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## Article

# Identification of Neotropical *Culex* Mosquitoes by MALDI-TOF MS Profiling

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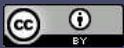
**Abstract:** The mosquito (Diptera: Culicidae) fauna of French Guiana encompasses 242 species, of which nearly half of them belong to genus *Culex*. Whereas several species of *Culex* are important vectors of arboviruses, only a limited number of studies focus on them due to the difficulties to morphologically identify field-caught females. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been reported as a promising method for the identification of mosquitoes. *Culex* females collected in French Guiana were morphologically identified and dissected. Abdomens were used for molecular identification using COI (cytochrome oxidase 1) gene. Legs and thorax of 169 specimens belonging to 13 *Culex* species, (i.e. *Cx. declarator*, *Cx. nigripalpus*, *Cx. quinquefasciatus*, *Cx. usquatus*, *Cx. adamesi*, *Cx. dunni*, *Cx. eastor*, *Cx. idottus*, *Cx. pedroi*, *Cx. phlogistus*, *Cx. portesi*, *Cx. rabanicolus* and *Cx. spissipes*) were then submitted to MALDI-TOF MS analysis. A high intra-species reproducibility and inter-species specificity of MS spectra for each mosquito body part tested were obtained. A corroboration of the specimen identification was revealed between MALDI-TOF MS, morphological and molecular results. MALDI-TOF MS protein profiling proves to be a suitable tool for identification of neotropical *Culex* species and will permit to enhance knowledge on this highly diverse genus.

**Keywords:** *Culex* species; neotropical mosquito; French Guiana; MALDI-TOF MS; proteomic tool; arbovirus; monitoring

## Introduction

The neotropical region is inventoried as a hotspot of mosquito (Diptera: Culicidae) species richness. French Guiana (hereafter FG) is a department of France situated in South America which harbors one of the highest relative species density of mosquitoes in the world [1,2]. Currently, 242 mosquito species, classified into 22 genera are known in this French territory [3]. Some species are medically important because they are proven vectors of human pathogens. For example, several *Anopheles* species are vectors of malaria parasitic infection in FG [4–7]. *Yellow Fever* (YFV), *dengue* (DENV), *chikungunya* (CHIKV) and *Zika* (ZIKV) viruses are transmitted by the day-biting *Aedes* (*Stegomyia*) *aegypti* (*Linnaeus*) in urban area [8–10].

Containing 788 species classified into 26 subgenera worldwide, the genus *Culex* (abbreviated Cx.) is one of the largest groups of the Culicidae family [11]. In FG, 104 species belong to the genus *Culex* [3] with a majority of species linked to two subgenera named *Culex* (abbreviated Cux.) and *Melanoconion*



(abbreviated *Mel.*). In the *Culex* subgenus many species are vectors of arboviruses and parasites. *Culex* (*Cux.*) *quinquefasciatus* Say was identified as vector of the nematode *Wuchereria bancrofti*, agent of lymphatic filariasis in FG [12] and as primary vector in northeast Brazil [13]. In a larger scale, this anthropophilic mosquito is also known to be vector of Western equine encephalitis virus (WEEV, *Alphavirus*) and many viruses from the *Flavivirus* genus, as Saint-Louis encephalitis virus (SLEV) and West Nile virus (WNV); besides, is also vector of nematode *Dirofilaria immitis*, that could infect both humans and dogs [14–17]. In South America, *Cx.* (*Cux.*) *declarator* Dyar & Knab is a vector of SLEV and Bussuquara virus (BSQV, *Flavivirus* genus) [18]. *Culex* (*Cux.*) *nigripalpus* Theobald allowed the isolation of Cabassou virus (CABV, *Alphavirus* genus) in FG [19] and is a recognized vector for members of *Flavivirus* genus with SLEV, WNV and ZIKV and for members of *Alphavirus* genus with Venezuelan equine encephalitis virus (VEEV) and Eastern equine encephalitis virus (EEEV) in America [20,21]. *Culex* (*Cux.*) *usquatus* Dyar belonging to the Coronator Complex is not a recognized as vector, but the females are indistinguishable from *Cx.* (*Cux.*) *coronator* Dyar & Knab, which is known as vector of WNV [22] and may participate in the transmission of SLEV, VEEV and Mucambo Virus (MUCV, *Alphavirus* genus) [23,24].

