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Assessment of MALDI-TOF MS for Arthropod Identification Based on Exuviae Spectra Analysis

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Abstract

MALDI-TOF MS is an innovative tool for identifying hematophagous and non-hematophagous arthropods at various life stages. However, identification by MALDI-TOF MS currently requires euthanizing of the specimen, hindering further phenotypic tests. All arthropods have a common factor, molting of their exoskeletons leaving a remaining structure known as the exuviae. This phenomenon is indispensable for their growth and can evidence past arthropod presence. This study assessed the performance of MALDI-TOF MS biotyping for arthropod identification using exuviae from nine distinct laboratory-reared species (*Aedes aegypti*, *Anopheles coluzzii*, *Cimex lectularius*, *C. hemipterus*, *Pediculus humanus humanus*, *Triatoma infestans*, *Rhodnius prolixus*, *Supella longipalpa* and *Blattella germanica*) compared its efficiency with a molecular identification approach using DNA sequencing. Molecular analysis showed low DNA quantity in exuviae ($n=108$) across species, resulting in low success of COI, 16s, and 18s amplification (50.0%), depending on the species and sequencing (10.2%). The establishment of an exuviae protocol for MS submission yielded spectra of high reproducibility and specificity per species. After upgrading a homemade reference MS database with exuviae spectra, a query with remaining spectra revealed that 100% of samples were correctly identified, with 85.8% (278/324) exceeding the threshold score value for reliable identification. MALDI-TOF MS showed high efficiency in identifying various arthropod species based on their exuviae. This approach is a groundbreaking development in the field of entomology underlining that MALDI-TOF outperformed traditional methods of exuviae identification, including morphological and molecular tools. It also prevents specimen sacrifice which could be used for complementary analyses.

Keywords Exuviae, Arthropods, Species identification, MALDI-TOF MS, Biotyping

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Introduction

Arthropods are the most diverse and populous phylum in the animal kingdom, representing about 79% of the animal species [1–3]. They encompass numerous groups, such insects, which have the largest number of orders, the arachnids including ticks and scorpions, or the crustaceans comprising shrimps, crabs and lobster, as well as myriapods [4]. Insects stand out as the most dominant group, accounting for over 80% of arthropod species, followed by mites, such as ticks [5].

Some of the arthropods are hematophagous, and a fraction of them act as vectors for the transmission of pathogenic agents responsible for human and animal diseases [6]. Mosquitoes are the primary vectors of infectious diseases, transmitting arboviral (e.g., dengue, chikungunya or zika viruses) [7], parasitic (e.g., plasmodia or filarioid helminths) [8] and potentially bacterial (e.g., *Rickettsia felis*) [9] pathogens. Ticks are considered the second most important order of pathogenic agent vectors (e.g., *Borrelia burgdorferi*, the agent of Lyme disease) [10]. Lice and triatomine bugs are other examples of human disease vectors transmitting bacterial agents (e.g., *Borrelia recurrentis*) and Chagas diseases (e.g., *Trypanosoma cruzi*), respectively [11, 12]. Other arthropods also require attention because they are considered pests such as bed bugs and cockroaches [13, 14].

The identification of arthropods to distinguish vectors from non-vectors remains the first step in the process of monitoring and controlling vector-borne diseases (VBDs). Currently, specimen identification is frequently done using morphological tools [15–17]. However, this method is time-consuming and depends on the availability of experienced entomologists and dichotomous keys [18]. As specimen identification is based on morphological criteria, damage on these delicate arthropods could hamper their taxonomic classification [19]. The decline of entomological expertise is another limiting factor [20]. To overcome the limitations of morphological identification, molecular identification methods have been widely developed for arthropod identification. These methods are widely recognized as accurate and reliable [21, 22] allowing precise identification even in the absence of distinctive morphological characteristics. Moreover, molecular biology techniques are independent of the specimen's developmental stage [23], making them particularly useful when specimens are partially degraded or immature. However, despite these advantages, certain limitations remain, particularly regarding the time required to obtain results and the high cost of reagents, which still represent significant obstacles to the widespread use of these molecular identification methods [24, 25].

Since the early 2010s, a rapid, accurate, and inexpensive proteomic approach has been successfully applied for arthropod identification [26, 27]. The principle of this proteomic approach is based on submitting a protein extract from arthropod samples to matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS). The resulting spectra are compared to a reference MS spectral database for specimen identification at the species level [18]. MALDI-TOF MS has proven effective for the identification of a wide range of arthropod families, such as mosquitoes [28], ticks [29], lice [30], fleas [31], sandflies [32, 33], bed bugs [34], and triatomines [35], using specific body parts according to the arthropod family and development stage [36, 37]. It is interesting to note that, unlike to genome, the protein repertoire of an arthropod changes according to the body part [37, 38]. Moreover, as arthropods are metamorphic organisms [39, 40], the protein repertoire can also change throughout their developmental cycle [41]. Therefore, establishing standardized protocols was essential for efficient arthropod identification at distinct developmental stage by MALDI-TOF MS [42].

Until now, few arthropod families have been analyzed by MALDI-TOF MS fot identification at different developmental stages, e.g., mosquitoes [43], ticks [44] and phlebotomine sandflies [33]. Mosquitoes are the only family that has been tested at pre-immature (eggs) [45, 46], immature (aquatic stages) [47] and imago (flying adult) [28] developmental stages. Unfortunately, for identification of the arthropod by MALDI-TOF MS, euthanasia of the specimen is necessary. In such conditions, complementary analysis on live specimens are not possible, such as assessing their insecticide susceptibility, vector competence or responses to environmental changes [48].

An alternative to sacrificing specimens is to identify them based on their exuviae [48]. Arthropods are characterized by a segmented body covered by a rigid cuticle forming the external skeleton [49], secreted by epidermal cells. The skeleton is primarily composed of two layers, the epicuticle, which includes a cement layer and a waxy layer, and the pro-cuticle, consisting of the exocuticle and endocuticle containing chitin embedded in a proteinaceous matrix [50]. During their life cycle, the arthropods molt, shedding their exoskeleton [4]. Metamorphosis is incomplete in heterometabolous species, where immature and adult stages resemble each other, differing mainly in size [51]. In holometabolous species, however, metamorphosis is complete, with marked morphological differences between immature and mature stages, as in mosquitoes. Growth occurs only during molting, when the rigid cuticle is renewed, allowing the arthropod to increase in size and/or transform. The discarded exoskeleton from the previous stage is known as the exuviae [52]. Recently, a proof concept demonstrated the application of MALDI-TOF MS for identifying two *Aedes* mosquito

species using exuviae [48]. Additionally, in the field, live arthropods can be highly mobile (e.g., flying insects) and can evade capture by hiding (e.g., bugs), whereas exuviae, which cannot escape collection, represent an interesting alternative.

The present study aimed to assess the feasibility of identifying several medically important arthropod species based on their exuviae using MALDI-TOF MS biotyping, as well as by conventional molecular biology tool. To establish this proof of concept, we tested exuviae from nine distinct laboratory-reared arthropod species, including two mosquito species (i.e., *Anopheles coluzzi* and *Aedes aegypti*), two bed bugs species (i.e., *Cimex hemipterus* and *Cimex lectularius*), one lice species (i.e., *Pediculus humanus humanus*), two species of triatomine bugs (i.e., *Triatoma infestant* and *Rhodnius prolixus*), and two cockroach species (i.e., *Blatella germanica* and *Supella longipalpa*).

For the analysis, the choice of the body part used depended on the size of the arthropods. For mosquitoes, given the small size of their pupal exuviae, the entire exuviae was used for analysis. For lice and bed bugs, exuviae from advanced developmental stages, starting from the third instar, were used to ensure sufficient protein content for analysis. In contrast, for the two larger species, namely triatomine bugs and cockroaches, two body compartments (legs and thorax + head) were evaluated to determine the most suitable for analysis. This process also involved assessing intra-species MS spectral variations based on the compartment analyzed, with the aim of identifying the most representative and suitable body part for this study.

For these experiments, exuviae from the pupal stage were used for both mosquito species, while third instar or later stages were selected for the other arthropod species. Whole exuviae were subjected to MS analysis, except for larger species like triatomine bugs and cockroaches, which produce large exuviae. For these larger species, the legs were selected for analysis. Additionally, intra-species MS spectral variations based on the body part analyzed were assessed.

Materials and methods

Ethical Considerations

No permits were required for the described study, because all arthropod specimens were laboratory reared (not collected in the field). Moreover, none of the species included in the present work were protected or considered as sensitive animals. Ethical approval for in vitro blood feeding of mosquitoes, triatomine bugs and bed bugs using human blood was obtained from the Laboratory Research Ethics Board for Molecular Hematology, of the French Blood Establishment (EFS). New Zealand white rabbits raised at IHU Méditerranée Infection and sourced from Charles River Laboratories were used to feed the lice. They were handled according to Decree No. 2013 – 118 of February 7, 2013, and in accordance with official experimental procedures (references APAFIS #42524-2023040414041603 v5). The methods were approved by the Ethics Committee "C2EA-14" of Aix-Marseille University, France, as well as by the French Ministry of National Education, Higher Education, and Research.

Arthropod Rearing and Exuviae Collection

Laboratory-reared specimens from nine arthropod species coming from eight genera and five families were selected. The exuviae collection was done immediately following arthropod molting. The modalities of management and storing for each arthropod family are indicated below. For each collection of exuviae, at least two counter species fresh specimens were collected, frozenly sedated and were used as controls of molecular and MALDI-TOF MS analyses.

