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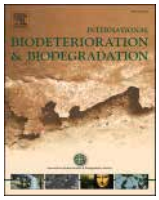

Assessment of MALDI-TOF MS for the identification of cultural heritage insect pests

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ABSTRACT

When heritage properties and contents are threatened by various harmful insects, especially coleopterans, rapid and reliable pest identification is required for effective management. While traditional methods have limitations, MALDI-TOF MS has recently emerged as a groundbreaking identification tool. In this study, our aim was to assess the effectiveness of MALDI-TOF MS in identifying cultural heritage insect pests belonging to the Coleoptera order. To do so, adult, larva, and exuviae from eight species of Coleoptera (*Lasioderma serricorne* ($n = 92$), *Attagenus smirnovi* ($n = 87$), *Trogoderma versicolor* ($n = 97$), *Reesa vespulae* ($n = 74$), *Anthrenus verbasci* ($n = 68$), *Anthrenus pimpinellae* ($n = 34$), *Anthrenus flavipes* ($n = 30$), and *Thylodrias contractus* ($n = 19$), were subjected to MALDI-TOF MS after optimisation of the sample preparation parameters. This optimisation was based on three key parameters influencing spectrum quality: the choice of body part, the buffer volume for protein extraction, and the method and duration of sample homogenisation. Reproducible intra-species MS spectra for each developmental stage were obtained. The species specificity of MS spectra for each developmental stage supported the use of MALDI- TOF MS for specimen classification. Spectra from 68 samples, including four specimens per species and stage, were added to our homemade reference arthropod MS database (DB). Among spectra classified as being consistent, 97.10% (335/345) of the samples were correctly classified with a relevant identification score. The morphological identification of all adult specimens in the reference MS spectra DB was confirmed by molecular biology, and the sequences were deposited in GenBank. This study is the first demonstration of the effective application of MALDI-TOF MS as a reliable tool for identifying various developmental stages of Coleoptera pests which pose a threat to the cultural heritage of France.

1. Introduction

Several insect pests threaten heritage properties and their contents. The Coleoptera order is one of them. This order possesses the largest number of known species, encompassing 176 families, 29 500 genera, and 386 500 species globally. (Zhi-Qiang, 2013).The Coleoptera order represents 25% of all described animal species (Cardé and Resh, 2009). Cigarette beetle (*L. serricorne*) belong to the Ptinidae family, and species of the Dermestidae family (*Attagenus* sp., *Anthrenus* sp., *Trogoderma* sp.), are serious economic pests of stored products, responsible for considerable damage to museum objects (Brimblecombe et al., 2024), libraries, archives, and historic buildings (Hagstrum et al., 2013; Querner, 2015). These two families can adopt two types of diets, xylophagous and polyphagous, and are the cause of significant economic losses (Crivellaro et al., 2022). To prevent the dissemination of insect pests and apply appropriate control measures, rapid and accurate identification of the specimen and its biology is essential (Querner, 2015).

Currently, cultural heritage pests are identified on the basis of on the

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observation of morphological characteristics using dichotomous keys (Ferret-Bouin, 1995; Háva, 2021). Although reliable, this technique has several limitations, including requiring an expert in entomology and the availability of appropriate identification keys (Zhou et al., 2022). Coleopterans can also be identified using molecular tools, by sequencing PCR products of specific genes such as mitochondrial cytochrome *c* oxidase I (CO1) (Amini et al., 2020; Hong et al., 2014), internal transcribed spacer 2 (ITS2) (Albo et al., 2019) or other nuclear ribosomal genes (*28S*, *18S*, *16S*) (Olson et al., 2014; Shull et al., 2001). A recent original study (Querner et al., 2024) demonstrated the potential to analyse dust collected in a museum environment by DNA metabarcoding to identify arthropod diversity, including pest species. This non-invasive approach offers the opportunity to identify pest species without detecting specimens. Despite the success of this approach, molecular identification remains a time-consuming and expensive method in terms of reagents and materials (Fang et al., 2002). The lack of a universal target sequence for the identification of all Coleoptera and/or the lack of respective sequences in molecular databases such as GenBank (https://www.ncbi.nlm.nih.gov/genbank/) or Barcode of Life Data System (BOLD, http://www.barcodinglife.org), are further factors hampering relevant specimen classification. Molecular tools do not, therefore, appear to be appropriate for routine specimen identification, particularly when there is a large number of samples to treat (Yssouf et al., 2016).

Over the course of the last decade, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) has emerged as an innovative approach for the identification of arthropods (Sevestre et al., 2021). The principle is based on the comparison of specific protein profiles originating from arthropod specimens submitted to MALDI-TOF MS, with reference MS spectra from a database. The spectra thus generated present a specific fingerprint signature of the sample tested (Yssouf et al., 2016). MALDI-TOF MS appears to be a robust tool which has demonstrated it efficiency for identifying species of many different arthropods, including ticks (Rothen et al., 2016; Yssouf et al., 2013), mosquitoes (Yssouf et al., 2014), fleas (Zurita et al., 2019), lice (Benyahia et al., 2021), and triatomines (Dos Santos Souza et al., 2020). This proteomic tool has also been successfully used to distinguish infected from non-infected arthropods (Laroche et al., 2017; Yssouf et al., 2015), as well as to identify the origin of host blood from engorged mosquitoes (Niare et al., 2018). Finally, MALDI-TOF MS has been demonstrated to be a rapid tool for the identification of arthropods and a relevant alternative to molecular biology to monitor pest species in the field (Nebbak et al., 2019; Suter et al., 2015).

Arthropods, including insects, undergo metamorphosis from the egg to adult stages (Hall and Martín-Vega, 2019). Hemimetabolic insects undergo incomplete metamorphosis with gradual changes across nymphal stages, resulting in less spectacular protein variation by MS according to stage than that observed for ticks (Karger et al., 2012) or Triatomae (Laroche et al., 2018) species. Conversely, holometabolic insects, such as coleopterans, undergo complete metamorphosis resulting in significant morphologic changes between the larval, pupal, and adult stages (Fedorenko, 2009). These morphological changes involve the selection of a distinct body part between the immature and adult stages, as previously reported for mosquitoes at the immature stages (Nebbak et al., 2018; Schaffner et al., 2014). It is paramount, therefore, to carefully optimise the protocol in order to obtain reliable and reproducible mass spectra (Bamou et al., 2022).

The purpose of this study was, for the first time, to assess how effective MALDI-TOF MS is at identifying insect pests which are harmful to cultural heritage. To test its effectiveness at identifying these metamorphic arthropods at various developmental stages, adult, larva, and exuviae from eight species of Coleoptera were subjected to MALDI-TOF MS after optimisation of the sample preparation parameters. The optimal conditions were evaluated based on the intra-species reproducibility and the inter-species specificity of the resulting MS spectra. Finally, the effectiveness of MALDI-TOF MS at correctly identifying these pest insects was assessed.