In the *Melanoconion* subgenus, *Cx.* (*Mel.*) *portesi* Senevet & Abonnenc and *Cx.* (*Mel.*) *spissipes* Theobald are highly suspected to be natural vectors of Tonate virus (TONV) that belongs to the *Alphavirus* genus in FG [25–27], but are also found infected by *Bunyavirus* (Caru, group Guama, Caraparu and group C) [19]. *Culex* (*Mel.*) *portesi* is a recognized natural vector for MUCV in Trinidad [28] and is a natural vector for the disease caused by El Huayo virus (a group C *Orthobunyavirus*) in Peru [29]. Ecological studies and the isolation of many arboviral species in *Cx.* (*Mel.*) *portesi* rank it as high potential invertebrate host of numerous viruses [19]. *Culex* (*Mel.*) *adamesi* Sirivanakarn & Galindo, *Cx.* (*Mel.*) *dunni* Dyar, and *Cx.* (*Mel.*) *pedroi* Sirivanakarn & Belkin are natural enzootic vectors of VEEV in Columbia, Peru and Panama [30–32]. *Culex* (*Mel.*) *dunni* was also found infected with Pacora virus (PCAV, *Bunyavirus*-like) in Panama [33]. A last example is *Cx.* (*Mel.*) *idottus* Dyar which is a caiman-biting mosquito involved in the transmission of *Hepatozoon caimani* and suggested as potential vector of WNV [34].

With global warming, deforestation and urbanization, the distribution areas of mosquitoes change, colonizing new territories. Such changes could promote the emergence of new mosquito vectors due to their exposure to disease agents and hosts, inducing potential outbreaks. To manage and to prevent epidemic emergence, the monitoring and accurate identification of *Culex* mosquitoes at the species level remains essential. Presently, morphological and molecular methods are the main strategies available for identification and classification of *Culex* species.

Morphological identification at species level of *Culex* mosquitoes requires the careful dissection and mounting of male genitalia by an entomological expert because of the presence of slight morphologic characters that allows the identification of sibling species of the genera [35]. Females identification is even harder because morphological characters may be either polymorphic or isomorphic among distinct species [36]. This approach is labor-intensive and time-consuming and, therefore, might not be adapted for routine identification of *Culex* females. Furthermore, field-caught specimens are rarely in perfect condition, which preclude any reliable morphological identification. For damaged mosquitoes or for immature stages, molecular tools can be an interesting alternative, especially to distinguish morphologically close species [37]. Barcoding and metabarcoding markers which combine DNA barcoding with high-throughput sequencing are sometimes used for delimitation and identification of species. However, species-level identification depends heavily on the choice of marker and the selected primer pair, often with a trade-off between successful species amplification and taxonomic resolution. Variation of a partial sequence of the cytochrome *c* oxidase subunit I (COI) gene is often used for the identification of mosquito species and contribute to discover cryptic diversity [38]. If on the one side, the COI barcode can be successfully used for delimiting and identifying numerous mosquito species, on the other hand recent studies on the *Culex* subgenus identification with this fragment indicate a poor resolution in separating species among complexes [36]. To improve species identification, multiple marker/primer pairs are often recommended and remains an expensive method for screening of large samples [39].

In the last decade, an innovative and cheaper proteomic tool named MALDI-TOF MS profiling, emerged for rapid mosquito species identification [40]. The principle is based on the matching of species-specific protein signatures of a specimen with reference spectra database. This promising tool has been reported to distinguish cryptic species of *Anopheles* genus with high efficiency [41]. Already used successfully for different taxonomic groups of insects, including culicids [42] and phlebotomids [43], standardization of protocols and optimized procedures could enable sharing all MS spectra reference and lead to the creation of an international MS database [44].

Thus, the aim of the present study was to assess whether this proteomic tool could distinct species from the neotropical *Culex* genus. In this way, field-caught mosquitoes from FG of the *Culex* genus were selected, classified by morphological criteria and confirmed by molecular barcoding. The legs and thoraxes of the selected *Culex* specimens were then submitted independently to MALDI-TOF MS to assess intra-species reproducibility and inter-species specificity of MS spectra. The establishment of such innovative tool for *Culex* mosquito identification should improve studies on this genus which are often hampered by the complexity of current methods for accurate classification.

## Materials and Methods

### 2. Mosquito Collection

Adult mosquitoes were collected from different sites of the coastal floodplain of FG. These mosquito collections occurred during entomological surveys conducted from November 2018 to January 2020, using backpack aspirators and CDC light traps baited (or not) with dry ice. Female specimens of *Culex* were sorted out and morphologically identified (whenever possible) using taxonomic keys [45–49] under a Leica M165 C stereomicroscope at a magnification up to  $\times 120$  (Leica Microsystems, Wetzlar, Germany). When specimens could not be identified until species level, they were grouped according to morphospecies using the genus name followed by the suffix 'sp.' combined with a number. Abdomen, thorax and legs of each mosquito specimen were dissected and kept individually at  $-20^{\circ}\text{C}$  for subsequent molecular and MALDI-TOF MS analyses. A total of 169 selected females were used in this study.