Mosquitoes

Two mosquito species, *Anopheles coluzzi* and *Aedes aegypti* (Bora strain) were reared in the laboratory, as previously described [48]. The exuviae from pupal stage were collected daily and rinsed once with alcohol and twice with water prior to transferring them individually in an Eppendorf tube. The remaining water was removed and the exuviae were either immediately processed for molecular or MALDI-TOF MS analyses or frozen at – 20 °C until future analysis.

Lices

Adult *pediculus humanus humanus* lice were reared in a climatic chamber (temperature: 25 °C; relative humidity: 80–90%) and successive generations were obtained by feeding lice on rabbits, as previously described [30]. Exuviae from the third larval stages were collected twice a week. The exuviae were either immediately processed for molecular or MALDI-TOF MS analyses or frozen at – 20 °C until future analysis.

Triatomine bugs and bed bugs

Two triatomine begs species, *Triatoma infestans* and *Rhodnius prolixus*, and two bed bug species, *Cimex hemipterus* and *Cimex lectularius*, were laboratory reared in jars and boxes in incubators. The *Triatomine* species required a constant temperature of 26 °C, with a relative humidity of 70% and a day cycle of 12 h light/12 h dark [53]. The *Cimex* species were maintained at a temperature of 26 °C and constant humidity of 60% with a day cycle of 12 h light/12 h dark. Details of rearing for *Triatomine* and *Cimex* species are available in previous works [53, 54]. Exuviae were recovered twice a week by

simple sorting. The exuviae sizes recovered from the triatomines and bed bugs measured, respectively at least 7 and 3 millimeters in length, corresponding to the 4th or 5th developmental stages.

Cockroaches

Two cockroach species, *Blatella germanica* and *Supella longipalpa*, were raised in climatic chambers at 24 °C and fed once a week with fish food (Fit + Fun Teichsticks, Krefeld, Germany) and jelly water (Delical Gelodiet, Torcé, France). The exuviae were collected after metamorphosis through a simple sorting process twice a week and were immediately analyzed by molecular biology or MALDI-TOF or stored at − 20 °C until future analysis. The exuviae recovered from the cockroaches measured at least 10 mm in length corresponding to 3rd instar or above larval stage.

DNA Extraction and Quantitative Analysis

Five specimens ($n = 45$) and twelve whole exuviae ($n = 108$) per arthropod species ($n = 9$) were used for DNA extraction using a NucleoSpin® 96 Tissue genomic DNA kit following the manufacturer's instructions (MACHEREY-NAGEL, Düren, Germany). The compartment and stage selected for DNA extraction per species are indicated in Table 1. Extracted DNA was quantified with Qubit® 2.0 Fluorometer (ThermoFisher Scientific, Waltham, USA) using the Qubit dsDNA Assay Kits (Thermo Fisher Scientific, Waltham, USA). Samples with too low DNA concentrations to be detected by the fluorometer (values below 0.50 ng/mL) were recorded as zero values for data analysis.

Molecular Analyses of Exuviae

PCR amplification was carried out using primer pairs of the mitochondrial cytochrome oxidase subunit I (COI), the ribosomal 16–18 S subunit sequences according to the arthropod family targeted (see Table S1 for details). PCR amplification was performed in 45 µL Master Mix composed of 25 µL of Amplitaq gold TM 360 Master Mix, 1 µL per primer and 18 µL of sterile water. Five microliters of extracted DNA were added to this Master Mix. DNA amplification was conducted by standard PCR conditions (Table S1) using an Applied Biosystems™ Veriti™ Thermal Cycler, 96-Well (Applied Biosystems, ThermoFisher Scientific, Foster City, CA, USA). The DNA extracted from five fresh specimens per species was used as positive controls of molecular experiments (Table 1). For negative controls, DNA was replaced by sterile water (PCR-grade water). PCR products were verified by electrophoresis using 1.5% agarose gel stained with SYBER Safe™ DNA gel dye (Thermo Fisher Scientific, Waltham, MA, USA), and visualized with the CheliDoc™ MP ultraviolet imager (Bio-Rad, Marnes-la-Coquette, France). The presence of a band at expected size for each exuviae sample per species was used to determine proportion of PCR success. The positive samples from PCR underwent purification and sequencing according to the previous procedures [55]. Sequencing was performed with a Sanger Sequencing 3500 Series Genetic Analyzer (Applied Biosystems®; Foster City, CA, USA). The quality of the obtained sequences was assessed using Geneious Prime 2024.0.5 (Dotmatics, Boston, USA). A sequencing was considered a success if a high-quality sequence was obtained according to geneious score (> 80%) (Dotmatics, Boston, USA). All obtained sequences were assembled and corrected on Geneious Prime 2024.0.5 (Technelysium Pty Ltd., Tewantin, Australia) and then were blasted against GenBank using the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/blast/Blast.cgi). Blast sequence query was considered as target sequence success if correct species identification was obtained with

Table 1 Comparison of molecular analysis effectiveness on exuviae samples per arthropod species

Species	*Fresh specimens* ($n = 5$)		*Exuviae* ($n = 12$)					
	Stage	Body part[a]	Stage	Body part[a]	Number of PCR success (%)[b]	Number of sequencing success (%)[b]	Number of target sequence success (%)[b]	Best Match Accession Number (NCBi)
P. humanus corporis	Adults	Whole	3th instars	Whole	0 (-)	/	/	/
Ae. aegypti	Pupae	Whole	Pupae	Whole	0 (-)	/	/	/
An. coluzzii	Pupae	Whole	Pupae	Whole	5 (41.6%)	3 (25.0%)	3 (25%)	MT375223.1
B. germanica	Adults	One leg	≥ 3th instars	Whole	7 (58.3%)	3 (25.0%)	3 (25%)	MT467295.1
S. longipalpa	Adults	One leg	≥ 3th instars	Whole	2 (16.6%)	1 (8.3%)	1 (8.3%)	KU543635.1
Cx. lectularius	Adults	Half[c]	4-5th instars	Whole	12 (100%)	3 (25.0%)	1 (8.3%)	MN088697.1
Cx. hemipterus	Adults	Half[c]	4-5th instars	Whole	12 (100%)	2 (16.7%)	0 (-)	/
T. infestans	Adults	One leg	4-5th instars	Whole	9 (75.0%)	4 (33.3%)	3 (25.0%)	NC_035547.1
Rh. prolixus	Adults	One leg	4-5th instars	Whole	7 (58.3%)	0 (-)	/	/
Total,n (%)[d]	**45**		**108**		**54 (50.0%)**	**16 (14.8%)**	**11 (10,2%)**	

[a]Body part used for DNA extraction. [b]Among the 12 exuviae per species. [c]The specimens were cut longitudinally, half was used for DNA extraction and the remaining part was stored at -20 °C. [d]The proportion among the total number tested, $n = 108$

proportions of identity and coverage, both upper than 90%.

Optimization of Sample Preparation Conditions for MALDI-TOF MS Analysis

To ensure the reproducibility of MS spectra within a species and specificity between species, several parameters were tested such as the exuviae compartment (whole or a specific part), the volume of homogenization buffer added and the duration of sample homogenization. The selection of the exuviae compartment was based on the size of the exuvia for each species (see Table S2). Each exuviae sample was individually placed in 1,5 mL Eppendorf tubes and mixed with homogenization buffer composed of a 50/50 (v/v) mix of formic acid (70%v/v) (Sigma, St. Louis, MO, USA) plus acetonitrile (50% v/v) (Fluka, Buchs, Switzerland). An adjustment of homogenization buffer volume was established for each kind of sample. The exuviae were homogenized using Tissue Lyser (Qiagen, Hilden, Germany) with a pinch of glass beads (Sigma, Lyon, France) as disruptor. The parameters for sample homogenization were a frequency of 30 Hz per cycle of 180 s. The number of cycles was also adjusted for each kind of sample. After homogenization, a quick spin (200 x g for 30 s) was performed and 1 µL of supernatant was deposited in quadruplicate spots onto the MALDI-TOF MS target plate (Bruker, Billerica, MA, USA). After drying, each spot was overlaid with 1 µL of CHCA matrix solution, composed of saturated α-cyano-4-hydroxycinnamic acid, 50% acetonitrile (v/v), 2.5% trifluoroacetic acid (v/v) and HPLC-grade water [21]. The thorax plus the head of a fresh *Ae. aegypti* specimens were homogenized together, while the legs were analyzed separately under the same conditions and used as quality control of sample preparation and MS spectra acquisition. Matrix solution was loaded in duplicate onto each MALDI-TOF target plate as negative controls.

MALDI-TOF MS Parameters

Protein mass spectra were obtained using a MicroFlex LT MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA). The laser frequency was set at 50 Hz within a mass range of 2–20 kDa, in a linear positive ion mode detection [56, 57]. Each spectrum represented ions generated from 240 laser shots performed in six regions of the same spot and acquired automatically using the AutoXecute method in FlexControl v2.4 (Bruker Daltonics, Billerica, MA, USA).

