Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as a useful tool for the rapid identification of wild flea vectors preserved in alcohol

Running title: MALDI-TOF MS identification of wild fleas

Antonio Zurita[1], Racha Djeghar[2], Rocío Callejón[1], Cristina Cutillas[1], Philippe Parola[3], Maureen Laroche[3]

[1]Department of Microbiology and Parasitology. Faculty of Pharmacy. University of Seville. Professor García González 2, 41012 Seville, Spain.

[2] Laboratoire d'Amélioration et Développement de la Production Végétale et Animale (LADPVA), Faculté des Sciences de la nature et de la vie, Ferhat Abbas University, Sétif 1, Sétif, Algérie

[3] Aix Marseille Univ, IRD, AP-HM, SSA, VITROME, IHU-Méditerranée Infection, Marseille, France

* Corresponding author: Maureen Laroche. VITROME - IHU Méditerranée Infection. 19-21 Boulevard Jean Moulin 13005 Marseille. E-mail: maureen.laroche972@gmail.com

Abstract

An increasing number of papers have reported the importance of flea identification since some species are considered as important vectors of several human pathogens that have emerged or re-emerged recently such as *Bartonella henselae* or *Rickettsia felis*. Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) has been evaluated in the last years for the identification of multicellular organisms, including arthropods. A preliminary study corroborated by the first time the usefulness of this technique for the rapid identification of fleas creating a preliminary database containing the spectra of 5 species of fleas. Nevertheless, long-term flea preservation in ethanol did not appear to be an adequate storage condition for specimen identification by MALDI-TOF MS profiling. The goal of the present work was to assess the performance of MALDI-TOF MS for the identification of seven fleas species (*Ctenocephalides felis*, *Ctenocephalides canis*, *Pulex irritans*, *Archaeopsylla erinacei*, *Leptopsylla taschenbergi*, *Stenoponia tripectinata* and *Nosopsyllus fasciatus*) collected in the field and stored in ethanol for different periods of time. Our results confirmed that MALDI-TOF MS can be used for identification of wild fleas stored in ethanol for different periods of time. Furthermore, this technique was able to discriminate not only different flea genera but also the two congeneric species *C. felis* and *C. canis*. Lastly, we updated the preliminary database with the spectra of seven flea species stored in ethanol.

Keywords: MALDI-TOF MS; flea identification; ethanol storage; Mediterranean

Introduction

In the last years a high number of studies have reported the importance of fleas and human flea-borne infections, especially in relation to zoonotic risk (Leulmi *et al*., 2014). Some of these flea-borne human pathogens have re-emerged recently (e.g., *Bartonella henselae*, *Rickettsia felis*) suggesting that much remains to be learned about the potential role of fleas as disease vectors (Eisen & Gage, 2012). We could assume that the cases of flea-borne diseases reported every year in the world may be underestimated. In this sense, health care professionals could not be aware of the presence of these flea-borne zoonoses and thus could fail to choose the most appropriate diagnosis method and treatment in each case. Furthermore, health authorities should be aware about the distribution and epidemiology of flea-borne diseases in order to be ready for potential outbreaks and to allocate adequate funding to the surveillance and control in these cases (Bitam *et al*., 2010).

Accurate vector identification at the species level is essential in order to guide clinicians regarding their patients' care. The classification of fleas is relatively advanced, thus we have a lot of knowledge about taxonomy of fleas, up to subspecies classification for several families (Whiting *et al*., 2008). Nevertheless, it requires extensive entomology knowledge of flea morphology and the availability of reference information (identification keys) since they show a complex quetotaxy and morphological specialization associated with ectoparasitism (Whiting *et al*., 2008). In the last years, molecular methods have emerged for the purpose of flea identification (Whiting *et al*., 2008; Marrugal *et al*., 2013; Lawrence *et al*., 2014; Zurita *et al*., 2015; Zurita *et al*., 2018), therefore, different molecular markers from nuclear ribosomal DNA (rDNA) or mitochondrial DNA (mtDNA) have been amplified and sequenced in order to characterize some species. However, despite the several advantages of molecular

methods, no consensus sequence exists for the identification of all flea species (Zurita *et al*., 2015). This absence of DNA sequence information for a larger number of flea genera and species restricts the use of molecular biology methods for flea identification (Fang *et al*., 2002; Feltens *et al*., 2010).

In the last years MALDI-TOF MS has been used for the identification of various taxonomic groups of arthropods including the *Culicoides* biting midges (Kaufmann *et al*., 2011; Kaufmann *et al*., 2012), ticks (Karger *et al*., 2012; Yssouf *et al*., *2013a), mosquitoes (*Muller *et al*., 2013; Yssouf *et al*., 2013b*;* Raharimalala *et al*., 2017; Mewara *et al*., 2018;), tsetse flies (Hoppenheit *et al*., 2013, 2014), sand flies (Dvorak *et al*., 2014), fleas (Yssouf *et al*., 2014) and Chagas disease triatomine vectors (Laroche *et al*., 2018). A preliminary study corroborated by the first time the usefulness of this technique for the rapid identification of fleas creating a preliminary database containing the spectra of 5 species of fleas (*Ctenocephalides felis*, *Ctenocephalides canis*, *Archaeopsylla erinacei*, *Xenopsylla cheopis* and *Stenoponia tripectinata*) (Yssouf *et al*., 2014). Afterwards, Nebbak *et al*. (2016) concluded that long-term arthropods preservation in ethanol did not appear to be an adequate storage condition for specimen identification by MALDI-TOF MS profiling since they observed differences at the spectral profile level between the fresh specimens and those from the same species stored in 70% ethanol. These differences were predominantly due to the disappearance of a number of peaks in the profile of specimens conserved in 70% ethanol for more than three months. Recently, (Diarra *et al*., 2017) proposed guidelines for the automation of sample preparation, including a dealcoholisation protocol for tick samples preserverd in 70% ethanol which could be used for another arthropod species.