2. Materials and methods

2.1. Coleopteran breeding, collection, and morphological identification

Coleopterans were provided by the Centre Interdisciplinaire de Conservation et de Restauration du Patrimoine (CICRP) in Marseille. A total of eight distinct species were provided by the CICRP (Table 1). Three of them (*A. flavipes*, *T. contractus,* and *A. pimpinellae*) originated from an ancient breed Only exuviae were available for *A. flavipes* and *T. contractus*, whereas for *A. pimpinellae*, exuviae and adult specimens were provided. The five remaining coleopterans were bred under conditions approaching those of their natural habitats, to ensure their well-being and promote their healthy development. They were maintained at room temperature, between 22 °C and 25 °C, with relative humidity between 35% and 55%. Semolina was used as the nutritional breeding source for *L. serricorne*, while for other species of Coleoptera, fish food (Tetra Pond Sticks ZO-396240, France) was provided to satisfy their needs. Samples were collected at the immature and adult developmental stages, as well as at the exuviae stage. Immature and adult stages were frozen at −20 °C while exuviae were stored at room temperature.

The species identity of each specimen at the adult and larval stages was confirmed by morphological identification. The detection of the morphological characters of each specimen was conducted under a binocular loupe (ZEISS Axio Zoom V16) and compared to identification keys (Hagstrum et al., 2013; Halstead, 1993; Háva, 2004, 2021). Details about collection of the species of Coleoptera are presented in Table 1.

2.2. Dissection and sample preparation

Live specimens were sedated and killed by storing them at −20 °C for 60 min. Before dissection, each sample (exuviae, larval, and adult stages) was rinsed for 2 min with 70% ethanol, followed by two successive baths of distilled water, and drying on sterile filter paper. The samples were dissected under a Leica ES2 10x/30x stereomicroscope using a sterile slide, scalpel, and pliers for each specimen. For adult coleopterans, several body parts were dissected to determine which compartment appeared to be the most appropriate for MALDI-TOF MS classification. Three species of Coleoptera were selected in order to establish the optimal conditions for MS analyses at the adult stage. These optimised conditions were then applied to the other remaining species. A total of five body parts were tested at the adult stage: the cephalothorax, the legs with the thorax, the legs without the thorax, the head, and the elytra. To avoid intra-species MS spectral variations due to their feeding regime, the abdomens of adult stage specimens were systematically excluded from MS analysis and were set aside for molecular biology experiments as required. For larvae, the cephalothorax was used for MS submission, and was separated from the abdomen by dissection. Once dissected, the selected body parts were either immediately submitted to MS or stored at −20 °C. No dissection was performed on exuviae, which were exclusively used for MS analyses.

2.3. MALDI-TOF MS protocol optimisation

Three parameters were taken into account when establishing the optimal protocol in order to obtain intra-species reproducible and inter-species-specific MS spectra. The three parameters tested were the body part selected, the volume of mix buffer, and the automatic homogenisation setting. For exuviae, the whole sample was submitted for MS analysis, For larvae, only the cephalothoraxes of *L. serricorne*, *A. smirnovi*, *T. versicolor*, *R. vespulae*, and *A. verbasci* were used and were homogenised with 25 μl of mix buffer in an automatic system (TissueLyser, Qiagen) for 6 min at 30 Hz (two homogenisation cycles). In contrast, for adult specimens, the five body parts mentioned earlierwere tested. Each sample was placed individually in 1.5 mL tubes and crushed

Table 1
Details of Coleoptera species.

Species	Number of samples	Number per stage			Collection site	Location (French postal code)	Collection date (month/year)	Breeding status[#]
		Adults[a]	Larvae	Exuviae				
L. serricorne	92	60 (20)	32	/	Factory	Vitrolles (13127)	February 2022	Continuously
A. smirnovi	87	41 (5)	14	32	Museum	Paris (75001)	January 2014	Continuously
T. versicolor	97	49 (10)	15	34	Museum	Marseille (13004)	July 2010	Continuously
R. vespulae	74	32	15	27	Museum	Paris (75001)	January 2014	Continuously
A. verbasci	68	26	15	27	Field	Rognac (13340)	February 2020	Continuously
A. pimpinellae	34	17	/	17	Field	Rognac (13340)	June 2021	Continuously
A. flavipes	30	/	/	30	Museum	Marseille (13004)	July 2010	Old breeding
T. contractus	19	/	/	19	Museum	Arles (13200)	March 2002	Old breeding
Total	**502**	**225**	**91**	**186**				

[a] the number of samples used for optimisation conditions are indicated into brackets. #All species were laboratory-reared and currently (breeding) or bred in the last two years and stored frozen (old breeding).

with TissueLyser (Qiagen, Germany) at a frequency of 30 Hz with a pinch of glass beads (1.0 mm) (Sigma-Aldrich, St. Louis, Missouri, USA) in a mix buffer composed of a 50/50 mixture of 70% (v/v) formic acid (Sigma-Aldrich, Lyon, France) and 50% (v/v) acetonitrile (Fluka, Buchs, Switzerland), as described previously (Nebbak et al., 2016). The duration of homogenisation and the volume of mix buffer were adjusted according to stage and body part, as indicated in Supplementary Table 1.

2.4. Sample loading on the target plate and MALDI-TOF MS settings

After the homogenisation step, a quick spin for 1 min at 2000×*g*, was performed. One microlitre of supernatant was spotted, in quadruplicate, onto a steel target plate (Bruker Daltonics, Wissembourg, France). After drying at room temperature, each spot was covered with 1 µL of the matrix solution, composed of saturated α-cyano-4-hydroxycinnamic acid, CHCA at 10 mg/mL (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 CHCA matrix solution was air-dried at room temperature. The target plate was then loaded in the mass spectrometer for analysis.

2.5. MALDI-TOF MS parameters

Protein mass profiles were generated using a Microflex LT MALDI-TOF Mass Spectrometer (Bruker Daltonics, Germany), with detection in a linear positive-ion mode at a laser frequency of 50 Hz within a mass range of 2 kDa–20 kDa. The accelerating voltage was 20 kV, and the extraction delay time was 200 ns (Kumsa et al., 2016). Each spectrum corresponded to ions obtained from 240 laser shots performed in six regions of the same spot, and automatically acquired using the AutoXecute method of the flexControl v2.4 software (Bruker Daltonics).