Analysis of MS Spectra

Flex Analysis v3.3 (Bruker Daltonics, Billerica, MA, USA) was used to visualize and to compare spectra, which were then exported to ClinProTools v2.2 and MALDI-Biotyper v3.0 (Bruker Daltonics, Billerica, MA, USA) for data processing including smoothing, baseline subtraction, peak selection and evaluation of intra-species reproducibility and inter-species specificity [58, 59]. The reproducibility and specificity of the exuviae MS spectra were examined using unsupervised statistical analyses of average spectral profiles (MSP, Main Spectrum Profile) obtained from the four spots for each sample, like cluster analyses (MSP dendrogram) using MALDI-Biotyper v3.0, or using supervised statistical analyses assessing the distribution of exuviae MS spectra from different compartments (legs and thorax + head) of triatomine and cockroach species, using Principal Component Analysis (PCA) from ClinProTools v2.2 with default settings. Cluster analyses (MSP dendrogram) tool from MALDI-Biotyper v3.0 was also used, to assess the spectral variations within and between each body part per triatomine and cockroach species, as previously described [38, 60]. CCI matrix was calculated using MALDI-Biotyper v3.0 with default settings (mass range 3.0–12.0 kDa; resolution 4; 8 intervals; auto-correction off). Higher correlation values (expressed by mean ± standard deviation – SD) reflecting higher reproducibility for the MS spectra, were used to estimate MS spectra distance between body parts per species.

Reference Database Creation

After checking intra-species reproducibility and inter-species specificity of exuviae MS profiles, two exuviae spectra per species were added to the homemade reference database. The reference MS spectra were created with an unbiased algorithm using information on the peak position, intensity and frequency using the MALDI-Biotyper v3.0 (Bruker Daltonics, Billerica, MA, USA) [61]. A total of 18 MSP from exuviae were included in the reference MS spectra DB (Table 2). The raw exuviae MS spectra added to the DB were deposited on the website of the University Hospital Institute (UHI) *Méditerranée infection* under the following web link (https://doi.org/10.35081/w84y-eg03), on September 2023.

Blind Tests and Experiment Validation

The MS spectra of the remaining exuviae were queried against our homemade arthropod reference spectra DB [43]. The reliability of exuviae species identification was estimated using the log score values (LSVs) obtained from the MALDI Biotyper v.3.0, which ranged from 0 to 3. LSVs greater than 1.8 were considered reliable for species identification, according to previous studies [57]. Data were analyzed with Prism software v.7.00 (GraphPad, San Di-ego, CA, USA).

Statistical Analysis

The comparison of DNA quantity or LSVs in each group (i.e., body parts) per species were computed using

Table 2 Exuviae submitted to MALDI-TOF MS analysis, including database creation, blind tests and results of correct and relevant identification for each species

Species	Number tested	Good spectra (%)	Added to DB	Blind tests	Correct identification (%)	LSVs range	Correct identification with LSVs ≥ 1.8 (%)
Ae. aegypti	98	90 (91.8%)	2	88	88 (100%)	1.62–2.57	81 (92.0%)
An. colluzzi	18	18 (100%)	2	16	16 (100%)	1.99–2.45	16 (100%)
C. lectularius	51	39 (76.5%)	2	37	37 (100%)	1.87–2.44	37 (100%)
C. hemipterus	46	44 (95.6%)	2	42	42 (100%)	2.05–2.50	42 (100%)
P. humanus corporis	45	32 (71.1%)	2	30	30 (100%)	1.65–2.56	28 (96.7%)
Tr. infestans	18	18 (100%)	2	16	16 (100%)	1.99–2.53	16 (100%)
Rh. prolixus	18	18 (100%)	2	16	16 (100%)	1.47–2.49	15 (93.8%)
B. germanica	23	23 (100%)	2	21	21 (100%)	1.77–2.64	20 (95.2%)
S. longipalpa	25	25 (100%)	2	23	23 (100%)	2.14–2.47	23 (100%)
Total, n (%)[a]	**342**	**307**	**18**	**289**	**289 (89.2%)**		**278 (85.8%)**

[a]The proportion among the total number submitted to MS after deduction of those included in the DB, $n = 324$ (i.e., $n = 342 - 18$). DB, database; LSVs, log score values

Mann–Whitney or Wilcoxon matched-pairs signed-rank tests when appropriate with GraphPad Prism 7.0.0 (GraphPad, San Diego, CA, USA). All differences were considered significant at $p < 0.05$.

Results

Collection of Exuviae from Various Arthropod Species

Exuviae of species selected for inclusion in this study, were collected exclusively from colonies reared within controlled laboratory environments. One exuviae per species was photographed (Fig. 1). The absence of dichotomy keys for exuviae did not allow the validation of their identity based on morphological analysis. Exuviae from mosquitoes, bed bugs and lice are three to ten-fold smaller than those from triatomines and cockroaches. To limit DNA and protein degradation, the exuviae were promptly frozen at -20 °C after collection.

Submission of Arthropod Exuviae to Molecular Biology Analyses

As all the exuviae used for molecular analyses come from laboratory-reared colonies, their identity at the species level was known. The effectiveness of molecular biology in identifying arthropods from exuviae has been assessed. Genomic DNA was extracted from the exuviae of the nine species ($n = 108$, 12 exuviae per species) and from paired fresh specimens ($n = 45$, five per species). DNA was quantified using Qubit dsDNA Assay Kits. As expected, the quantities of DNA extracted from fresh specimens (mean ± standard deviation (SD): 10.81 ± 10.61 ng/mL) were significantly higher (Mann–Whitney test, $p < 0.0001$) than those from the exuviae (mean ± SD: 0.22 ± 1.53 ng/mL, Fig. 2). To control whether the DNA extraction from exuviae was sufficient for molecular identification, the amplification of *COI* genes for mosquitoes, lice, and cockroaches, *16 S* for triatomines, and *18 S* for bed bugs, was successful for 100% ($n = 45$) of the fresh samples. In contrast, PCR success was obtained for 50% ($n = 54/108$) of the exuviae tested (Table 1). No PCR product was detected for exuviae from *P. humanus humanus* and *Ae. aegypti*. Among the 54 exuviae samples with PCR success, high-quality sequences were obtained for 16 of them (Table 1). Among the 16 exuviae samples with sequencing success, 11 matched the target taxonomic groups, representing only 10,2% ($n = 11/108$) of the exuviae samples submitted to molecular identification. Conversely, the rate of sequencing success and target sequencing success were both 100% for the DNA extracted from one fresh specimen per species used as positive controls ($n = 9$).

MALDI-TOF MS Analyses

Optimization of Sample Preparation Conditions

As the size of the exuviae differed according to arthropod families, two strategies were used. For mosquitoes, bed bugs and lice, whole exuviae were analyzed by MALDI-TOF MS, whereas for triatomines and cockroaches, leg and thorax plus head exuviae were submitted independently to MS to select the best body part. With the exception of *Ae. aegypti* species, it is the first time that these exuviae have been submitted to MALDI-TOF MS [48]. It was then necessary to establish the most appropriate protocols to obtain the best protein profiles. The criteria used to determine the better sample preparation conditions were the reproducibility and intensity of resulting MS spectra. The parameters tested were the body parts for large samples, the volume of homogenization mix buffer and the conditions of homogenization. For triatomines and cockroaches, leg exuviae were selected for MS submission. The optimal volume of mix buffer was adjusted from 15µL to 40µL according to the size of the exuviae samples (Table S2). The homogenization time was set at two cycles of three minutes at a frequency of 30 Hz with the TissueLyser apparatus. These setting conditions were applied for future submission of exuviae samples to MS analysis.