The goal of the present work was to characterize for the first time seven fleas species (*C. felis*, *C. canis*, *Pulex irritans*, *A. erinacei*, *Leptopsylla taschenbergi*, *S. tripectinata*

and *Nosopsyllus fasciatus*) stored in ethanol (70% and 96%) for different periods of time by MALDI-TOF MS collected on Corse Island, East of Algeria, the French Pyrenees area and the South West of Spain, using MALDI-TOF MS. Firstly, optimized sample preparation protocol for fleas stored in ethanol for MALDI-TOF MS analysis was established. Secondly, a MS reference spectra database was created based on morphological and molecular identification of fleas.

Material and Methods

Ethical statement

The maintenance of laboratory colony of *C. felis* fleas has been approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine at Aix-Marseille University, France. The collection of all the samples for this study in the field did not involve national parks or other protected areas and endangered or protected species.

Catching and handling procedures of animals in this study were carried out according to the guidelines provided by the Institutional Animal Care of the University of Seville based on RD 53/2013 of 1st January, 2013 (BOE number 34 of 8th February, 2013) of the Spanish government. Rodents were trapped using Sherman traps and released into its natural habitat after collecting fleas procedures. Dogs belonged to owners and public dog kennels. The consent of owners and dog kennels chairman was obtained for the inclusion of the dogs in the study. In Algeria, we obtained the approval of the municipality of Constantine to include the dogs from the kennels. Rodents and dogs were exhaustively and manually examined for fleas on the head, neck, body, sides, tail, and ventral regions of each animal. No specific approval of any Institutional Animal

115 Care and Use Committee were needed in this study since any invasive procedure nor
116 anesthesias were required for animal handling.

117 *Sample collection and morphological identification*

118 Fleas belonging to *C. felis* and *C. canis* species were collected between 2011 and 2016
119 from dogs (*Canis lupus familiaris*) from three localities of the South West of Spain:
120 Villamanrique de la Condesa, Seville (37°14′47″N 6°18′23″W), Dos Hermanas, Seville
121 (37°17′01″N 5°55′20″W), Sanlúcar de Barrameda, Cádiz (36°46′44″N 6°21′14″W) as
122 well as Constantine, Algeria (36°21′54″N 6°36′52″E). Specimens identified as *P.*
123 *irritans* were collected from an abandoned horse stable with a flea infestation from
124 Seville, Spain (37°23′00″N 5°59′00″W) whereas, specimens identified as *A. erinacei*
125 were collected from hedgehogs (*Erinaceus europaeus*) killed on roads from Almonte,
126 Spain (37°16′00″N 6°31′00″W) and Dos Hermanas, Spain. Lastly, some samples were
127 collected from different species of rodents (see Table 1) from Corse Island (42°09′00″N
128 9°05′00″E) and Conat, France (42°36′51″N 2°21′27″E). All of flea specimens were
129 preserved in 70% and 96% ethanol at room temperature until morphological, molecular
130 and MALDI-TOF MS analyses. Some of them (*C. felis*, *C. canis*, *P. irritans* and *A.*
131 *erinacei*) were obtained through our own collecting efforts whereas others were
132 obtained through the assistance of colleagues (see Acknowledgements). The first ones
133 were identified by us whereas those fleas provided by our colleagues were first
134 identified by them and then morphologically corroborated by us in our laboratory.
135 Morphological identification to the species level was carried out according to different
136 publications and taxonomic keys (Marrugal *et al*., 2013; Jordan & Rothschild, 1912; Gil
137 Collado, 1949; Hopkins & Rothschild, 1953; Jordan, 1958; Lewis, 1967, 1993; Hastriter
138 & Tipton, 1975; Beaucournu & Launay, 1990; Giannetto *et al*., 1997; Beaucournu &
139 Menier, 1993, Menier & Beaucournu, 1998; Acosta & Morrone, 2013). For

morphological analysis, all specimens were examined using a Nikon microscope equipped with a camera lucid system and a photomicroscope. All fleas included in this study were not engorged.

MALDI-TOF MS analyses

According to Diarra *et al*. (2017) previously published dealcoholisation protocol of arthropods for MALDI-TOF MS, each sample (flea legs and cephalothorax) was introduced in a sterile Eppendorf and dried overnight at 37º C, prior to homogenization in order to remove all the ethanol. Secondly, a sterile tungsten bead (Qiagen, Hilden, Germany) was added to each sample plus 20 µL of a mix of 70 % (v/v) formic acid and 50 % (v/v) acetonitrile (Fluka, Buchs, Switzerland). The samples were then homogenized using the TissueLyser device (Qiagen, Hilden, Germany) by 3 cycles of 30 s at a frequency of 30 Hz. The homogenized cephalothorax and legs of fleas were centrifuged at 10000 r.p.m. for 30 s and 1 µL of the supernatant from each sample was carefully dropped onto the MALDI-TOF target plate as previously described (Nebbak *et al*., 2017). Each spot was then recovered with 1 µL of CHCA matrix solution composed of saturated α-cyano-4-hydroxycynnamic acid (Sigma, Lyon. France), 50 % acetonitrile (v/v), 2.5 % trifluoroacetic acid (v/v) (Aldrich, Dorset, UK) and HPLC-grade water (Yssouf *et al*., 2016) The target plate, after drying for several minutes at room temperature, was introduced into the Microflex LT MALDI-TOF Mass Spectrometer device (Bruker Daltonics, Germany) for analysis. The loading of the MS target plate, the matrix quality, and the performance of the MALDI-TOF were performed as previously described (Nebbak *et al*., 2017). For each target plate we used one specimen of *C. felis* from the University Hospital Institute Méditerranée Infection of Marseille laboratory rearing as a positive control and 2 µL of CHCA matrix solution as a negative control. Protein mass profiles were obtained using a Microflex LT MALDI-TOF mass

