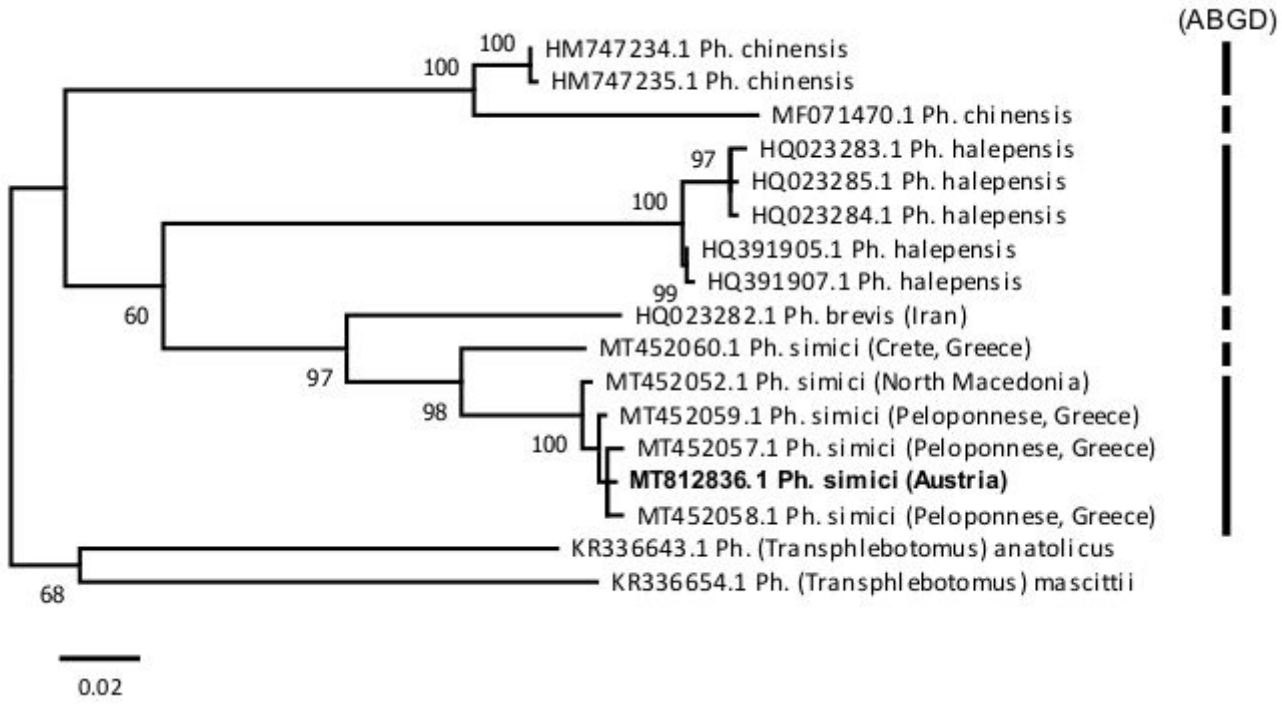

Figure 6

Maximum likelihood (ML) tree calculated based on cytb sequences of Adlerius spp. Ph. (Transphlebotomus) mascittii and Ph. (Transphlebotomus) anatolicus were used as out-group. Vertical bars represent hypothetical species calculated by ABGD. Bootstrap values higher than 50 % are shown.

Supplementary Files

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