2.6. Spectral analysis and creation of the reference database

The quality of all MS spectra obtained by each protocol and by body part was estimated, firstly, by a visual inspection of the MS spectra using ClinProTools v2.2 and flexAnalysis v.3.3 software (Bruker Daltonics) (Benkacimi et al., 2020). The intra-species reproducibility and inter-species specificity of the spectra were evaluated by principal component analysis (PCA) and cluster analysis (MS dendrogram) using ClinProTools v2.2 and MALDI-Biotyper v3.0. software (Bruker Daltonics), respectively. Reference MS spectra were created using the optimal body part and protocol for each species and developmental stage.

MS spectra from four distinct specimens per species and developmental stage were selected and added to our homemade reference MS spectra database containing more than 2000 spectra of various arthropods (Ahamada M'madi et al., 2022), using MALDI-Biotyper v3.0. software (Bruker Daltonics). MS spectra included in the database were created with an unbiased algorithm using information on peak position, intensity, and frequency (Hamlili et al., 2021).

2.7. Blind tests

The efficiency of MS profiling for sample identification was assessed by blind testing the spectra of the remaining spectra of coleopteran samples at the adult (thorax and legs), larval (cephalothorax), and exuviae (whole) stages against the MS reference database. The reliability of species identification was assessed using log score values (LSVs) ranging from 0 to 3, obtained from the MALDI-Biotyper v3.0 software (Bruker Daltonics). Each sample spectrum generated one LSV. The percentage of MS spectra with an identification score of 1.8 or higher was determined.

2.8. DNA extraction and molecular identification of coleopterans

Four specimens per species at the adult stage added into the MS reference database were subjected to molecular biology identification. Their respective abdomens were used for DNA extraction with the NucleoMag Pathogen Kit from the KingFisher Flex system (Thermo Fisher Scientific, Waltham, MA, USA), in accordance with the manufacturer's recommendations. Standard PCR was performed to validate specimen identity in an automated DNA thermal cycler (Applied Biosystems, 2720, Foster City, CA, USA) using Folmer's universal *COI* (cytochrome *c* oxidase subunit I) barcoding (primers, LCO1490, and HCO2198), targeting a 710 base-pair region, and/or the *16S* gene (primers, LRJ12961, and LRN13398), targeting a 500 base-pair region. The settings for the PCR product were similar to those previously described (Folmer et al., 1994; Olson et al., 2014). The PCR mix without DNA was used as negative control and DNA extracted from laboratory-reared *Aedes albopictus* mosquitoes was used as positive control. The amplification products were then subjected to electrophoresis through a 1.5% agarose gel stained with SYBR Safe™ and visualised with the ChemiDoc™ MP ultraviolet imager (Bio-Rad, Marnes-la-Coquette, France) (Benkacimi et al., 2020). After purifying positive samples, the PCR products were sequenced using the BigDye Terminator v1.1, v3.1 5x Sequencing Buffer (Applied Biosystems, Warrington, UK). Sequencing was performed with Sanger sequencing using Applied Biosystems® Sanger Sequencing 3500 Series Genetic Analyzers. Sequence chromatograms were assembled and edited using Chromas Pro1.77 (Technelysium Pty. Ltd, Tewantin, Australia). The sequences obtained were compared to sequences available in GenBank (Benson et al., 2018). This comparison was made using the BLAST algorithm.

2.9. Nucleotide sequence accession numbers

The sequences obtained were corrected and formatted according to the GenBank submission recommendations, using BankIt as the

recommended submission tools (Benson et al., 2018). After validation, GenBank assigns unique accession numbers to each sequence. The corresponding accession numbers using the *16S* gene are: *L. serricorne* (PP474525), *A. smirnovi* (PP474515, PP474516, PP474517, PP474518), *T. versicolor* (PP239566, PP239565), *R. vespulae* (PP474519, PP474520), *A. pimpinellae* (PP474521, PP474522, PP474523, PP474524). And with *COI* gene: *L. serricorne* (PP600566, PP600567), *A. smirnovi* (PP600568), *R. vespulae* (PP600562, PP600563, PP600564, PP600565), *A. verbasci* (PP702081, PP702082, PP702083, PP702084), and *A. pimpinellae* (PP600558, PP600559, PP600560, PP600561).

3. Results

3.1. Morphological identification

All the coleopterans provided by the CICRP were bred in laboratory boxes containing specimens and feeding substrate, with one species per box. In these conditions, the identity of all specimens, whatever the developmental stage, was known. Nevertheless, the identity of all specimens at the adult and larval stages was confirmed by individual morphological analysis under the supervision of an entomological coleopteran specialist. Eight distinct species of Coleoptera were included in this study (Fig. 1). The larval, exuviae, and adult stages were available for four species, namely *A. smirnovi*, *T. versicolor*, *R. vespulae* and *A. verbasci*. For L. *serricorne*, the larval and adut stages were collected, while the exuviae stage was missing. Exuviae from L. *serricorne* were not recovered from the breeding equipment due to their fragility. For *A. pimpinellae*, only the exuviae and adult stages were provided, as they originated from an old breeding collection. Finally, for the last two species, *A. flavipes* and *T. contractus*, only the exuviae were available (Table 1). For these species, validation of their identity using morphological tools was not possible due to the lack of taxonomic keys for the exuviae stages. As exuviae from *A. flavipes* and *T. contractus* came from reared colonies, no doubt exists as to their identity.

3.2. Optimisation and validation of standardised protocols for MS identification of coleopterans

- *Adult stage*

The reproducibility and specificity of the MS spectra per species were the main criteria used for the selection of body parts and the sample preparation conditions. The first tests were conducted on *L. serricorne,* which was the species for which the largest number of specimens was available. Body parts from five *L. serricorne* specimens were selected per condition (Supplementary Table 1). The protocol P1 applied to the elytra and cephalothorax body parts generated MS spectra of low intensity and were heterogeneous per compartment (Supplementary Fig. 1A). The detection of large particles in the tubes following automatic destruction indicated a partial homogenisation of the samples. To improve the quality of the MS spectra, the duration of the homogenisation was extended, and different volumes of mix buffer were tested according to the protocols P2 and P3 (Supplementary Table 1). The results of protocol P2 (Supplementary Fig. S1B) and protocol P3 (Supplementary Fig. 1C) applied to the elytra, head, and thorax plus legs were presented. The problem of homogenisation was solved by extending the duration of sample grinding from 3 min to 9 min, which resulted in the disappearance of large particles. Nevertheless, MS spectra heterogeneities were noted among samples from the same body parts as well as among replicates (Supplementary Figs. 1B and 1C). The increase of mix buffer in protocol P3 induced a decrease in the intensity of the MS spectra, which is likely to correspond to a dilution of the samples.

Finally, protocol P4 (Supplementary Table 1) was applied to the elytra and thorax plus legs body parts and the volume of mix buffer was adjusted to 30 μL. An improvement in the reproducibility and intensity (≥3000 au) of MS spectra was obtained for both body parts by applying protocol P4 (Supplementary Fig. 1D). MS spectra from *L. serricorne* obtained with protocol P4 on the thorax plus legs were considered as suitable quality for MS analysis.