spectrometer (Bruker Daltonics, Germany) using parameters previously described by (Kumsa *et al*., 2016). The spectrum profiles obtained were visualized with Flex analysis v.3.3 software and exported to ClinProTools software v.2.2 and MALDI-Biotyper v.3.0. (Bruker Daltonics, Germany) for analysis and data processing (smoothing, baseline subtraction and peak picking). MSPs of each species were created as previously described (Nebbak *et al*., 2017). Only the high quality and reproducible spectra were loaded into the MALDI Biotyper v.3.0 to create a spectral database with 2–6 specimens for each species as previously described (Diarra *et al*., 2017). To upgrade the database, MSP reference spectra were created using spectra from at least two specimens per species by the automated function of the MALDI-Biotyper software v3.0. (Bruker Daltonics). MS spectra were created with an unbiased algorithm using information on the peak position, intensity and frequency. This database was formerly composed of a total of five flea species (*C. felis*, *C. canis*, *A. erinacei*, *X. cheopis* and *S. tripectinata*) plus others arthropods species described in (Boucheikhchoukh *et al*., 2017). The spectra files are available on request and transferable to any Bruker MALDI-TOF device.

For the blind test analysis, 148 good quality spectra from the seven species of fleas tested were selected to query the upgraded database. Since the protein extracted from each specimen was spotted on the MALDI-TOF target plate in quadruplicate, each specimen was associated with four spectra. The results of the database queries are presented as log score values (LSVs) for each spectrum, corresponding to a matched degree of signal intensities of mass spectra of the query and the reference spectra. LSVs range from 0 to 3. LSVs allow good evaluation of the reproducibility between a queried spectrum and a reference spectrum as it is the result of thorough comparison of peaks position and intensity between those two spectra. A LSV was obtained for each

189 spectrum of the samples tested blindly. For each specimen, the spectrum with the
190 highest LSVs was selected for identification (Laroche *et al*., 2018).

191 On the other hand, in order to visualize MS profiles similarity and distances,
192 hierarchical clustering of the mass spectra of 3-4 specimens of each tested species was
193 performed using the dendrogram function of MALDI Biotyper, version 3.0.
194 Dendrograms are based on the results of Composite Correlation Index (CCI) matrix.
195 CCIs are calculated by dividing spectra into intervals and comparing these intervals
196 across a dataset. The composition of correlations of all intervals provides the CCI,
197 which is used as a parameter that defines the distance between spectra. A CCI match
198 value of 1 represents complete correlation, whereas a CCI match value of 0 represents
199 an absence of correlation (Laroche *et al*., 2018).

200 *Molecular identification*

201 For molecular purposes the abdomens of each specimen were cut off and transferred to
202 a 1.5 mL tube containing 180 µL of G2 lysis buffer (Qiagen, Hilden, Germany), and 20
203 µL of proteinase K (Qiagen, Hilden, Germany), and incubated at 56º C overnight. DNA
204 extraction was performed with an EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany)
205 according to manufacturer recommendations. Flea DNAs were then eluted in 100 µL of
206 Tris EDTA buffer using the DNA extracting EZ1 Advanced XL Robot (Qiagen, Hilden,
207 Germany). The DNA was either immediately used or stored at −20° C until molecular
208 analysis. The DNA extracting EZI Advanced XL Robot was disinfected after each batch
209 of extraction as per the manufacturer's recommendations, to avoid cross-contamination.

210 The nuclear marker ITS1 of two to six samples for each flea species was amplified by
211 standard PCR using a Bio-Rad Thermocycler (Bio-Rad Laboratories, Hercules,
212 California, USA). For this purpose, we used the DNA of those specimens added to the

MALDI-TOF database. The following conditions were applied: 95 °C at 15 min (denaturing), 35 cycles at 94 °C at 30 seconds (s) (denaturing), 58 °C at 30 s (annealing), 72 °C at 90 s (primer extension), followed by 5 min at 72 °C. Forward primer used was NC5 (5´-GTAGGTGAACCTGCGGAAGGATCATT-3´) (Gasser *et al*., 1996) whereas reverse primer used was ITS1rev (5´-GCT GCG TTC TTC ATC GACCC-3´) (Vobis *et al*., 2004). DNA from *C. felis* specimens reared at the laboratory was used as positive control. The PCR products were visualized by electrophoresis through a 1.5 % agarose-tris-borate-EDTA gel containing SYBR Safe. PCR products of the positive samples were purified as previously described Kumsa *et al*. (2016). Then, the purified DNA products were sequenced in both directions using a Big Dye Terminator kit and an ABI PRISM 3130 Genetic Analyzer (Applied BioSystems, Courtabeauf, France). The sequences were analysed using the ABIPRISM DNA Sequencing Analysis software version 3.0 (Applied BioSystems) and compared, when it was possible, with sequences available in the GenBank database using the BLAST algorithm (http://blast.ncbi.nlm. nih.gov/Blast.cgi). All sequences were aligned using the ClustalW tool of the BioEdit software (http://www.mbio.ncsu.edu/BioEdit/page2.html) in order to confirm obtaining a consensus sequence for each species, and one sequence per species was submitted to the NCBI GenBank database.