### 2. Molecular Identification of Mosquitoes

DNA was individually extracted from the abdomen of each mosquito specimen using the QIAamp DNA tissue extraction kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Sequences of a fragment of the cytochrome c oxidase subunit I (COI) gene from extracted DNA of each mosquito were amplified using DNA-barcode primers forward LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and reverse HC02198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'), according to protocols previously described [50]. After expected amplified DNA fragment size (658pb) verified by electrophoresis (1.5% agarose gel), samples were sent for sequencing. Sequences were edited using the Molecular Evolutionary Genetics Analysis (MEGA) X software, assembled and aligned in Clustal  $\omega$ 2 algorithm using the default parameters.

A reference database of *Culex* COI sequences obtained from sequences accessible in the Barcode of Life Data Systems (BOLD) [51] and in GenBank® [52] was used for identification and to construct a Maximum Likelihood tree using a General Time Reversible (GTR) model +G+I (Gamma distribution ; evolutionarily Invariable). Support for internal nodes was estimated using the nonparametric bootstrap method with 1000 replicates. By using the Nucleotide Basic Local Alignment Search Tool (BLAST) [53], the identity percentage and query coverage parameter was also extracted for each sample. Only specimens with identity  $>97\%$  were selected for this study. Each specimen was then assigned to a mosquito species name. Species identification through DNA-barcoding was used for all specimens including undetermined *Culex* female specimens but also for confirmation of specimens already identified by morphology.

### 2. Phylogenetic tree analyses based on COI sequences for MALDI-TOF comparison

COI sequences from specimens included in the MALDI-TOF reference database, were used for the creation of phylogenetic trees. Sequences were aligned through Clustal  $\omega$ 2 algorithm in MEGA X and applied to construct a Maximum Likelihood tree for thoraxes with similar analyses used for identification of the COI sequences to species level.

## 2. Sample homogenization and MALDI-TOF MS analysis

Thorax and legs of each mosquito specimen were prepared for MALDI-TOF MS as previously described [54,55]. After homogenization of each sample with mix buffer (1:1 solution of 70% (v/v) formic acid and 50% (v/v) acetonitrile), 1  $\mu$ L of the supernatant was spotted on the MALDI-TOF steel target plate (Bruker Daltonics, Wissembourg, France) in quadruplicate and air-dried. The spots were covered with 1  $\mu$ L of matrix solution containing saturated  $\alpha$ -cyano-4-hydroxycinnamic acid, accordingly to previous work [56]. To control matrix quality (*i.e.* absence of MS peaks due to matrix buffer impurities) and MALDI-TOF apparatus performance, matrix solution was loaded in duplicate onto each MALDI-TOF plate alone. Likewise, thorax and legs from one fresh specimen of *Ae. (Stg.) aegypti* Bora-Bora strain was loaded on each plate as quality control of sample preparation and MS spectra acquisition.

## 2.1 MALDI-TOF MS parameters

Protein mass profiles were obtained using a MicroFlex LT MALDI-TOF Mass Spectrometer (Bruker Daltonics, Germany), with detection in the linear positive-ion mode at a laser frequency of 50 Hz within a mass range of 2–20 kDa. The setting parameters of the MALDI-TOF MS apparatus were identical to those previously used [57]. MS spectra were acquired automatically using the AutoXecute of the Flex Control v.2.4 software (Bruker Daltonics).

## 2.2 MS spectra analysis

MS spectra profiles were controlled visually first with flexAnalysis v.3.3 software (Bruker Daltonics), then exported to ClinProTools v.2.2 and MALDI-Biotyper v.3.0 software (Bruker Daltonics) for data processing (smoothing, baseline subtraction, peak picking). MS spectra reproducibility was assessed by the comparison of the average spectral profiles (MSP, Main Spectrum Profile) obtained from the four spots for each specimen according to body part with MALDI-Biotyper (Bruker Daltonics). Additionally, mosquito body part MS spectra reproducibility and specificity were assessed using cluster analyses (MSP dendrogram) and the composite correlation index (CCI) tool. The MSP dendograms were performed from clusters of the specimens, based on comparison of their protein mass profile (*i.e.* their mass signals and intensities). The CCI tool from MALDI-Biotyper was also used to assess the spectral variations within and between each sample group, as previously described [58]. Higher correlation values (expressed as the mean  $\pm$  standard deviation, SD) reflecting higher reproducibility for the MS spectra, were used to estimate MS spectra distance between species for each body part. In addition, ClinProTools was used to identify discriminatory peaks among the 13 mosquito species for each body part. The top five of the most intense MS peaks, per mosquito species and per body part, were analyzed with ClinProTools to estimate their performance to discriminate the *Culex* species. The default parameter settings in ClinProTools for spectrum preparation were applied as previously described [54]. Based on the peak list obtained per species for each body part, the top five of the most intense m/z peaks were selected to include them into the genetic algorithm (GA) model. The selected peaks by the operator provided a recognition capability (RC) value bind to the highest cross-validation (CV) value. The presence or absence of all discriminating peak masses generated by the GA model was controlled by comparing the average spectra from each species per body part.