To determine whether *L. serricorne* MS spectra were species-specific, two other species, *A. smirnovi* and *T. versicolor,* were submitted for MS analysis. As neither of these species had previously been tested by MALDI -TOF MS, an optimisation of the sample preparation parameters

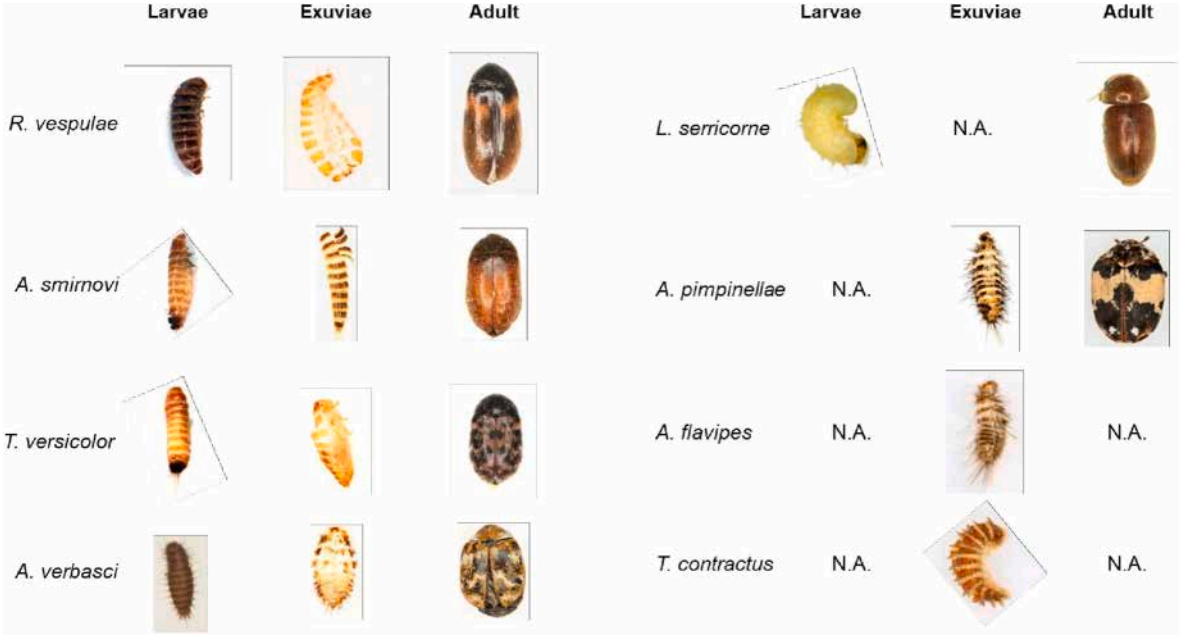

Fig. 1. Photographs of coleopterans at different developmental stages. Images of the eight beetle species at larval, exuviae, and adult stages are presented when available. The species name and the developmental stage are indicated respectively, at the top and left side of the panel. Specimens were photographed with Canon MP-E 65 mm at magnification x5. NA = species not available at this stage.

per species was required. These two species were approximately double the size of *L. serricorne*. For *T. versicolor,* the thorax plus legs body parts were then mixed with two distinct volumes of mix buffer, 100 µL and 50 µL (Supplementary Table 1). Although reproducible spectra were obtained with both protocols (P5 and P6), more intense MS profiles were generated with the lower volume of mix buffer in protocol P6 (Supplementary Fig. 1E).

Application of the P6 protocol to *A. smirnovi* on the thorax plus legs body part also produced high-quality protein profiles which were reproducible among specimens (Supplementary Fig. 1F). Additionally, a visual comparison of MS spectra per species appeared to indicate species specificity. To standardise the protocol applied to species of Coleoptera at the adult stage for MS analysis, the thorax plus legs, homogenised for three automatic cycles in 40 µL of mix buffer, were the conditions selected for future analyses. Application of this protocol P7 to *L. serricorne, A. smirnovi,* and *T. versicolor*, as well as to the other three species of Coleoptera (*R. vespulae, A. verbasci,* and *A. pimpinellae*), made it possible to obtain MS spectra with high intensity (≥3000 au) and intra-species reproducibility (Fig. 2A).

- *Larval stage*

Because these cultural heritage insect pests can fed on a variety materials, their gut contents could generate MS spectra heterogeneity, according to the origin of the food and level of digestion, as previously reported for engorged mosquitoes (Niare et al., 2016). To overcome this difficulty, the abdomen was excluded from the analysis. With the exception of *L. serricorne*, these conditions made it possible to obtain high intensity MS spectra (≥3000 au) with intra-species reproducibility for the four other species tested (Fig. 2B). For L. *serricorne*, despite using the same body part and the same sample preparation conditions, low intensity mass spectra with high heterogeneity across specimens were obtained (Fig. 2B). Even after making modifications to the protocol, such as using pistons for manual grinding, we were unable to improve the quality of the MS spectra (Supplementary Fig. 2). While we do not have a definitive explanation for this issue, it could be due to the hardness and rigidity of the *L. serricorne* cephalothorax compared to other species.

- *Exuviae stage*

Because exuviae, which correspond to the arthropod exoskeleton, are breakable and their dissection was not straightforward, the entire exuviae were used for MS analysis. Nevertheless, the cuticle remained hard to destroy. Three cycles of homogenisation (each 3 min at 30 Hz) with TissueLyser (Qiagen) were needed to sufficiently destroy the cuticles prior to submission to MS. Two volumes of mix buffer, 30 µL and 40 µL, were tested. As the MS spectral profiles were comparable independently of the mix buffer volume, 40 µL of mix buffer was selected in order to apply the same parameters used for the adult stages (protocol P7, Supplementary Table 1). Application of this protocol to exuviae from *A. smirnovi, T. versicolor, A. verbasci, R. vespulae, A. pimpinellae, A. flavipes*, and *T. contractus*, generated high intensity MS spectra with intra-species reproducibility and low background noise (Fig. 2C). Interestingly, for all species, a predominant peak at around 4 kDa was present in each species.