Results

Morphological identification

A total of 176 fleas were collected for this study. Seven different species of fleas were identified in our study (*C. felis*, *C.canis*, *P. irritans*, *A. erinacei*, *L. taschenbergi*, *N. fasciatus* and *S. tripectinata*). All specimens collected from dogs from Villamanrique de

237 la Condesa, Dos Hermanas, Sanlúcar de Barrameda as well as six samples collected
238 from Seville showed specific morphological characteristics of *C. felis*. *P. irritans* was
239 only collected from Seville, while specimens collected from Corse Island showed
240 morphological characteristics of *N. fasciatus* and *S. tripectinata.* On the other hand, all
241 the specimens collected from Constantine were identified as *C. canis*, whereas, all the
242 fleas collected from hedgehogs from Almonte and Dos Hermanas showed specific
243 morphological characteristics of *A. erinacei*. Finally specimens collected from rodents
244 from Conat were identified as *L. taschenbergi* (Table 1).

245 *MALDI-TOF MS analyses*

246 Using the sample homogenization and preparation protocol described in material and
247 methods section, we were able to obtain high quality spectra (high reproducibility and
248 high intensity of MS spectra) from cephalothorax and legs of each flea species assessed
249 in this study. Thus, reproducible and specific MALDI-TOF MS spectra profiles were
250 obtained for each flea species. Furthermore, we upgraded the database with twenty eight
251 new reference spectra from seven species *C. felis* (n=6), *C. canis* (n=4), *P. irritans*
252 (n=3), *A. erinacei* (n=2), *L. taschenbergi* (n=3) *S. tripectinata* (n=5) and *N. fasciatus*
253 (n=5). Representative MS profiles obtained for each flea species with high intensities
254 peaks in the range of 2–20 kDa are presented in Fig. 1.

255 All the 148 specimens used from blind test were queried successively against the
256 upgraded MS reference database. Using this database, the blind test yielded 100%
257 correct identification at the species level for the specimens tested, indistinctly of their
258 storage conditions (70 % ethanol or 96 % ethanol) and geographical origin in the case of
259 *C. felis* and *A. erinacei*. For the blind test of *C. felis*, 32 specimens were used of which
260 29 (90.6 %) were correctly identified with high LSVs ranging from 1.80 to 2.54 while 3

of them were correctly identified with low LSVs ranging from 1.31 to 1.66. For *C. canis´* blind test, all specimens (n=21) were correctly identified with high LSVs ranging from 2.07 to 2.426. Regarding *P. irritans* and *A. erinacei* a total of 20 and 16 samples, respectively, were queried and were all identified with high LSVs (1.84-2.51). Similar results were observed for *L. taschenbergi* (24 samples), *S. tripectinata* (17 samples) and *N. fasciatus* (18 samples) blind tests. Thus, for these species, we observed high LSVs ranging from 1.87 to 2.49. It is noteworthy that the spectra of specimens preserved in alcohol always showed a correct identification with low LSVs or a wrong identification when they were first compared with MS spectra of fresh specimens of the same species which had already been added at the database. Ultimately, the results of the blind test showed 100% concordance among morphological, molecular and MALDI-TOF MS identification.

On the other hand, dendrogram based on the seven flea species MS profile showed all the species provided highly specific spectra. Furthermore *P. irritans*, *C. felis*, *C. canis* and *A. erinacei* (Pulicidae), are clustering together and separated from *N. fasciatus*, *L. taschenbergi* (Ceratophyllidae) and *S. tripectinata* (Stenoponiidae) (Fig. 2). Furthermore, within the Pulicidae family, the two congeneric species *C. felis* and *C. canis* are on two distinct branches (Fig. 2).

Molecular study

In order to carry out a molecular identification, we sequenced the ITS1 of 26 specimens from each species assessed in this work: *C. felis* (n=6), *C. canis* (n=4), *P. irritans* (n=3), *A. erinacei* (n=2), *L. taschenbergi* (n=3) *S. tripectinata* (n=5) and *N. fasciatus* (n=3). The molecular results obtained were in concordance with morphological identification, thus BLAST analysis showed that six of seven fleas species had high sequence

similarity with their respective homologue species available in GenBank ranging from 99 % (*P. irritans* and *N. fasciatus*) to 100 % (*C. felis*, *C. canis*, *A. erinacei* and *S. tripectinata*). The accession numbers of the GenBank reference species used for BLAST analysis were: EU169198 (*P. irritans*), LT158057 (*N. fasciatus*), HF583247 (*C. felis*), HF563590 (*C. canis*), LT604113 (*A. erinacei*) and LK937052 (*S. tripectinata*). All the ITS1 sequences obtained in this study were submitted to the NBCI Genbank database (accession No. AN: LN827902, LT797457, LS992461, LS992468, LS992469, LS992470). In the case of *L. taschenbergi*, we submitted the first ITS1 sequence of GenBank in order to update the database (AN: LS992202).

Discussion

MALDI-TOF MS technique has emerged as an efficient approach for the rapid identification of fleas but with certain limitations (Yssouf *et al*., 2014, 2016). Thus, to fully exploit the use of MALDI-TOF MS for the identification of arthropods such as fleas by sharing databases between laboratories, a standardization of operational protocols is required, including sample preparation (Nebbak *et al*., 2017). Ethanol is widely used in the field for arthropod conservation and transport, as it is less restrictive than frozen methods (Reis *et al*., 2011; Bessas *et al*., 2016). However, previous studies revealed some limitations when samples preserved in alcohol were used for MALDI-TOF MS analyses, especially when they have been stored for long periods (Yssouf *et al*., 2014; Nebbak *et al*., 2017). Nevertheless, Diarra *et al*. (2017) have shown that these limitations could be circumvented on ticks by specific dealcoholisation protocols. Therefore, in this work we provided by the first time a reliable MALDI-TOF MS sample preparation protocol for fleas stored in ethanol for different periods of time. This new method includes the dealcoholisation steps proposed by Diarra *et al*. (2017) but is combined with a new crushing method with tungsten beads, which provides higher

quality spectra for flea preserved in alcohol than the reference method with glass powder (Yssouf *et al*., 2016). On the other hand, fleas were dissected as previously suggested by Yssouf *et al*. (2014). The choice of the arthropod's body part is a crucial step in the development of a MALDI-TOF MS protocol for arthropod identification (Yssouf *et al*., 2016). The exclusion of the abdomen prevents the impact of the host blood on the resulting spectra; therefore this protocol was validated for this study.