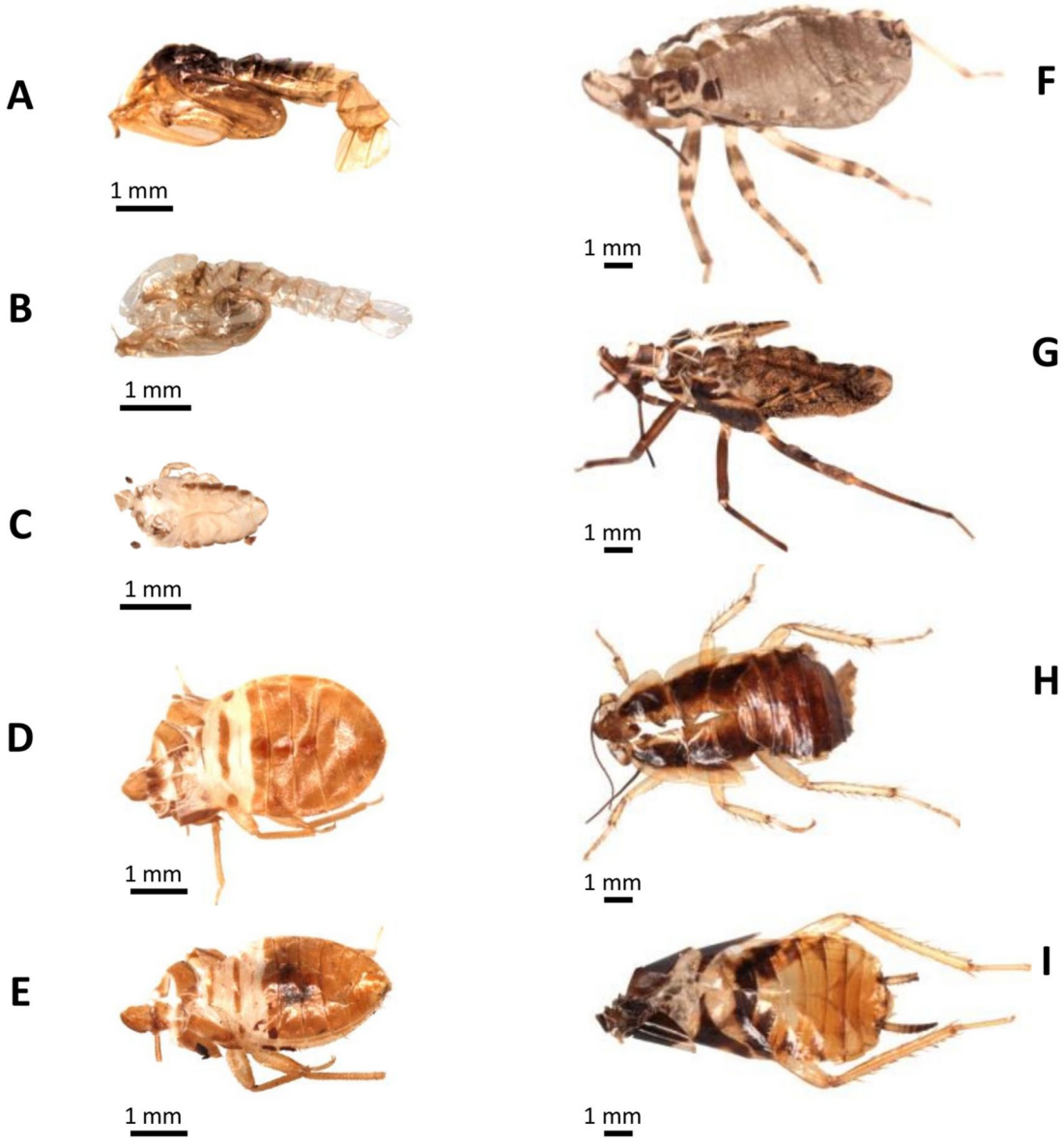

Fig. 1 Exuviae of various arthropod species tested. Exuviae of *Aedes aegypti* pupae (**A**), *Anophele coluzzi* pupae (**B**), *Cimex lectularius* larvae (**C**), *Cimex hemipterus* larvae (**D**), *pediculus humanus humanus* pupae (**E**), *Triatoma infestans* larvae (**F**), *Rhodnius prolixus* larvae (**G**), *Blatella germanica* larvae (**H**), *Supella longipalpa* larvae (**I**) photographed with DigitalCanon E05 7D supplied with a Canon MP-E 65 mm at magnification x5. To compare exuviae sizes according to species, a bar scale corresponding to 1 mm was included on each panel

Assessment of MS Spectra Reproducibility and Specificity According to Species

A total of 342 exuviae samples were subjected to MALDI-TOF MS analysis (Table 2). Among them, high-intensity MS spectra were obtained for 89.8% (307/342) of the samples tested. The 35 MS spectra which did not reach the inclusion criteria (Intensity > 3000 a.u., background lower than 15-fold of the more intense peak), were considered as unconfirmed and excluded from analysis (Table 2). A visual comparison of the MS profiles indicated an intra-species reproducibility and inter-species specificity, respectively, between exuviae from the same species and between different species (Fig. 3(i)). These results were confirmed by the comparison of the MS profiles from nine exuviae per species using ClinProTools gel view (Fig. 3(ii)). To evaluate the reproducibility and specificity of the MS spectra of exuviae, a clustering analysis was conducted. An MSP dendrogram was performed with two MS spectra of each species. The spectra of exuviae from the same species were clustered in the same branch of the MSP dendrogram, confirming the reproducibility and specificity of the protein profiles (Fig. 3(iii)). It is interesting to note that species from

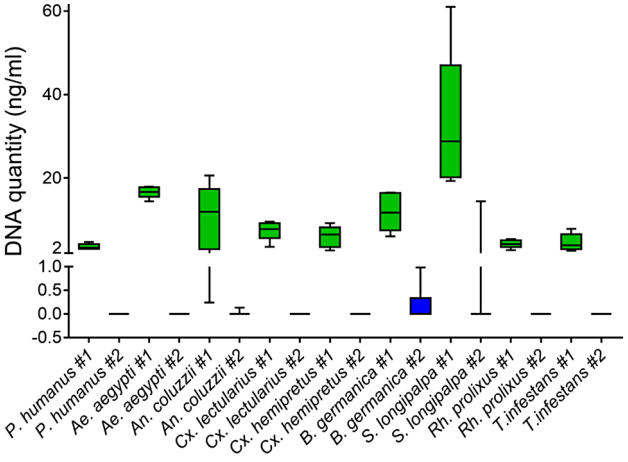

Fig. 2 Comparison of DNA quantity (ng/mL) extracted from exuviae and paired species specimens. Extractions were done on 5 fresh specimen (#1) and 12 exuviae (#2) samples per species. The results of exuviae and fresh specimens are indicated respectively by green (#1) and blue (#2) box indicating 25th and 75th percentiles and the whiskers the Min to Max. Black lines indicate the median. Sample names are indicated below the graph. The ordinate axis was split in two segments to visualize very low DNA quantity

the same family were grouped in the same part of the dendrogram.

Evaluation of MALDI-TOF MS for Arthropod Species Identification Based on Exuviae MS Spectra

After checking the reproducibility and specificity of the MS spectra, a database was created using MALDI-Biotyper 3.0 with two representative MS spectra of high intensity and quality for each species. MS spectra of each species, with the exception of those added to the database ($n = 18$), were queried against our updated reference MS database. Of the 289 MS spectra queried against the database, 100% of them were correctly identified (Table 2). The LSVs ranged from 1.62 to 2.64 (mean ± SD: 2.25 ± 0.19). For relevant arthropod identification at the species level, LSV should be higher than 1.8 [48]. Here, more than 89.8% (307/342) of exuviae that were correctly classified reached this threshold, underlining the reproducibility and specificity of these arthropod exoskeleton spectra. Among the eleven spectra that did not reach the threshold, they originated from exuviae of *Ae. Aegypti* (seven), *P. humanus humanus* (two), *Rh. Prolixus* (one) and *B. germanica* (one), respectively (Fig. 4).

Similarity of Exuviae MS Spectra from Distinct body Parts of the Same Species

For species producing large exuviae, such as triatomines or cockroach, two compartments were evaluated (i.e., legs and thorax + head), and the legs were selected for MS identification. However, since these samples are highly fragile, the loss of leg exuviae is possible, compromising their identification. As arthropod exoskeletons are composed essentially of chitin and associated proteins [62], we investigated whether MS spectra from exuviae of the same species were similar independently of the body part tested. To this end, we evaluated the reproducibility of the MS spectra between legs and thorax plus head per species from the two species of triatomines or cockroach.

The visual comparison of the paired MS profiles between legs and thorax plus head per species, including *B. germanica* ($n = 23$), *S. longipalpa* ($n = 25$), *Tr. Infestans* ($n = 8$) and *Rh. prolixus* ($n = 8$) using Flex analysis software indicated a similarity of MS profiles per species (Fig. 5(i)). An accurate comparison of spectra from these two body parts per species using PCA, revealed a clustering of the spectra per body part (Fig. 5(ii)), suggesting the presence of a specific signature in the MS profiles associated with the compartments for each of the four species. However, despite the presence of specific peaks distinguishing exuviae body parts from the same species, numerous peaks were shared between leg and thorax plus head spectra, as confirmed by the CCI analysis (Additional Figure S1). As expected, higher CCI values were obtained between spectra from the same body per species, ranging from 0.57 to 0.85. Nevertheless, the relatively high CCI values obtained between leg and thorax plus head of paired species highlighted the proximity of the spectra.

To evaluate the similarity of spectra per species between body parts, blind tests were performed. In this way, thorax plus head MS spectra of exuviae from these four species were queried against our homemade DB containing, among others, only reference MS spectra of exuviae legs from these four species (i.e., no reference spectra from exuviae of thorax + head). Although the legs LSVs were significantly higher (Wilcoxon matched-pairs signed-rank tests) than LSVs of thorax plus head for *B. germanica* ($p < 0.001$), *S. longipalpa* ($p < 0.001$), *T. infestans* ($p < 0.01$) and *Rh. prolixus* ($p < 0.01$), all the spectra (100%, $n = 120$) were correctly identified at the species level. Nevertheless, among the spectra of the thorax plus head exuviae, 20% (12/60) did not reach the threshold for relevant identification, with LSVs ranging from 1.43 to 2.39 (mean ± SD: 1.94 ± 0.19). Only one leg spectra did not reach the threshold (LSV = 1.77 from *B. germanica*). Among the 12 thoraxes plus head exuviae spectra that did not reach the threshold, nine originated from *S. longipalpa* (Fig. 5(iii)).

Discussion

Unfortunately, the use of MALDI-TOF MS for arthropod identification requires sacrificing the specimen under study. This constraint precludes carrying out additional analyses on live specimens, which could provide essential information on various aspects of their biology and behavior. For instance, it is currently not possible to test the susceptibility of arthropods to insecticides or