3.3. Species-specific MS spectra

Visual comparison of the MS spectra revealed a reproducibility of protein profiles by species, and the singularity of these profiles between species from one stage to another (Fig. 2). To affirm the species

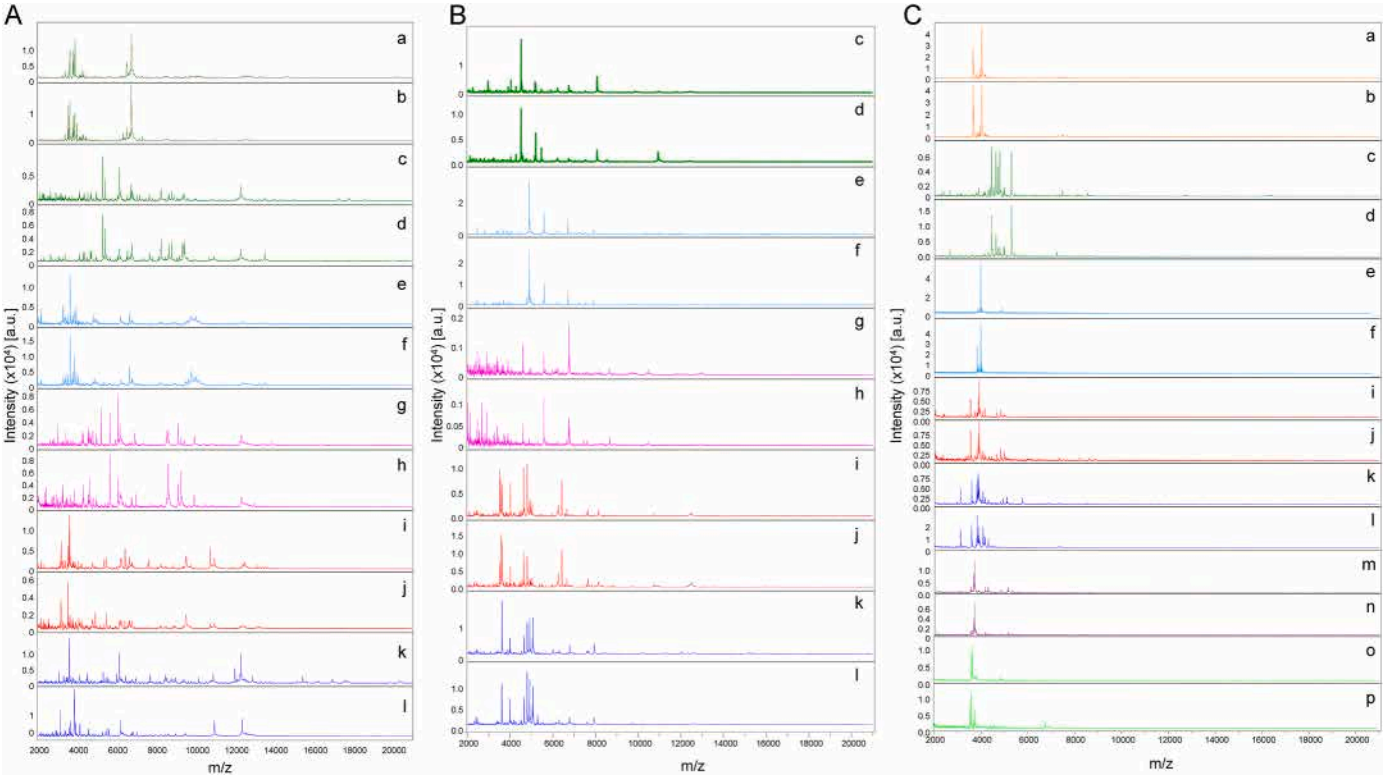

Fig. 2. Representative MS spectra from different species of Coleoptera using flexAnalysis v.3.3 (**A**) Representative MS spectra of *A. pimpinellae* (a,b), *A. smirnovi* (c,d), *A. verbasci* (e,f), *L. serricorne* (g,h), *R. vespulae* (i,j) and *T. versicolor* (k,l), at adult stages. (**B**) Representative MS spectra of *A. smirnovi* (c,d), *A. verbasci* (e,f), *L. serricorne* (g,h), *R. vespulae* (i,j) and *T. versicolor* (k,l), at larval stages. (**C**) Representative MS spectra of A. *pimpinellae* (a,b), *A. smirnovi* (c,d), *A. verbasci* (e,f), *R. vespulae* (i,j) *T. versicolor* (k,l), *A. flavipes* (m, n) and *T. contractus* (o, p) at exuviae stagse. The same colour code and letters are used to present spectra of the same species. MS spectra from two distinct specimens per species are presented. au = arbitrary units; m/z = mass-to-charge ratio.

specificity of the MS profiles, MSP dendrograms were generated from the MS spectra of four specimens per species for each development stage. The cluster analyses revealed no intertwining and MS spectra from specimens of the same species were grouped on the same branch for each developmental stage (Fig. 3). It is interesting to note that MS spectra from the two species of the *Anthrenus* genera were not found in the same part of the dendrogram at the adult and exuviae stages (Fig. 3A and C). To assess the specificity of the MS spectra according to developmental stage per species, PCA was performed for *A. smirnovi, T. versicolor, A. verbasci*, and *R. vespulae*, for which the three stages were available. The high separation of the dot on PCA confirmed the specificity of the MS profiles per developmental stage (Supplementary Fig. 3).

3.4. Molecular validation of specimen identities

As all specimens were laboratory-reared, their identities were already known. However, as some specimens were included in our MS spectra database and would be used as references for coleopteran identification, a molecular confirmation of this classification was felt to be necessary. Abdomens from four adult specimens per species (n = 6) were thus subjected to molecular biology analyses. Among the 24 samples tested, the rate of successful sequencing was 54.1% (n = 13) and 62.5% (n = 15) for the *16S* and *COI* genes, respectively (Supplementary Table 2). At least one sequence was obtained from both genes for all species with the exception of *T. versicolor* and *A. verbasci*, for which only the *16S* and *COI* sequences were obtained, respectively (Supplementary Table 2). For *T. versicolor*, the *16S* sequences matched with *Trogoderma* sp. (NCBI acc. Number: #MZ571643), while a confirmation of the *A. verbasci* classification was obtained by sequencing their *COI* (#MN609311).

BLAST of *16S* and *COI* sequences against GenBank confirmed the morphological identifications for both gene sequences only for the *L. serricorne* samples, with relevant proportions of coverage and identity (Supplementary Table 2). For *A. smirnovi*, *T. versicolor*, *R. vespulae*, and *A. pimpinellae*, the query of *16S* sequences against GenBank did not confirm the morphological identification. The *16S* sequences of *A. smirnovi* specimens blasted with high identity and coverage rates with the *Trogoderma simplex* sequence (#KJ930456), whereas the *COI* gene sequence confirmed the morphological determination (*A. smirnovi*, #KU494072; Supplementary Table 2). Similarly, inconsistent identifications were obtained between the *16S* and *COI* sequences of *A. pimpinellae* although, once again, *COI* gene sequences validated the morphological determination (*A. pimpinellae*, #KU494047). For both target gene sequences, the proportions of identity were lower than 95% which did not allow us to reach a definitive conclusion as to their identities. Finally, for *R. vespulae* specimens, the sequencing of the *16S* and *COI* genes obtained both high coverage and identical rates with *Eurhopalus vespulae* (#MZ571653 and #OP669194, respectively). The 13 and 15 sequences of the *16S* and *COI* genes obtained in the present study have been deposited in GenBank in accordance with morphological classification, and the access numbers assigned to them are listed in Supplementary Table 2.