## 2.3 Database creation and blind tests

The MS reference database (DB) was created using spectra from paired legs and thorax of one to four specimens per species using MALDI-Biotyper (Bruker Daltonics). MS spectra were created with

an unbiased algorithm using information on the peak position, intensity and frequency. A total of 58 MSP from thoraxes or legs, were included in the reference MS spectra DB (Table 1). The raw MS spectra from legs and thoraxes of *Culex* mosquitoes are provided for free use (Supplementary file S1). MS spectra from mosquito legs and thoraxes of the 111 remaining specimens were tested against this in-house MS reference spectra DB. The reliability of species identification was estimated using the log score values (LSVs) obtained from the MALDI Biotyper software v.3.0, which ranged from 0 to According to previous studies [42,56,57], LSVs greater than 1.8 were considered reliable for species identification. Data were analyzed with Prism software v.7.00 (GraphPad, San Diego, CA, USA).

**Table** Overview of *Culex* mosquito origins and identification by COI molecular typing.

Collection			BOLD# accession number (number of associated sequences)	COI gene sequence coverage (%) / identity (%)	Number of specimens included in the reference MS DB per body part (Thoraxes/Legs)§
Species	Site	Year			
<i>Cx. (Mel.) adamesi</i>	Mac.	2018	1	FGMOS2220-20 (1)	99% / 97% 1 / 1
<i>Cx. (Cux.) declarator</i>	Mac.	2018, 2019	7	FGMOS2272-20 (7)	100% / 99– 100% 2 / 1
<i>Cx. (Mel.) dunni</i>	Mac.	2018, 2019	30	FGMOS2748-20 (20); FGMOS2750-20 (10)	100% / 98– 100% 4 / 3
<i>Cx. (Mel.) eastor</i>	Mac.	2018, 2019	3	FGMOS2695-20 (1); FGMOS2743-20 (1); FGMOS2752-20 (1)	100% / 99– 100% 1 / 1
<i>Cx. (Mel.) idottus</i>	Mac.	2018	2	FGMOS3098-23 (2)	100% / 100% 1 / 0
<i>Cx. (Cux.) nigripalpus</i>	Mac.	2018, 2019	12	FGMOS225-16 (12)	100% / 100% 3 / 2
<i>Cx. (Mel.) pedroi</i>	Mac.	2018, 2019	15	FGMOS2700-20 (12); FGMOS2758-20 (3)	100% / 100% 4 / 2
<i>Cx. (Mel.) phlogistus</i>	Mac.	2019	1	FGMOS1542-20 (1)	100% / 99% 1 / 1
<i>Cx. (Mel.) portesi</i>	Mac.	2018	28	FGMOS2416-20 (28)	100% / 100% 4 / 1
<i>Cx. (Cux.) quinquefasciatus</i>	Cay.	2019, 2020	34	FGMOS2275-20 (34)	100% / 100% 4 / 4
<i>Cx. (Mel.) rabanicolus</i>	Mac.	2018	5	FGMOS2744-20 (5)	100% / 100% 2 / 2
<i>Cx. (Mel.) spissipes</i>	Mac.	2018, 2019	9	FGMOS2701-20 (9)	100% / 97– 99% 3 / 2
<i>Cx. (Cux.) usquatus</i>	Mac., Rem.	2018, 2019	22	FGMOS046-16 (9); FGMOS049-16 (1); FGMOS2284-20 (12)	100% / 99– 100% 4 / 4
<b>Total</b>			<b>169</b>		<b>34 / 24</b>

#BOLD: Barcode of Life Data Systems; COI: cytochrome oxidase subunit 1; §Number of specimens used to create the reference MS database per body part; *Cx.*: *Culex*; *Cux.*: *Culex*; *Mel.*: *Melanoconion*; Mac.: Macouria; Cay.: Cayenne; Rem.: Remire-Montjoly.