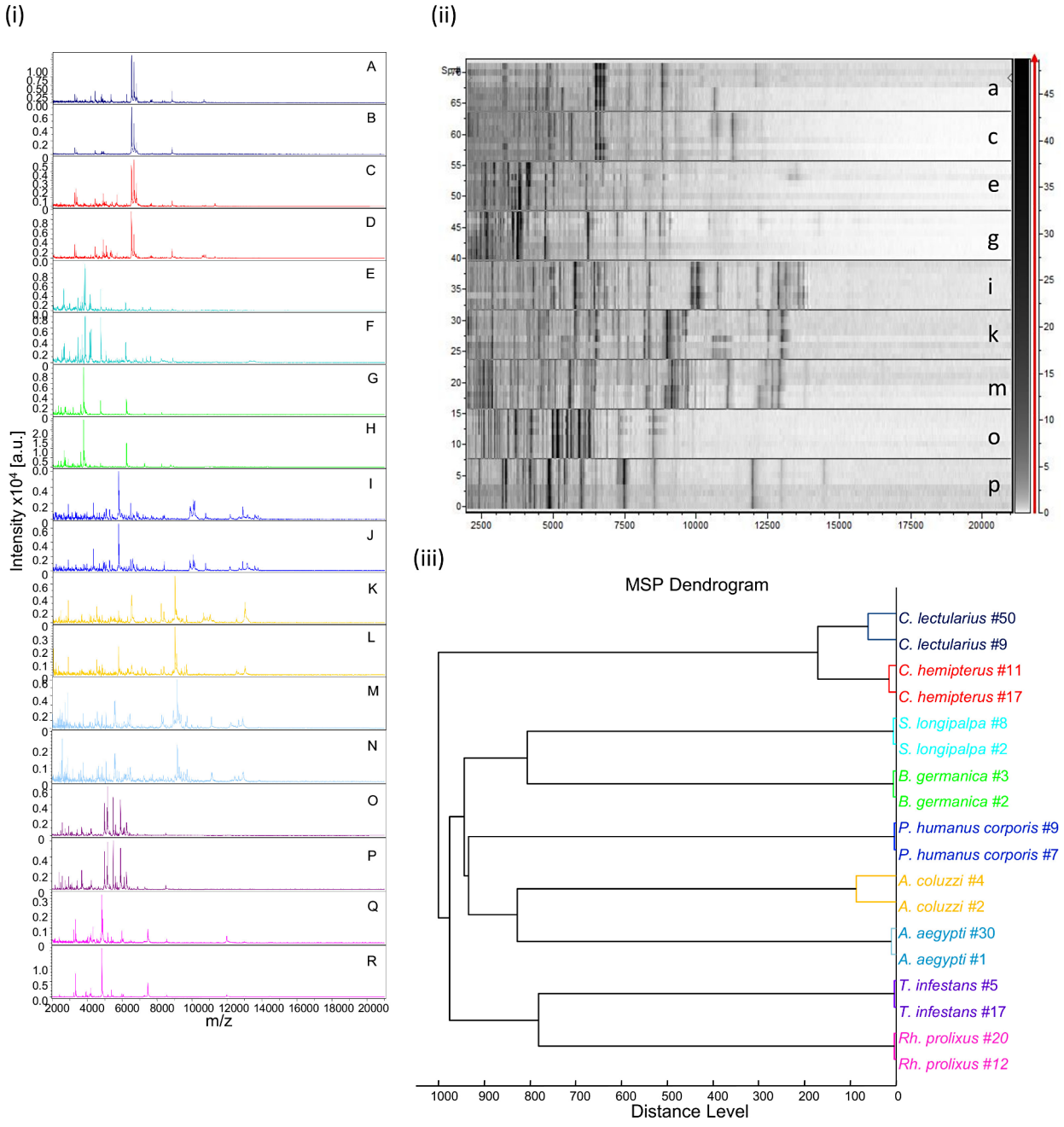

Fig. 3 Assessment of intra-species reproducibility and inter-species specificity of MS spectra of arthropod exuviae. (**i**) Representative of two exuviae MS spectra per species, including *C. lectularius* (A, B), *C. hemipterus* (C, D), *S. longipalpa* legs (E, F), *B. germanica* legs (G, H), *P. humanus humanus* (I, J), *An. coluzzi* (K, L), *Ae. aegypti* (M, N), *Tr. infestans* legs (O, P) *Rh. prolixus* legs (Q, R). (**ii**) Comparison of MALDI-TOF MS spectra from exuviae of each species using gel view of ClinProTools v.2.2. *C. lectularius* (a), *C. hemipterus* (c), *S. longipalpa* legs (e), *B. germanica* legs (g), *P. humanus humanus* (i), *An. coluzzi* (k), *Ae. aegypti* (m) *Tr. infestans* legs (o) *Rh. prolixus* legs (p). (**iii**) MSP dendrogram constructed with two representative exuviae spectra from the nine arthropod species. The dendrogram was created by Biotyper 3.0.

investigate their vector competence on specimens collected in the field while simultaneously subjecting them to MS identification [48]. The analysis of their exuviae, which occur during molting processes in these metamorphosing specimens, appears as another option.

Currently, the identification of arthropods based on the morphological analysis of exuviae remains scarcely applied and involves a meticulous examination of their structures [63]. The requirement of well-preserved fragile samples and experienced specialists to carry out relevant identification are factors that limit its broader use

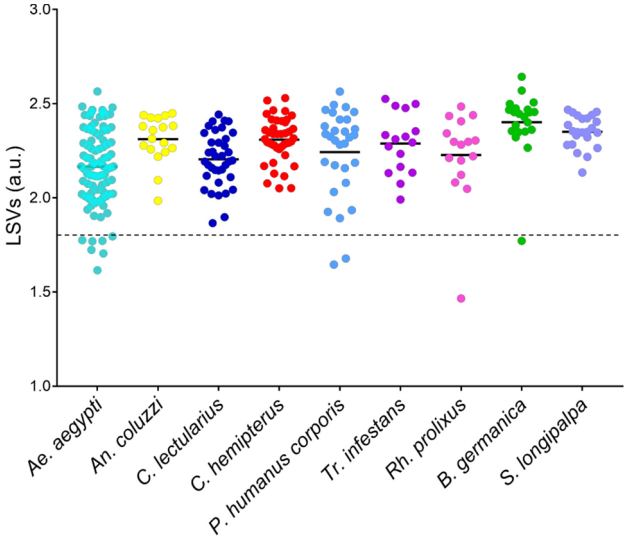

Fig. 4 LSVs of MS spectra exuviae query against homemade MS reference database. The exuviae of each species was indicated at the bottom of the graphic. The dashed line represents the threshold value (LSV ≥ 1.8), for relevant identification. a.u., arbitrary units; LSVs, log score values

[20]. Moreover, the availability of dichotomous keys and the rapid degradation of exuviae in the field, notably for species with aquatic stages [48], present additional challenges to its application [64]. Here, the failing to find morphological identification keys for exuviae of the nine species included confirmed the limitations of this approach [64, 65]. Effectively, arthropod identification based on the morphological analysis of their exoskeleton remains niche and restricted to a limited number of species, such as dragonflies [65], chironomid [67] or cicadinae [68, 69].

DNA-based identification methods offer a promising avenue to address the challenges associated with identifying sister species, variations in traits within the same species that cannot be morphologically distinguished, and samples that have undergone alterations during field collection [48]. Previous studies have shown that exuviae can be subjected to molecular biology techniques specimen identification [66, 70]. Nonetheless, the molecular identification of exuviae proved challenging. Here, solely half of the exuviae tested produced positive PCR products and less than 11% were successfully sequenced with correct target taxonomy. The low rate of sequencing success revealed that this approach is not a reliable method for identifying species from their exuviae. The very low quantity of DNA detected could explain the failure of amplification. The quantity of DNA extracted from exuviae was ten- to several hundred-fold lower than that in fresh specimens of the same species. These results corroborate previous work done on arthropod exuviae [70]. For instance, chironomid pupal exuviae, a bioindicator of water quality, collected in Norwegian lakes were submitted to molecular identification [71]. Although the proportion of positive COI amplification products overtook 82% in chironomid pupal exuviae, the rate of correct sequencing was below 20% [71]. In a recent study, the application of molecular biology for the identification of mosquito species from pupal exuviae succeeded in distinguish two *Aedes* sibling species (i.e., *Ae. coluzzii* from *Ae. detritus*) [72]. However, the correct sequencing success rate reached nearly 60%, which could be problematic for monitoring closely related species. The low amount of DNA in arthropod exuviae, which are mainly composed of non-cellular epicuticle and exocuticle, explains the high difficulty of their identification molecularly [72]. The failure of amplification could be attributed to the rapid degradation of the exuviae and the attached epithelial cells, which may also not be present in sufficient quantity for successful amplification [70]. It is also possible that the DNA extraction method used was not ideal, and an optimized one could improve the amplification success rate [71].

The use of mass spectrometry for the identification of arthropod exuviae will enable to overcome the limitations of morphological and molecular identification methods. Applying MALDI-TOF MS to a range of arthropod exuviae families was essential to validate its use as an innovative and alternative method for arthropod identification. Until now, only a limited number of arthropod families have been subjected to MALDI-TOF MS analysis for identification at various developmental stages [33, 43, 44]. For heterometabolous species, the same body part can be used for specimen identification by MS at various developmental stages [44]. In contrast, for holometabolous species, the complete metamorphosis occurring at one step of the life cycle, leads to morphological changes inducing evolution protein repertory of the specimen's tissues [1, 43]. These metamorphic species require the creation of specific reference MS spectra according to developmental stages and body parts [28, 73]. Interestingly, the analysis of immature aquatic stages of certain Culicidae using MALDI-TOF MS showed that species identification was more successful for late instar larvae than the pupal stage [47]. Effectively, during the pupal stage, mosquito metamorphosis occurred leading to important changes in protein composition during this step. These protein repertory changes altered the identification rate of these pupal stages in mosquito species collected in the field, hampering the application of this tool to monitor the last aquatic stage of mosquitoes [41]. In contrast, pupal exuviae, corresponding to the exoskeleton, are composed essentially of chitin and cuticle proteins, which remain stable within a species [74]. Until now, only one study has demonstrated the suitability of MALDI-TOF MS for the identification of two mosquito species, *Ae. albopictus* and *Ae. aegypti* using their exuviae