The phylogenetic analysis with the *COI* and *16S* gene sequences of these specimens indicated clustering according to genera or that the same subgenus clustered together (Supplementary Figs. 4 and 5).

3.5. Blind tests

A total of 190, 91, and 186 coleopteran samples at the adult, larvae, and exuviae stages, respectively, were submitted for MS analysis (Table 2). Among them, spectra from 13 (6.8%), 32 (35.2%), and nine (4.8%) coleopteran samples at the adult, larvae, and exuviae stages, respectively, were excluded from the analysis due to the low intensity (<3000 au). Interestingly, the 32 specimens from larvae stage corresponded to all *L. serricorne* species. Of the remaining 413 MS spectra used as reference MS spectra, 68 new MS spectra were added to the database to enrich the existing collection (Table 2). These new spectra were integrated into the database under the following DOI: (https://doi.org/10.35081/ykng-z643). The remaining 345 MS spectra attest to the efficiency of this tool to classify MS spectra at correct species level, whatever the stage.

All spectra selected to create the MSP dendrogram at the adult (n = 24), larval (n = 16), and exuviae (n = 28) stages were included in our homemade MS spectra database using MALDI-Biotyper 3.0. No MS spectra from *L. serricorne* specimens at the larvae stage were added to the MS spectra database, due to their low intensity and the low reproducibility among samples.

All the spectra (100%) queried against the MS spectra database were correctly identified at the species level whatever the stage. The LSVs ranged from 1.70 to 2.73 for the thorax plus leg MS spectra at the adult stage, from 1.89 to 2.70 for the cephalothorax MS spectra at the larval stage, and from 1.77 to 2.68 for the whole exuviae MS spectra (Fig. 4). Here, 94.77% (145/153) of MS spectra from adults, 100% (43/43) from larvae, and 98.65% (147/149) from exuviae had a threshold LSV greater than 1.8 (Table 2).

4. Discussion

Over the past decade, MALDI-TOF MS has proven to be a robust proteomic tool due to its high performance and low operating cost, once

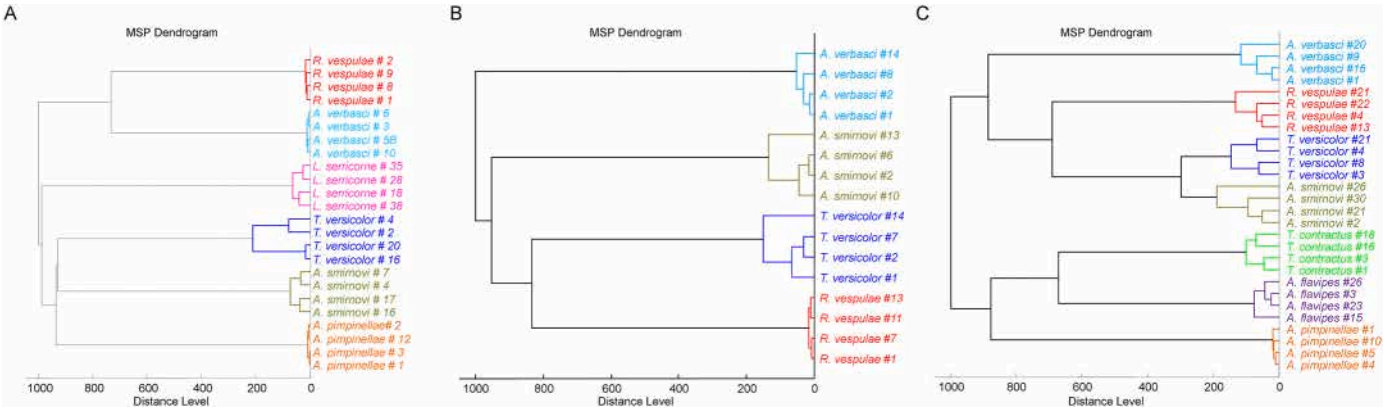

Fig. 3. Assessment of species specificity of MS profiles with MSP dendrogram from the MS spectra of four specimens per species for each development stage. (**A**) MSP dendrogram constructed using four representative MS spectra of adult specimens (**B**) MSP dendrogram using four representative MS spectra of larvae specimens (**C**) MSP dendrogram using four representative MS spectra of exuviae specimens. The dendrogram was created using Biotyper v3.0 software. An arbitrary sample number, attributed to each specimen, is indicated after the species names (#XX).

Table 2
Results of blind tests per Coleoptera species and per developmental stage.

Stage	Species	Number of samples	Spectra included[a]	Spectra used as reference	Spectra blind tested	Correct ID (%)	Score range[b]	Relevant ID (LSVs ≥1.8)
Adult	*L. serricorne*	40	32	4	28	100	[1.70–2.53]	75.0%
	A. smirnovi	36	35	4	31	100	[1.84–2.42]	100%
	T. versicolor	39	39	4	35	100	[1.79–2.48]	97.1%
	R. vespulae	32	28	4	24	100	[1.88–2.73]	100%
	A. verbasci	26	26	4	22	100	[1.89–2.51]	100%
	A. pimpinellae	17	17	4	13	100	[1.93–2.37]	100%
Larvae	*L. serricorne*	32	/	/	/	/	/	/
	A. smirnovi	14	14	4	10	100	[2.04–2.47]	100%
	T. versicolor	15	15	4	11	100	[1.89–2.50]	100%
	R. vespulae	15	15	4	11	100	[2.37–2.70]	100%
	A. verbasci	15	15	4	11	100	[2.19–2.57]	100%
Exuviae	*A. smirnovi*	32	32	4	28	100	[2.00–2.54]	100%
	T. versicolor	34	34	4	30	100	[1.89–2.68]	100%
	R. vespulae	27	27	4	23	100	[2.09–2.54]	100%
	A. verbasci	27	27	4	23	100	[1.94–2.47]	100%
	A. pimpinellae	17	14	4	10	100	[1.77–2.59]	80.0%
	A. flavipes	30	30	4	26	100	[1.86–2.47]	100%
	T. contractus	19	13	4	9	100	[1.83–2.15]	100%
Total		**467**	**413**	**68**	**345**	**100%**	[1.70–2.73]	**97.10%**

[a] 54 MS spectra were excluded due to their low intensity (<3000 au).
[b] Details of LSVs per sample are available in Fig. 4.