The application of this technique to the specific identification of fleas is very interesting because this Order exhibit a great complexity in its morphological characters which sometimes become the morphological identification of fleas in a very hard task.

Another advantage of this technique is that it is useful to identify fleas that could be damaged, in which case the morphological characteristics would be deformed or even disappear, making morphological identification very challenging. On the other hand, with the use of MALDI-TOF MS, we could circumvent the main drawbacks of molecular biology method such as the length, high costs or even the lack of reference data in the case of Siphonaptera order. Indeed, as shown by this study, a specific marker (ITS1) had to be sequenced to distinguish *C. canis* from *C. felis* due to its unique divergence compared to other genes frequently used to identify arthropods (Rodríguez-Pérez., 2006). ITS1 reference sequences were either absent from GenBank, as it was the case for *L. taschenbergi*, or only recently added for *S. tripectinata*, *N. fasciatus* and *A. erinacei* (Zurita *et al*., 2015, 2017, 2018). MALDI-TOF MS allows discrimination of all the included flea species in a single step with the same protocol, whereas molecular biology can be limited by the choice of the targeted sequences.

We agree with previous studies reporting that the use of the body of a flea without the abdomen is the best sample for distinguishing flea species by the MALDI-TOF MS

approach (Yssouf *et al*., 2014). Nevertheless, based on the results obtained in this study, we were able to set a suitable homogenization protocol in order to obtain higher quality and more reproducible spectra for fleas preserved in 96% and 70% ethanol for different periods of time. Therefore, using this new homogenization protocol we were able to correctly identify seven different flea species preserved in ethanol for less of one or two year (*A. erinacei*, *L. taschenbergi*, *N. fasciatus*, *P. irritans*, *S. tripectinata* and *C.canis*) to more than six years (*C. felis*). This fact was corroborated by blind test which results showed that all specimens were correctly identified by MALDI-TOF MS and only 2 % of them were well identified with low LSVs. In addition, we did not find notable differences among the same species spectra when specimens were collected from different geographical areas, preserved for different periods of time or under different storage conditions (70% ethanol or 96% ethanol).

On the other hand, MALDI-TOF MS results were in agreement with morphological identification as well as molecular results based on ITS1 sequences. This marker has thoroughly been used in the last years to approach different taxonomic and phylogenetic questions within Siphonaptera Order (Vobis *et al*., 2004; Marrugal *et al*., 2013; Zurita *et al*., 2018). This choice could be based on the fact that has been proved that morphological variation in the same flea species do not correspond with different ITS sequences (Marrugal *et al*., 2013; Zurita *et al*., 2018) as well as the presence of flea-common symbionts as *Wolbachia pipientis* do not affect the ITS sequences variability (Zurita *et al*., 2016). Additionally, this molecular marker has showed to have diagnostic value for specific determination of two congeneric species such as *C. felis* and *C. canis* (Marrugal *et al*., 2013) which remain very closely each other from a morphological point of view (Linardi & Santos, 2012). *C. felis* and *C. canis* are not always readily taxonomically distinguishable, thus, in some studies; these species have been

misidentified on the basis of their morphological characters included in taxonomic keys. Particularly the classification of the subspecies of *C. felis* in the Afrotropical zone (*C. felis strongylus*) has often been misidentified as *C. canis* based on the sole criterion of the cephalic profile; and likewise, *C. orientis* in Asia (Beaucournu & Kock, 1990). Recently, Linardi & Santos (2012) and Marrugal *et al*. (2013) concluded that the shape of the head and the chaetotaxy of the tibia and metepisternite showed significant intra-specific variations and, therefore, these two characteristics should be cautiously used for interspecific diagnosis between *C. felis* and *C. canis*. Based on these results, they argued that some others morphological criteria (relative size of the first and second genal spines or male and female genitalia) must be considered in order to discriminate between these two species (Fig. 3). Moreover, although both species are recognized vectors of *R. felis* and several *Bartonella* spp. pathogens (Lawrence *et al*., 2015), numerous authors reported much lower prevalence of these bacteria in *C. canis* (Horta *et al*., 2006; Kumsa *et al*., 2014; Nasereddin *et al*., 2014). These studies confirm the need for an unambiguous identification of these two species. As illustrated on the dendrogram created with MS spectra of the seven included flea species, we could suggest the usefulness of this technique to discriminate different flea families (Pulicidae, Ceratophyllidae and Stenoponiidae) but also closely related species such as the two congeneric species *C. felis* and *C. canis*. Nevertheless, although results are encouraging for the distinction of closely related species, this robustness would be confirmed with more specimens, particularly *C. orientis*, the major *Ctenocephalides* species in South East Asia, closely related to *C. felis* and *C. canis* (Linardi & Santos, 2012).

MALDI-TOF MS has been developed during the last years as an important tool not only for the taxonomic identification but also for the phylogenetic classification of microorganisms (Freiwald & Sauer, 2009). In our study, dendrogram results are in

concordance with former phylogenetic studies which reported a close phylogenetic relation between *Ctenocephalides* and *Archaeopsylla* genera. Thus, in these studies, specimens belong to both genera clustered together composing the Archaeopsyllini subfamily within Pulicidae family (Zhu *et al*., 2015; Zurita *et al*., 2017). However, we agree with Yssouf *et al*. (2013b) who argued that the MALDI-Biotyper software cannot yet be considered to be a reliable tool for studying the phylogeny of arthropods. The number of flea is still too limited in our study to hypothesize that MS spectra could be used as a taxonomic tool as it is already the case for bacteria. Therefore, although MALDI-TOF MS has opened new doors for phylogenetic studies in this phylum, additional data are still needed, and detailed studies should be carried out, especially within Siphonaptera order where the MS data are still limited.