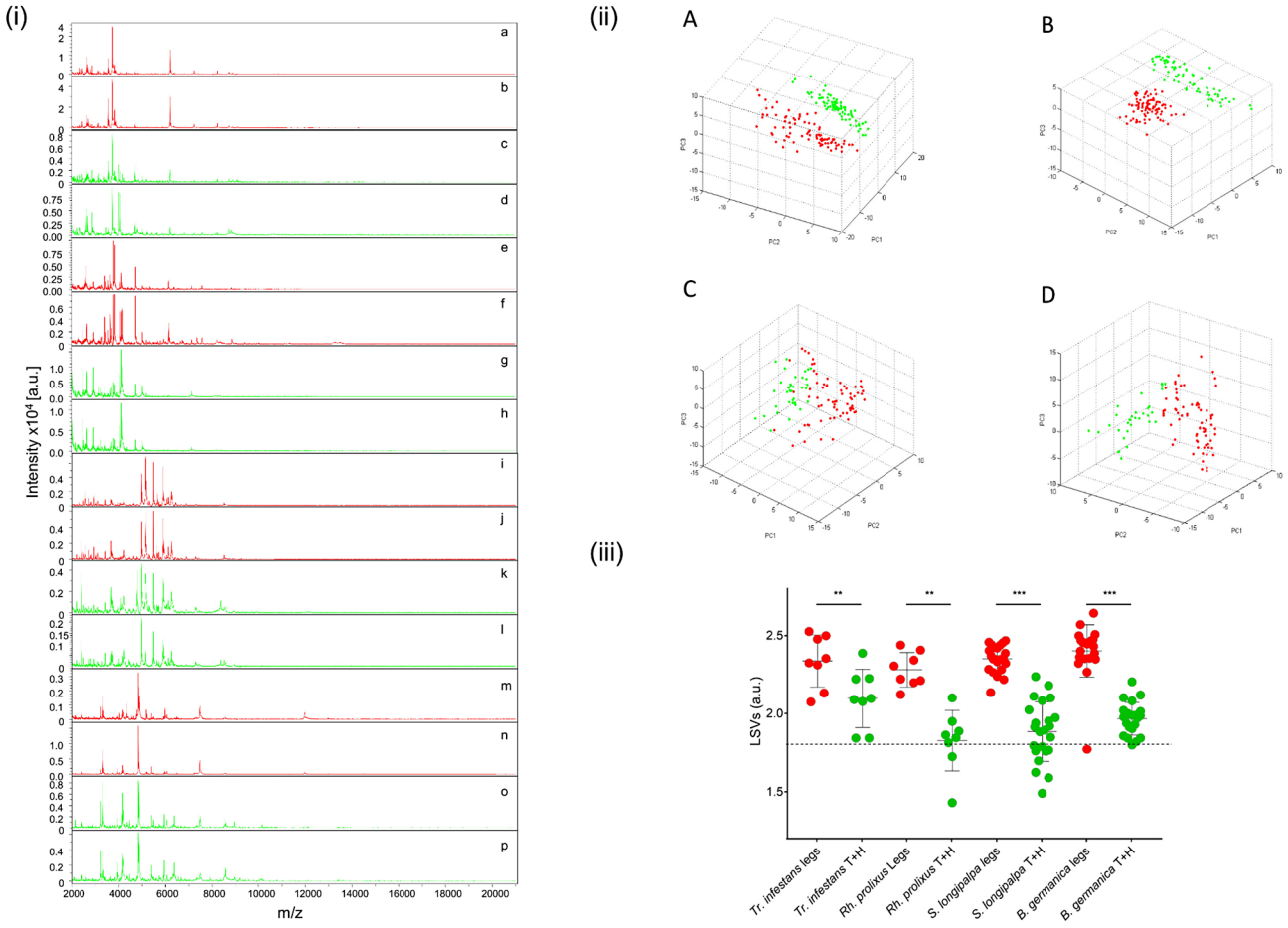

Fig. 5 Comparison of exuviae MS spectra from legs as well as those from the thorax + head of triatomines and cockroach. (**i**) Two exuviae MS spectra of legs (red) and thorax + head (green) from *B. germanica* (a-d), *S. longipalpa* (e-h), *Tr. infestans* (i-l) *Rh. prolixus* (m-p) are presented. (**ii**) Principal components analysis (PCA) dimensional image from exuviae MS spectra of *B. germanica* (A), *S. longipalpa* (B), *Tr. infestans* (C) *Rh. prolixus* (D) comparing legs (red) with those of the thorax + head (green) protein profiles. (**iii**) LSVs of exuviae leg and those from the thorax + head of the exuviae MS spectra query against homemade MS reference database. The database did not contain thorax + head exuviae MS reference spectra from triatomine or cockroach species. The samples queried were indicated at the bottom of the graphic. The dashed line represents the threshold value (LSV ≥ 1.8), for relevant identification. Comparisons of LSVs done using Wilcoxon matched-pairs signed-rank tests. a.u., arbitrary units; LSVs, log score values; ns, not significant; **, $p < 0.01$; ***, $p < 0.001$

from the fourth instar larval and pupal stages [48]. The protein signatures differed between the two species, as well as between larval and pupal stages within the same species, demonstrating the specificity of the MS spectra and the distinction of representative peaks of cuticular proteins compared to those obtained from specimens of respective stages and species. The profiles of the larval exuviae exhibited limited diversity, and their handling proved challenging due to their fragility [48]. Here, for species with aquatic immature stages (mosquitoes), it was opted to analyze uniquely pupal exuviae. MS submission revealed reproducible MS spectra for mosquito exuviae pupal stage per species (*Ae. aegypti* and *An. coluzzii*) confirming the applicability of this tool for their identification. In the future, the enlargement of other culicid species will be necessary to confirm its application.

The present study revealed that MALDI-TOF MS biotyping can be applied to identify a whole range of arthropod families, including mosquitoes, bed bugs, lice, triatomines and cockroaches, based on their exuviae. Although around 10% of the sample were excluded because their spectra were classified as non-compliant, the analysis of the remaining protein profiles from exuviae revealed inter-species specificity and intra-species reproducibility. The creation of an exuviae database enabled the correct identification of 100% of the samples, among with 85.8% (278/324) exceeding the threshold value (LSV > 1.8), ensuring reliable identification as previously established [58, 75]. As direct identification of the specimen can be challenging, the collection of exuviae could serve as a relevant alternative to detect arthropod infestation [76, 77]. The advantages of identifying arthropods from their exuviae are particularly pertinent

when the arthropod is in a state of active dispersal, either moving locally or attempting to escape [76]. The search of arthropod traces, such as exuviae, can be more easily found.

The large size of cockroach and triatomine exuviae led us to select their legs for MS identification. However, as exuviae can be damaged during their collection or store due to their extreme fragility, leading to the loss of the target body part, another body part was tested for sample identification without upgrading the reference MS database. The comparison of leg exuviae MS spectra and the combined thorax and head exuviae MS spectra within triatomine and cockroach species revealed intra-species specificity between the compartments, even though numerous peaks were shared among compartments. Collectively, these results revealed a sharing of exuviae MS spectra between body parts per species. However, despite the reduction in LSVs, which could compromise the acquisition of relevant identification scores for some samples, correct species identification was achieved. Nonetheless, the lower LSVs of exivuae from the thorax plus head compared to legs do not rule out the risk of misidentification that could occur during this cross-body part query. In contrast to fresh specimens, for which highly specific MS spectra were obtained for each body part, as with tick [44] or mosquito [38] specimens, thorax plus head profiles from triatomine and cockroach exuviae remained sufficiently similar to those of their respective legs to allow correct species identification. As the profiles are not radically different, in the case of damaged samples, an exchange of the body part tested could be done. The results obtained show that, despite some specificity between compartments of the exuviae, they remain similar enough to allow correct species identification. This consistency is explained by the composition of the exuviae which remains closely related regardless of the body part [50, 78].

Conclusion

Identifying arthropods from their exuviae using MALDI-TOF mass spectrometry is an innovative and promising approach for entomological research, as well as for monitoring and managing arthropod disease vectors and pests. This study demonstrates that MALDI-TOF biotyping overcomes the limitations of morphological identification of exuviae, which is often infeasible due to the unavailability of appropriate identification keys for numerous arthropod families. Additionally, molecular biology faces limitations due to the low quantity of DNA extractable from exuviae. This study represents the first attempt to establish a broader and diverse reference database for identifying arthropods from their exuviae. Furthermore, assessing this tool on exuviae collected in the field, considering the risk of MS profile alteration due to protein degradation appears essential to determine whether it could become a powerful technique and a promising method for the reliable identification of arthropod exuviae.

Abbreviations

CCI	Composite Correlation Index
CHCA	Cyano, Hydroxy, Cinnamic, Acid (α-cyano-4-hydroxycinnamic acid)
COI	Cytochrome oxidase subunit I
DB	Database
DNA	Deoxyribonucleic Acid
HPLC	High-Performance Liquid Chromatography
LSV	log score values
MALDI-TOF MS	Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12575-024-00260-3.

Supplementary Material 1: Primer pairs used for species identification according to arthropod family [79, 80].

Supplementary Material 2: Comparison of MALDI-TOF MS spectra from leg and thorax+head exuviae of the two Triatomines and two Cockroach species. Composite Correlation Index (CCI) matrix value representing the levels of body part MS spectra reproducibility among specimens of the same species and between species (Triatoma infestans, Rhodnius prolixus, Supella longipalpa, and Blatella germanica). Five specimens per species were tested per body part. The levels of MS spectra reproducibility are indicated in red and blue, revealing relatedness and incongruence between spectra, respectively. The values correspond to the mean coefficient correlation and respective standard deviations obtained for paired condition comparisons. CCI was calculated with MALDI-Biotyper v.3.0. T+H, Thorax plus head.

Supplementary Material 3: Parameters used for sample preparation.