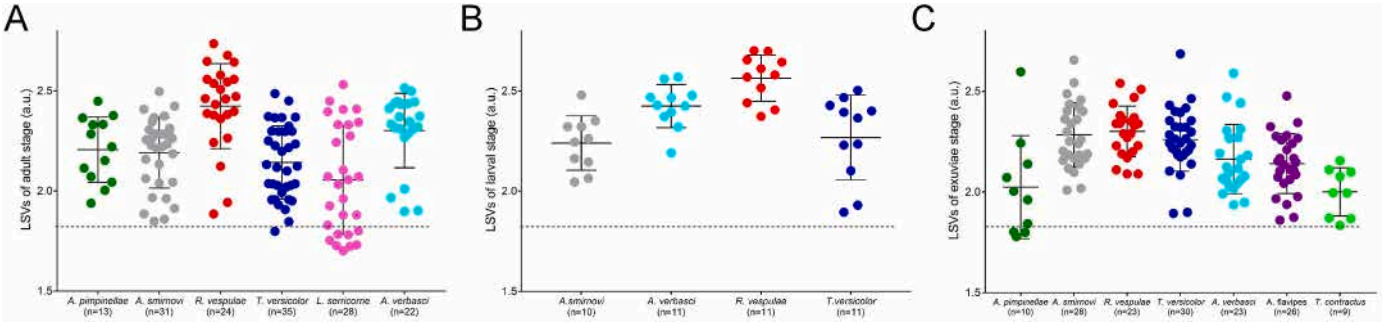

Fig. 4. LSV of MS spectra from various developmental stages compared to an internal MS reference database. (A) LSVs of MS spectra adults queried against homemade MS reference database **(B)** LSVs of MS spectra larval queried against homemade MS reference database. **(C)** LSVs of MS spectra exuviae queried against homemade MS reference database. The samples queried are indicated at the bottom of the graphic. The same colour code is used per species. The dashed line represents the threshold value (LSV ≥1.8), for relevant identification. au = arbitrary units; LSVs = log score values.

the device has been acquired as part of building technological platforms. MALDI-TOF MS has revolutionised the field of clinical microbiology, becoming one of the most valuable and useable tools for microbial identification in clinical microbiology laboratories (Nomura, 2015; Schubert and Kostrzewa, 2017). Despite the relative initial high cost of the instrument, the investment is offset by the low cost of the analyses (around a few cents) (Jang and Kim, 2018). Its reliability and accurate application in the domains of microbiology, mycology, and now medical entomology (Sevestre et al., 2021), have been demonstrated, offering the opportunity to use it in shared technological platforms (Jang and Kim, 2018). It has been shown that arthropods cause considerable damage in the field of cultural heritage (Querner et al., 2018), and in order to effectively combat this threat, rapid and accurate identification of pests is essential (Fohrer, 2011). This study assessed the potential of MALDI-TOF-MS for the identification of insect pests of cultural heritage. Eight species of Coleoptera were tested, at the immature, adult, and exuviae stages when available.

The quality of the obtained spectra was influenced by several parameters, including the choice of body part of the arthropod, the volume setting of the buffer used for protein extraction, the method and duration of sample homogenisation, the matrix preparation, and the storage conditions (Nebbak et al., 2016, 2017). Here, for the first time, sample preparations were optimised for the MS identification of coleopteran specimens of the three different species. These protocols were evaluated on three different adult stage species of Coleoptera to determine which protocol would give the best quality of MS spectra (intensity ≥3000 au, low background noise) with intra-species reproducibility and inter-species specificity.

Among the parameters tested, the choice of body part to submit for MS was a key factor. Effectively, in contrast to molecular approach, the protein repertoire differs according to the compartment, while the genome is unchanged (Nabet et al., 2021). For instance, in previous studies, different body parts were subjected to MALDI- TOF MS analysis and the quality and reproducibility of the MS spectra were compared (Nebbak et al., 2018). The body part generating MS spectra with the best quality were systematically selected for future analysis, such as legs for ticks (Ngnindji-Youdje et al., 2023) and thoraxes for mosquitoes (Costa et al., 2023). For other arthropod families, other body parts may be selected, such as the cephalothoraxes for fleas (Yssouf et al., 2014), and heads for bedbugs (Benkacimi et al., 2020). Concerning the selection of the body part, four distinct compartments were submitted to MALDI-TOF MS analysis, including the head, the elytra and the legs with or without the thorax. Among them, the thorax with legs appeared to be the body part which generated reproducible intra-species MS spectra for the three species tested. Moreover, this compartment made it possible to obtain MS spectra of high intensity with relative diversity in the peak

position among the species. This protocol was successfully applied to identify the remaining species of Coleoptera. For larval stages of coleopterans, because MS spectral variations could occur due to gut contents, as previously mentioned (Karger et al., 2012; Nabet et al., 2021), the cephalothoraxes were selected. The small size and difficulty of dissecting the exuviae led us to submitting the entire sample for MS. Here, the abdomens from adult and larval stages of all coleopteran specimens were excluded from MS analyses. The presence of food and its state of digestion in the digestive tract can introduce unwanted MS spectral variability and noise (Nabet et al., 2021). One such example is hematophagous arthropods. The high abundance of host blood proteins in the abdomens of freshly engorged mosquitoes have been revealed by MS, hiding mosquito proteins from the abdomen (Niare et al., 2016).

The second parameter assessed was the quantity of mix buffer added to each sample to avoid dilution. The volume of mix buffer may differ from one arthropod family to another and from one body part to another from the same species (Sevestre et al., 2021). For instance, the quantity of mix buffer is adjusted to the compartment size of adult mosquitoes between the legs and the thorax (Vega-Rúa et al., 2018), but also between the aquatic developmental stages (Dieme et al., 2014). Here, the challenge was to determine the appropriate mix buffer volume providing intense and reproducible MS spectra, particularly for adult specimens, for which significant differences in size between species of Coleoptera can occur (Crowson, 1981). Effectively, *A. smirnovi* and *T. versicolor*, at the adult stage, are nearly twice the size of *L. serricorne*. Despite this variation in size, we succeeded in determining a unique volume of mix buffer which was suitable for all species. The use of the same quantity of mix buffer for Coleoptera exuviae makes it possible to homogenise the sample preparation conditions for these two coleopteran stages. To obtain the best quality of MS spectra, it is necessary to reduce the sample volume for coleopteran cephalothoraxes, due to their small size.