In conclusion, our results confirm that MALDI-TOF MS could be used for identification of fleas preserved in alcohol. It requires the creation of a specific database with specimens stored in ethanol since spectra from fresh specimens differ from those obtained from ethanol-preserved specimens. Accordingly, we updated the preliminary database with the spectra of seven flea species stored in ethanol (*C. felis*, *C.canis*, *P. irritans*, *A. erinacei*, *L. taschenbergi*, *N. fasciatus* and *S. tripectinata*). This protocol was developed and validated on seven flea species. Our reference spectra are available on request for laboratories equipped with Bruker MALDI-TOF MS devices to upgrade their own databases. The rapid identification of wild flea species by MALDI-TOF MS in only one step would constitute a relevant discover in epidemiology and entomology field since this technique shows some advantages compared with classic and molecular methods such as the low consumable costs, the minimal time required for sample preparation or the rapid availability of the results. For this reason, this study should encourage other scientific teams about the necessity of setting up an updated MALDI-

TOF MS database. This tool is also important for clinicians as this study illustrates that wild fleas collected from a patient or at his house could be preserved in alcohol allowing a fast and reliable subsequent identification, which is critical information for a medical doctor.

Conclusion

The present study confirms that the drawbacks of ethanol preservation for MALDI-TOF MS identification of arthropods can be circumvented with the appropriate sample preparation. This approach is fast, with low running costs and validated on field samples. It can be used by operators with no entomological training, and opens numerous perspectives such as high throughput vector monitoring and identification of fleas collected from patients.

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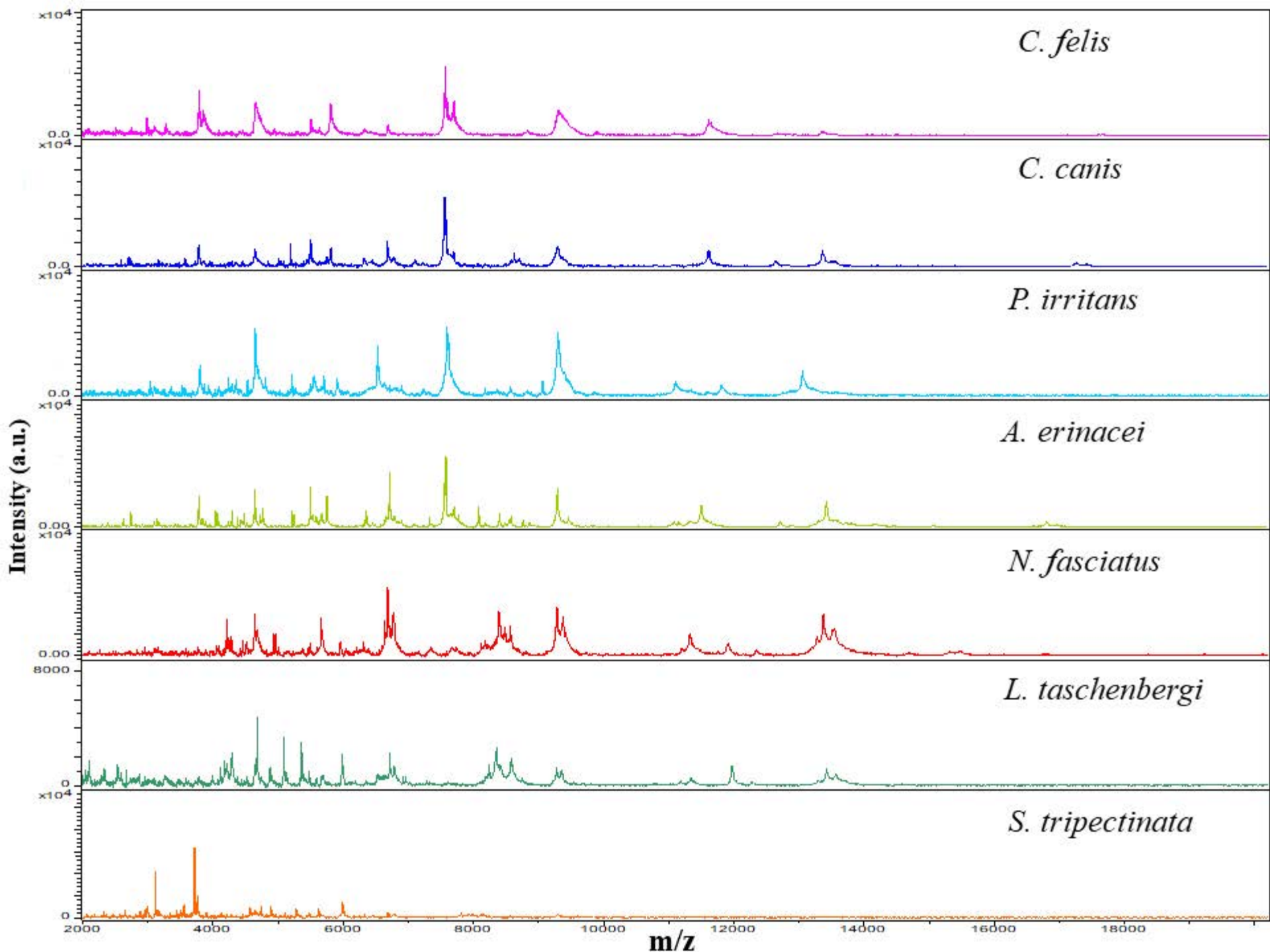
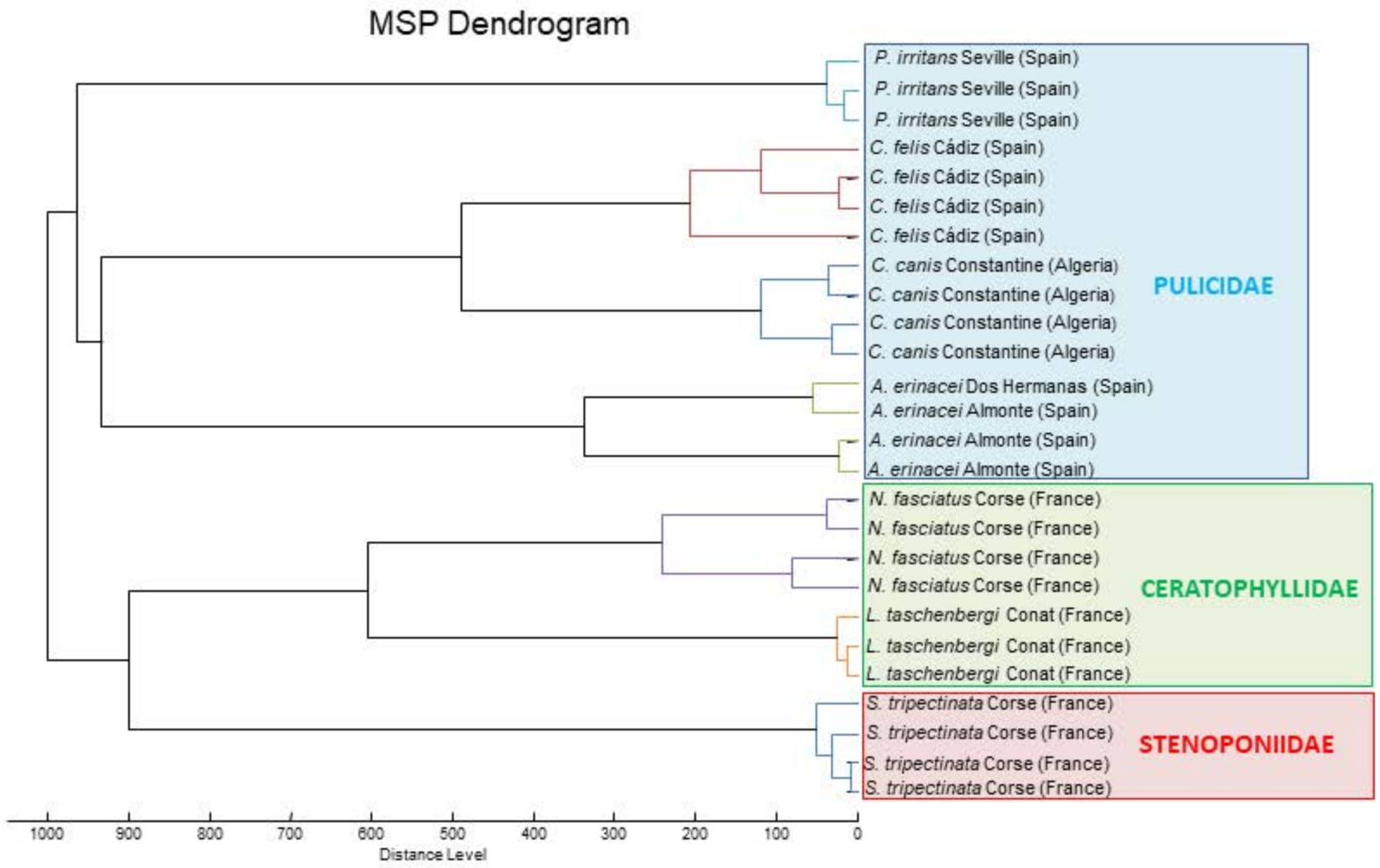
Figure captions