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Author Contributions

Conceived and designed the experiments: LA. Performed the experiments: RB, RA, LA. Analyzed the data: RB, AZD, LA. Contributed reagents/materials/analysis tools: RB, AZD, RA, JMB, AB, PP. Investigation: RB, AZD, JMB. Drafted the paper: RB, LA. Revised critically the paper: all the authors.

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Data Availability

The MS reference spectra included in the database of this study are freely accessible and can be downloaded via the provided DOI (https://doi.org/10.35081/w84y-eg03).

Declarations

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication
Not applicable.

Competing Interests
The authors declare no competing interests.

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References

1. Zhang ZQ. Animal biodiversity: an outline of higher-level classification and survey of taxonomic richness (Addenda 2013). Zootaxa. 2013;3703:1–82.
2. Giribet G, Edgecombe GD. The phylogeny and evolutionary history of Arthropods. Curr Biol CB. 2019;29(12):R592–602.
3. Giribet G, Ribera C. A review of Arthropod Phylogeny: New Data based on ribosomal DNA sequences and direct character optimization. Cladistics. 2000;16(2):204–31.
4. Nakagawa Y, Henrich VC. Arthropod nuclear receptors and their role in molting: Arthropod nuclear receptors. FEBS J. 2009;276(21):6128–57.
5. Stork NE. How many species of insects and other Terrestrial arthropods are there on Earth? Annu Rev Entomol. 2018;63:31–45.
6. Fontaine A, Diouf I, Bakkali N, Missé D, Pagès F, Fusai T, et al. Implication of haematophagous arthropod salivary proteins in host-vector interactions. Parasit Vectors. 2011;4(1):187.
7. Zeller H, Marrama L, Sudre B, Bortel WV, Warns-Petit E. Mosquito-borne disease surveillance by the European Centre for Disease Prevention and Control. Clin Microbiol Infect. 2013;19(8):693–8.
8. Rydzanicz K, Golab E, Rozej-Bielicka W, Masny A. Screening of mosquitoes for filarioid helminths in urban areas in south western Poland-common patterns in European Setaria tundra xenomonitoring studies. Parasitol Res. 2019;118(1):127–38.
9. Dieme C, Bechah Y, Socolovschi C, Audoly G, Berenger JM, Faye O, et al. Transmission potential of *Rickettsia felis* infection by *Anopheles gambiae* mosquitoes. Proc Natl Acad Sci. 2015;112(26):8088–93.
10. Sertour N, Cotté V, Garnier M, Malandrin L, Ferquel E, Choumet V. Infection kinetics and tropism of Borrelia burgdorferi Sensu Lato in Mouse after Natural (via Ticks) or Artificial (needle) infection depends on the bacterial strain. Front Microbiol. 2018;9:1722.
11. Zumla A, Ustianowski A. Tropical diseases: definition, geographic distribution, transmission, and classification. Infect Dis Clin North Am. 2012;26(2):195–205.
12. Vieira CB, Praça YR, Bentes KL, da Santiago S, Silva PB, Silva SMM. Triatomines: trypanosomatids, Bacteria, and viruses potential vectors? Front Cell Infect Microbiol. 2018;8:405.
13. Parola P, Izri A, Bedbugs. N Engl J Med. 2020;382(23):2230–7.
14. Mehainaoui A, Menasria T, Benouagueni S, Benhadj M, Lalaoui R, Gacemi-Kirane D. Rapid screening and characterization of bacteria associated with hospital cockroaches (*Blattella germanica* L.) using MALDI-TOF mass spectrometry. J Appl Microbiol. 2021;130(3):960–70.
15. Rahola N, Günay F, Öztürk M, Alten B, Aqeehal HA, Saadawi WK, et al. Assessment of expertise in morphological identification of mosquito species (Diptera, Culicidae) using photomicrographs. Parasite Paris Fr. 2022;29:45.
16. Wijerathna T, Gunathilaka N. Morphological identification keys for adults of sand flies (Diptera: Psychodidae) in Sri Lanka. Parasit Vectors. 2020;13(1):450.
17. Erlank E, Koekemoer LL, Coetzee M. The importance of morphological identification of African anopheline mosquitoes (Diptera: Culicidae) for malaria control programmes. Malar J. 2018;17(1):43.
18. Sevestre J, Diarra AZ, Laroche M, Almeras L, Parola P. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: an emerging tool for studying the vectors of human infectious diseases. Future Microbiol. 2021;16(5):323–40.
19. Torres-Sangiao E, Leal Rodriguez C, García-Riestra C. Application and perspectives of MALDI-TOF Mass Spectrometry in Clinical Microbiology Laboratories. Microorganisms. 2021;9(7):1539.
20. Harrup LE, Bellis GA, Balenghien T, Garros C. Culicoides Latreille (Diptera: Ceratopogonidae) taxonomy: current challenges and future directions. Infect Genet Evol J Mol Epidemiol Evol Genet Infect Dis. 2015;30:249–66.
21. Yssouf A, Almeras L, Raoult D, Parola P. Emerging tools for identification of arthropod vectors. Future Microbiol. 2016;11(4):549–66.
22. Hebert PDN, Cywinska A, Ball SL, deWaard JR. Biological identifications through DNA barcodes. Proc Biol Sci. 2003;270(1512):313–21.
23. Zajac BK, Martin-Vega D, Feddern N, Fremdt H, Castro E, Szpila CP. Molecular identification and phylogenetic analysis of the forensically important family Piophilidae (Diptera) from different European locations. Forensic Sci Int. 2016;259:77–84.
24. Kaur R, Singh D, Molecular markers a valuable tool for, species identification of insects. Rev. 2020;38:1–20.
25. Lamoril J, Bogard M, Ameziane N, Deybach JC, Bouizegarène P. Biologie moléculaire et microbiologie clinique en 2007. Immuno-Anal Biol Spéc. 2007;22(1):5–18.
26. Müller P, Pflüger V, Wittwer M, Ziegler D, Chandre F, Simard F et al. Identification of Cryptic Anopheles Mosquito Species by Molecular Protein Profiling. Moreira LA, editor. PLoS ONE. 2013;8(2):e57486.
27. Yssouf A, Flaudrops C, Drali R, Kernif T, Socolovschi C, Berenger JM, et al. Matrix-assisted laser desorption ionization–time of Flight Mass Spectrometry for Rapid Identification of Tick vectors. J Clin Microbiol. 2013;51(2):522–8.
28. Mewara A, Sharma M, Kaura T, Zaman K, Yadav R, Sehgal R. Rapid identification of medically important mosquitoes by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Parasit Vectors. 2018;11(1):281.
29. Karger A, Kampen H, Bettin B, Dautel H, Ziller M, Hoffmann B, et al. Species determination and characterization of developmental stages of ticks by whole-animal matrix-assisted laser desorption/ionization mass spectrometry. Ticks Tick-Borne Dis. 2012;3(2):78–89.
30. Benyahia H, Ouarti B, Diarra AZ, Boucheikhchoukh M, Meguini MN, Behidji M et al. Identification of Lice Stored in Alcohol Using MALDI-TOF MS. Severson D, editor. J Med Entomol. 2021;58(3):1126–33.
31. Zurita A, Djeghar R, Callejón R, Cutillas C, Parola P, Laroche M. 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. Med Vet Entomol. 2019;33(2):185–94.
32. Chavy A, Nabet C, Normand AC, Kocher A, Ginouves M, Prévot G et al. Identification of French Guiana sand flies using MALDI-TOF mass spectrometry with a new mass spectra library. Al-Salem WS, editor. PLoS Negl Trop Dis. 2019;13(2):e0007031.
33. Halada P, Hlavackova K, Dvorak V, Volf P. Identification of immature stages of phlebotomine sand flies using MALDI-TOF MS and mapping of mass spectra during sand fly life cycle. Insect Biochem Mol Biol. 2018;93:47–56.
34. Ngnindji-Youdje Y, Lontsi-Demano M, Diarra AZ, Makaila AM, Tchuinkam T, Berenger JM, et al. Morphological, molecular, and MALDI-TOF MS identification of bed bugs and associated Wolbachia species from Cameroon. Acta Trop. 2024;249:107086.
35. Dos Santos Souza É, Fernandes RP, Galvão C, de Paiva VF, da Rosa JA. Distinguishing two species of Cavernicola (Hemiptera, Reduviidae, Triatominae) with matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Acta Trop. 2019;198:105071.
36. Briolant S, Costa MM, Nguyen C, Dusfour I, Pommier De Santi V, Girod R et al. Identification of French Guiana anopheline mosquitoes by MALDI-TOF MS profiling using protein signatures from two body parts. Banoub J, editor. PLOS ONE. 2020;15(8):e0234098.
37. Nabet C, Kone AK, Dia AK, Sylla M, Gautier M, Yattara M, et al. New assessment of Anopheles vector species identification using MALDI-TOF MS. Malar J. 2021;20(1):33.
38. Vega-Rúa A, Pagès N, Fontaine A, Nuccio C, Hery L, Goindin D, et al. Improvement of mosquito identification by MALDI-TOF MS biotyping using protein signatures from two body parts. Parasit Vectors. 2018;11(1):574.
39. Truman JW. The evolution of insect metamorphosis. Curr Biol CB. 2019;29(23):R1252–68.
40. Belles X. Origin and Evolution of Insect Metamorphosis. In: Encyclopedia of Life Sciences [Internet]. 1st ed. Wiley; 2011 [cited 2024 May 22]. Available from: https://onlinelibrary.wiley.com/doi/https://doi.org/10.1002/9780470015902.a0022854
41. Nebbak A, Koumare S, Willcox AC, Berenger JM, Raoult D, Almeras L, et al. Field application of MALDI-TOF MS on mosquito larvae identification. Parasitology. 2018;145(5):677–87.
42. Bamou R, Costa MM, Diarra AZ, Martins AJ, Parola P, Almeras L. Enhanced procedures for mosquito identification by MALDI-TOF MS. Parasit Vectors. 2022;15(1):240.
43. Nebbak A, Willcox AC, Bitam I, Raoult D, Parola P, Almeras L. Standardization of sample homogenization for mosquito identification using an innovative proteomic tool based on protein profiling. Proteomics. 2016;16(24):3148–60.
44. Boyer PH, Almeras L, Plantard O, Grillon A, Talagrand-Reboul É, McCoy K, et al. Identification of closely related Ixodes species by protein profiling