The last parameter tested was the quality of sample homogenisation by adjusting the duration of grinding. In previous studies, it was effectively demonstrated that optimisation of the conditions of homogenisation of the sample could improve the quality of resulting MS spectra for mosquitoes (Nebbak et al., 2016) as well as ticks or fleas (Nebbak et al., 2017). These insects possess a very hard cuticle, requiring longer homogenisation for adult and exuviae specimens. That why, the number of homogenisation cycles was multiplied by three compared to the protocol used for larvae (Anbutsu et al., 2017). For all the species tested, the MALDI-TOF MS technique proved to be extremely effective. This remarkable performance can be attributed to the ability of MALDI-TOF MS to generate intense and reproducible MS spectra.

In this study, standardised protocols were established to achieve optimal results for each developmental coleopteran stage, regardless of species. For adult specimens, the most effective conditions for the thorax and leg sample preparation involved 40 μL of buffer mix and three automatic cycles of 3 min of homogenisation. For larvae, the cephalothorax was homogenised in 25 μL of buffer mix for 6 min (two cycles), while for exuviae, 40 μL of buffer mix with three homogenisation cycles was recommended. These protocols ensured consistent and reproducible MS spectra.

The reproducibility and specificity of the species were confirmed with cluster analysis, clearly demonstrating a distinction between different species of Coleoptera in this study. In addition, the validity of the database was evaluated through a blind test of the remaining specimens, in which all specimens of all species were correctly identified (100%). Among the 345 samples from different developmental stages that were analysed, 97.10%, corresponding to 335 specimens, exceeded the LSV threshold of 1.8. Exceeding the LSV threshold of 1.8 is a crucial indicator of the reliability and quality of the identification performed, in accordance with the standards established in previous research (Bamou et al., 2022; Yssouf et al., 2014). This excellent performance ensures a reliable and confident identification of the specimens. One of the main advantages of the MALDI-TOF MS method is its reliability across all stages of insect development. Whether at the larval or adult stages, or even with exuviae, the technique ensures precise and rapid identification, which is a considerable advantage. Accurate identification of various developmental stages is particularly important for several reasons. Firstly, larvae are widely known to cause significant damage to heritage objects (Trematerra and Pinniger, 2018). However, in the field, larvae are difficult to find as they often hide. Additionally, adults may be constantly on the move or have already left the area, further complicating their detection and identification (Trematerra and Pinniger, 2018). Heritage sites may also display subtle signs of insect activity, such as exuviae and frass, indicating the insects' progression through different growth stages. Their presence can indicate past or ongoing infestations, offering important clues to evaluating the level of risk. It is essential to stress that identifying exuviae based on morphological characteristics is often impossible due to the deformation or alteration of these structures after the insects emerge (Nebbak and Almeras, 2020). Even when molecular methods are employed, they may not be as effective as hoped (Dhananjeyan et al., 2010; Yumoto et al., 2021).

Molecular biology techniques were used to improve the accuracy and reliability of identifying the six adult species before they were added to the database. The success rate for sequencing was relatively low, ranging from 50% to 60% for both the *COI* and *16S* genes. While the *COI* gene is widely considered to be a universal marker for insect identification (Hebert et al., 2003), and Coleoptera in particular (Raupach et al., 2016), its effectiveness may be limited for certain pests such as those of the Trogoderma genus, due to insufficient information on inter- and intra-specific variations (Olson et al., 2014). In our study, accurate identification was achieved for most species using the *COI* gene, with the exception of *T. versicolor*, for which viable sequences could not be obtained. In response to this challenge, we turned to the *16S* gene, as previous research has employed the *16S* gene for DNA amplification in the Trogoderma genus, successfully obtaining sequences from various species within the genus (Olson et al., 2014). Similarly, in our study, we successfully amplified the DNA of *T. versicolor* and obtained high-quality sequences using the *16S* gene.

The molecular biology results confirmed our morphological identifications for certain species such as *L. serricorne* and *A. smirnovi*, either through the use of both genes or solely through the *COI* gene, for which homologous sequences were accessible on GenBank. However, it is important to note that the identification and coverage percentages were relatively low, as observed in the case of *A. pimpinellae*. This divergence could be due to the incorrect or low-quality of the sequences available on GenBank. It is essential, therefore, to maintain and improve the quality of public genetic databases in order to ensure reliable identification results using molecular biology. This is why we submitted our sequences to GenBank. However, for other species, morphological identification did not match the molecular identification. The most probable explanation is the updates to the systematics of Coleoptera, with changes in the species names, as in the case of *R. vespulae* which was changed to *E. vespulae* (Zhou et al., 2022).

MALDI-TOF MS offers a reliable, cost-effective, and rapid alternative to morphological and molecular methods, allowing identification of coleopterans at the species level whatever the developmental stage, with minimal sample preparation and entomological expertise. One recent article (Wang et al., 2024) emphasised the importance of MALDI-TOF MS for rapid and reliable species identification, especially for pests like *Hylurgus ligniperda*. The authors suggest extending the application of this method to on-site species identification, particularly during customs interceptions and field surveys.

This identification method is particularly crucial for harmful organisms, as it allows for the rapid and accurate identification of pest species, thereby providing strong support for prevention and control measures (Venette and Hutchison, 2021). In the future, this method could also be applied to identifying other pest species within the order Coleoptera, further enhancing its role in pest management.

5. Conclusion

Our study is a pioneering step forward, demonstrating the use of MALDI-TOF mass spectrometry to identify various insect pests threatening cultural heritage in France, with a focus on Coleoptera. It established optimal protocols prior to submitting species of Coleoptera to MS. For adult, larval, and exuviae samples, the thorax with legs and the whole exoskeleton were, respectively, the more appropriate compartment to obtain species-specific MS spectra for their successful identification. This innovative approach overcomes the limitations of other tools, including the time constraints of morphological identification and the limitations of molecular biology. In the future, it would be interesting to evaluate the reliability of MALDI-TOF MS by conducting tests on specimens collected directly from heritage sites.

CRediT authorship contribution statement

Dikra Hamadouche: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Adama Zan Diarra:** Writing – review & editing, Visualization, Validation, Project administration, Methodology, Formal analysis. **Fabien Fohrer:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation. **Jean-Michel Bérenger:** Writing – review & editing, Visualization, Resources, Investigation. **Ahmed Benakhla:** Writing – review & editing, Visualization, Resources, Project administration. **Lionel Almeras:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Philippe Parola:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition.

Availability of data and materials

The MS reference spectra included in the database of this study are freely accessible and can be downloaded via the DOI provided.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Declaration of competing interest

The authors declare that there are no conflicts of interest. Funding played no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.

List of abbreviations

CHCA	α-cyano-4-hydroxycinnamic acid
CICRP	Centre Interdisciplinaire de Conservation et de Restauration du Patrimoine.
COI	cytochrome oxidase subunit I
DNA	Deoxyribonucleic Acid
HPLC	High-Performance Liquid Chromatography
ITS	Internal transcribed spacer
LSV	log score value
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibiod.2025.106033.

Data availability

Data will be made available on request.

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