Figure 1. Representative spectra from cephalothorax of *C. felis, C. canis, P. irritans, A. erinacei, L. taschenbergi, S. tripectinata* and *N. fasciatus*. a.u., arbitrary units; m/z, mass-to-charge ratio.

Figure 2. Dendrogram constructed using some representative MS spectra of each flea species studied in this work.

Figure 3. Morphological differences between *C. felis* and *C. canis*. A. Genal ctenidium with a long first spine (arrow) of *C. felis*. B. Apex of manubrium (arrow) of *C. felis* male. C. Spermatheca with a short hilla (arrow) of *C. felis* female. D. Genal ctenidium with a short first spine (arrow) of *C. canis*. E. Apex of manubrium (arrow) of *C. canis* male. F. Spermatheca with a long hilla (arrow) of *C. canis* female.


C. felis
C. canis
P. irritans
A. erinacei
N. fasciatus
L. taschenbergi
S. tripectinata
Intensity (a.u.)
m/z
2000
4000
6000
8000
10000
12000
14000
16000
18000


MSP Dendrogram
P. irritans Seville (Spain)
P. irritans Seville (Spain)
P. irritans Seville (Spain)
C. felis Cádiz (Spain)
C. felis Cádiz (Spain)
C. felis Cádiz (Spain)
C. felis Cádiz (Spain)
C. canis Constantine (Algeria)
C. canis Constantine (Algeria)
C. canis Constantine (Algeria)
C. canis Constantine (Algeria)
PULICIDAE
A. erinacei Dos Hermanas (Spain)
A. erinacei Almonte (Spain)
A. erinacei Almonte (Spain)
A. erinacei Almonte (Spain)
N. fasciatus Corse (France)
N. fasciatus Corse (France)
N. fasciatus Corse (France)
N. fasciatus Corse (France)
L. taschenbergi Conat (France)
L. taschenbergi Conat (France)
L. taschenbergi Conat (France)
CERATOPHYLLIDAE
S. tripectinata Corse (France)
S. tripectinata Corse (France)
S. tripectinata Corse (France)
S. tripectinata Corse (France)
STENOPONIIDAE
1000
900
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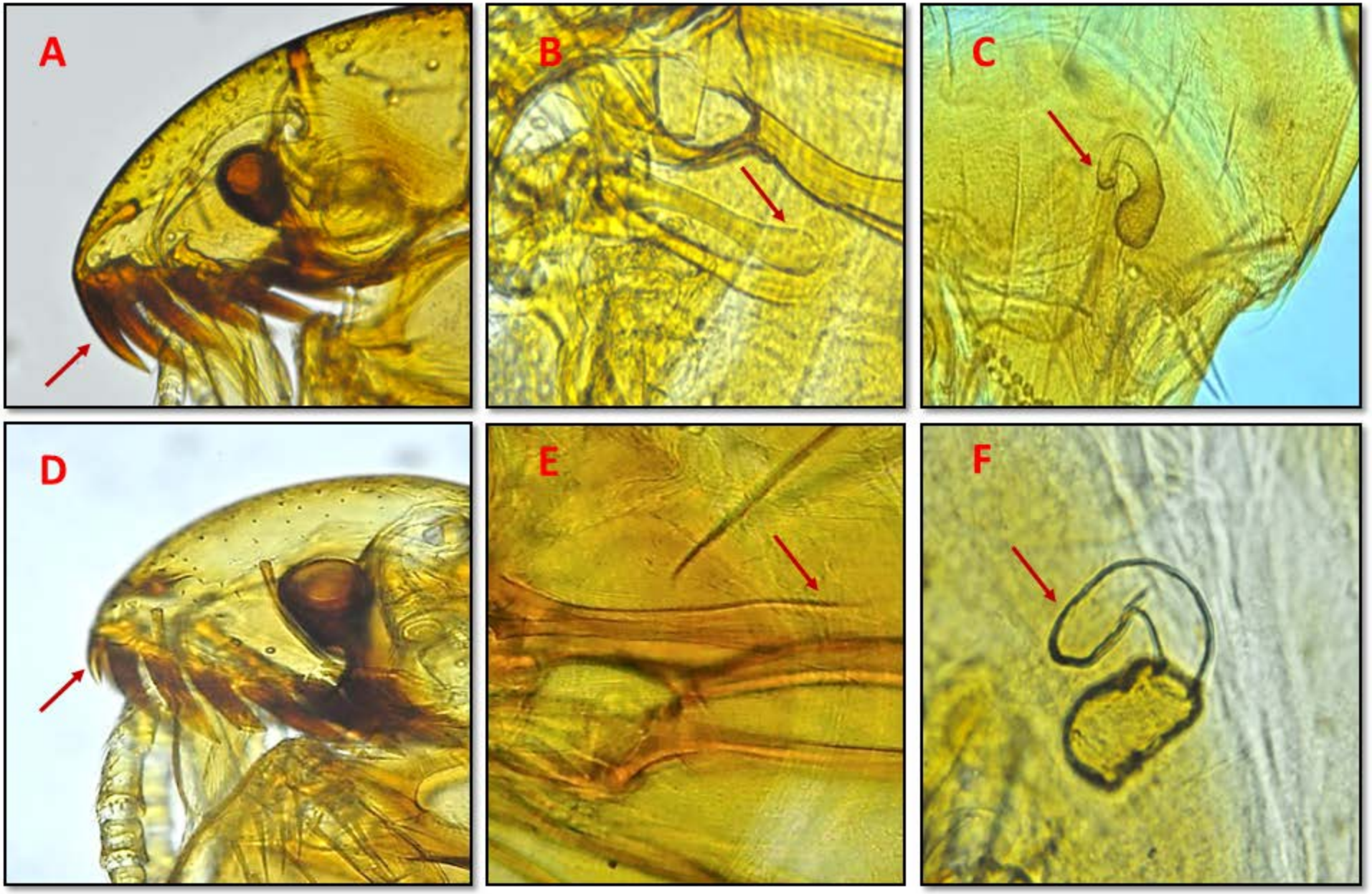
200
100
0
Distance Level


A
B
C
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E
F

Table 1. Geographical origin, preservation method, date of collection and hosts of seven different species of fleas used in this study.

Species	Geographical area	Number of samples (male/female)	Preservation method	Date of collection	Host
C. felis	Seville (Spain)	6 (1/5)	96 % Ethanol	May 2016	*C. l. familiaris*
	Villamanique de la Condesa (Spain)	7 (1/6)	96 % Ethanol	August 2014	*C. l. familiaris*
	Dos Hermanas (Spain)	3 (0/3)	96 % Ethanol	January-June 2016	*C. l. familiaris*
	Sanlúcar de Barrameda (Spain)	22 (1/21)	70 % Ethanol	March-September 2011	*C. l. familiaris*
C. canis	Constantine (Algeria)	25 (6/19)	70 % Ethanol	June-November 2016	*C. l. familiaris*
P. irritans	Seville (Spain)	23 (10/13)	96 % Ethanol	May 2016	-
A. erinacei	Almonte (Spain)	16 (5/2)	96 % Ethanol	April 2018	*E. europaeus*
	Dos Hermanas (Spain)	2 (0/2)		Mars 2017	
*L. taschenbergi**	Conat (France)	27 (11/16)	70 % Ethanol	June-September 2016	*Apodemus* sp.
*N. fasciatus**	Corse (France)	23 (5/18)	70 % Ethanol	January-June 2016	*Rattus* sp.
*S. tripectinata**	Corse (France)	22 (1/21)	70 % Ethanol	January-June 2016	*Rattus* sp., *Mus* sp., *Apodemus* sp.

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