with MALDI-TOF mass spectrometry. Munderloh UG. Editor PLOS ONE. 2019;14(10):e0223735.
45. Suter T, Flacio E, Fariña BF, Engeler L, Tonolla M, Müller P. First report of the invasive mosquito species Aedes Koreicus in the swiss-italian border region. Parasit Vectors. 2015;8(1):402.
46. Schaffner F, Kaufmann C, Pflüger V, Mathis A. Rapid protein profiling facilitates surveillance of invasive mosquito species. Parasit Vectors. 2014;7(1):142.
47. Dieme C, Yssouf A, Vega-Rúa A, Berenger JM, Failloux AB, Raoult D et al. Accurate identification of Culicidae at aquatic developmental stages by MALDI-TOF MS profiling. Parasit. Vectors. 2014; 2(7):544.
48. Nebbak A, Almeras L. Identification of aedes mosquitos by MALDI TOF MS biotyping using protein signatures from larval and pupal exuviae. Parasites Vectors. 2020; 13(1):161.
49. Thomas GWC, Dohmen E, Hughes DST, Murali SC, Poelchau M, Glastad K, et al. Gene content evolution in the arthropods. Genome Biol. 2020;21(1):15.
50. Balabanidou V, Grigoraki L, Vontas J. Insect cuticle: a critical determinant of insecticide resistance. Curr Opin Insect Sci. 2018;27:68–74.
51. Haug JT, Haug C, Garwood RJ. Evolution of insect wings and development - new details from Palaeozoic nymphs. Biol Rev Camb Philos Soc. 2016;91(1):53–69.
52. Zong R, Gong Y. Allopatric molting of devonian trilobites. Sci Rep. 2022;12(1):13851.
53. Laroche M, Bérenger JM, Gazelle G, Blanchet D, Raoult D, Parola P. MALDI-TOF MS protein profiling for the rapid identification of Chagas disease triatomine vectors and application to the triatomine fauna of French Guiana. Parasitology. 2018;145(5):665–75.
54. Benkacimi L, Gazelle G, El Hamzaoui B, Bérenger JM, Parola P, Laroche M. MALDI-TOF MS identification of Cimex lectularius and Cimex hemipterus bedbugs. Infect Genet Evol [Internet]. 2020 Nov [cited 2022 Nov 29];85(104536). Available from: http://www.scopus.com/inward/record.url?scp=85091251712&partnerID=8YFLogxK
55. Tahir D, Socolovschi C, Marié JL, Ganay G, Berenger JM, Bompar JM, et al. New Rickettsia species in soft ticks Ornithodoros hasei collected from bats in French Guiana. Ticks Tick-Borne Dis. 2016;7(6):1089–96.
56. Fotso Fotso A, Mediannikov O, Diatta G, Almeras L, Flaudrops C, Parola P et al. MALDI-TOF mass spectrometry detection of pathogens in vectors: The Borrelia crocidurae/Ornithodoros sonrai Paradigm. Foley J, editor. PLoS Negl Trop Dis. 2014;8(7):e2984.
57. Kumsa B, Laroche M, Almeras L, Mediannikov O, Raoult D, Parola P. Morphological, molecular and MALDI-TOF mass spectrometry identification of ixodid tick species collected in Oromia, Ethiopia. Parasitol Res. 2016;115(11):4199–210.
58. Nebbak A, El Hamzaoui B, Berenger JM, Bitam I, Raoult D, Almeras L, et al. Comparative analysis of storage conditions and homogenization methods for tick and flea species for identification by MALDI-TOF MS. Med Vet Entomol. 2017;31(4):438–48.
59. Costa MM, Benoit N, Saby F, Pradines B, Granjeaud S, Almeras L. Optimization and standardization of Human Saliva Collection for MALDI-TOF MS. Diagn Basel Switz. 2021;11(8):1304.
60. Diarra AZ, Almeras L, Laroche M, Berenger JM, Koné AK, Bocoum Z et al. Molecular and MALDI-TOF identification of ticks and tick-associated bacteria in Mali. Coburn J, editor. PLoS Negl Trop Dis. 2017;11(7):e0005762.
61. Yssouf A, Parola P, Lindström A, Lilja T, L'Ambert G, Bondesson U, et al. Identification of European mosquito species by MALDI-TOF MS. Parasitol Res. 2014;113(6):2375–8.
62. Togawa T, Nakato H, Izumi S. Analysis of the chitin recognition mechanism of cuticle proteins from the soft cuticle of the silkworm. Bombyx mori. 2004;34(10):1059–67.
63. Nevill H, Venter GJ, Meiswinkel R, Nevill EM. Comparative descriptions of the pupae of five species of the Culicoides Imicola complex (Diptera, Ceratopogonidae) from South Africa. Onderstepoort J Vet Res. 2007;74(2):97–114.
64. Noh P, Kim WJ, Song JH, Park I, Choi G, Moon BC. Rapid and simple species identification of Cicada Exuviae using COI-Based SCAR assay. Insects. 2020;11(3):168.
65. Adambukulam SP, Kakkassery FK. Taxonomical studies of dragonfly nymphs (Odonata, Libellulidae) using their exuviae. | Entomon | EBSCOhost [Internet]. Vol. 48. 2023 [cited 2024 May 22]. p. 497. Available from: https://openurl.ebsco.com/contentitem/doi:10.33307%2Fentomon.v48i4.983?sid=ebsco:plink:crawler&id=ebsco:doi:10.33307%2Fentomon.v48i4.983
66. Sittenthaler M, Fischer I, Chovanec A, Koblmüller S, Macek O, Sattmann H, et al. DNA barcoding of exuviae for species identification of central European damselflies and dragonflies (Insecta: Odonata). J Insect Conserv. 2023;27(3):435–50.
67. Bouchard RW, Ferrington LC. The effects of subsampling and sampling frequency on the use of surface-floating pupal exuviae to measure Chironomidae (Diptera) communities in wadeable temperate streams. Environ Monit Assess. 2011;181(1–4):205–23.
68. Song JH, Kim WJ, Cha JM, Yang S, Choi G, Moon BC. Comparative morphological, ultrastructural, and Molecular studies of Four Cicadinae species using Exuvial Legs. Insects. 2019;10(7):199.
69. Lee HY, Oh SY, Jang Y. Morphometrics of the final instar exuviae of five cicada species occurring in urban areas of central Korea. J Asia-Pac Entomol. 2012;15(4):627–30.
70. Dhananjeyan KJ, Paramasivan R, Tewari SC, Rajendran R, Thenmozhi V, Leo SVJ, et al. Molecular identification of mosquito vectors using genomic DNA isolated from eggshells, larval and pupal exuvium. Trop Biomed. 2010;27(1):47–53.
71. Kranzfelder P, Ekrem T, Stur E. Mol Ecol Resour. 2016;16(1):353–63.
72. Berger A, Le Goff G, Boussès P, Rahola N, Ferré JB, Ayala D, et al. Using a pupal exuvia to designate the undamaged neotype of a species belonging to a complex of sibling species - the case of Aedes coluzzii (Diptera, Culicidae). Parasite Paris Fr. 2022;29:19.
73. Nebbak A, Willcox AC, Koumare S, Berenger JM, Raoult D, Parola P, et al. Longitudinal monitoring of environmental factors at Culicidae larval habitats in urban areas and their association with various mosquito species using an innovative strategy. Pest Manag Sci. 2019;75(4):923–34.
74. Magkrioti CK, Spyropoulos IC, Iconomidou VA, Willis JH, Hamodrakas SJ. cuticleDB: a relational database of Arthropod cuticular proteins. BMC Bioinformatics. 2004;5:138.
75. Yssouf A, Socolovschi C, Leulmi H, Kernif T, Bitam I, Audoly G, et al. Identification of flea species using MALDI-TOF/MS. Comp Immunol Microbiol Infect Dis. 2014;37(3):153–7.
76. Delaunay P, Blanc V, Del Giudice P, Levy-Bencheton A, Chosidow O, Marty P, et al. Bedbugs and infectious diseases. Clin Infect Dis off Publ Infect Dis Soc Am. 2011;52(2):200–10.
77. Gürtler RE, Cecere MC, Canale DM, Castañera MB, Chuit R, Cohen JE. Monitoring house reinfestation by vectors of Chagas disease: a comparative trial of detection methods during a four-year follow-up. Acta Trop. 1999;72(2):213–34.
78. Zhou Y, Badgett MJ, Bowen JH, Vannini L, Orlando R, Willis JH. Distribution of cuticular proteins in different structures of adult Anopheles gambiae. Insect Biochem Mol Biol. 2016;75:45–57.
79. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994;3(5):294–9.
80. Weirauch C, Munro JB. Molecular phylogeny of the assassin bugs (Hemiptera: Reduviidae), based on mitochondrial and nuclear ribosomal genes. Mol Phylogenet Evol. 2009;53(1):287–99.

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