## Results

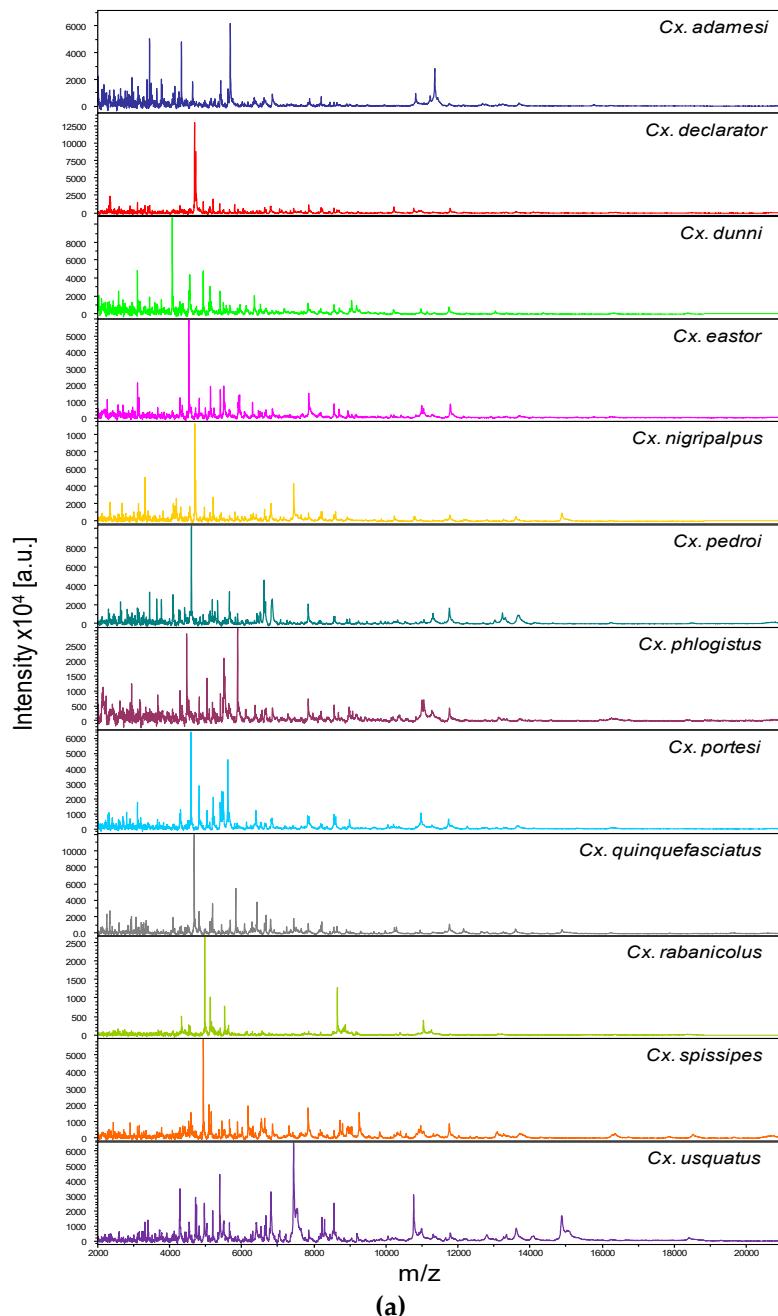
### 3. Morphological and molecular identification of *Culex* specimens

Field mosquitoes catching were performed during 2018 to 2020 in three distinct areas from FG (**Table 1**). Among the mosquitoes captured, only *Culex* female specimens were selected for the present study. The morphological identification was applied to 205 females. However, 29% (60/205) of the specimens were unsuccessfully classified to species level. All *Culex* specimens were then subjected to molecular analysis by DNA barcoding, using COI gene marker, and each COI sequence was queried against the BOLD system. Reliable species identifications were obtained for 169 females with identity and coverage ranges of 97–100% and 100%, respectively (**Table 1**). *Culex* females belonged to 13 distinct species classified in two subgenera. Four species were categorized in the *Culex* subgenus (*Cx. declarator*, *Cx. nigripalpus*, *Cx. quinquefasciatus* and *Cx. usquatus*), and the nine remaining species were classified in the *Melanoconion* subgenus (*Cx. adamesi*, *Cx. dunni*, *Cx. eastor*, *Cx. idottus*, *Cx. pedroi*, *Cx. phlogistus*, *Cx. portesi*, *Cx. rabaniculus* and *Cx. spissipes*). According to our data set availability, 1 to 34 specimens per species were acquired. Thirty four *Culex* specimens encompassing the 13 species and used for MALDI-TOF MS reference database were selected for the phylogenetic analysis based on COI gene sequences (**Supplementary file S2**). Specimens are successfully assembled and classified by species.

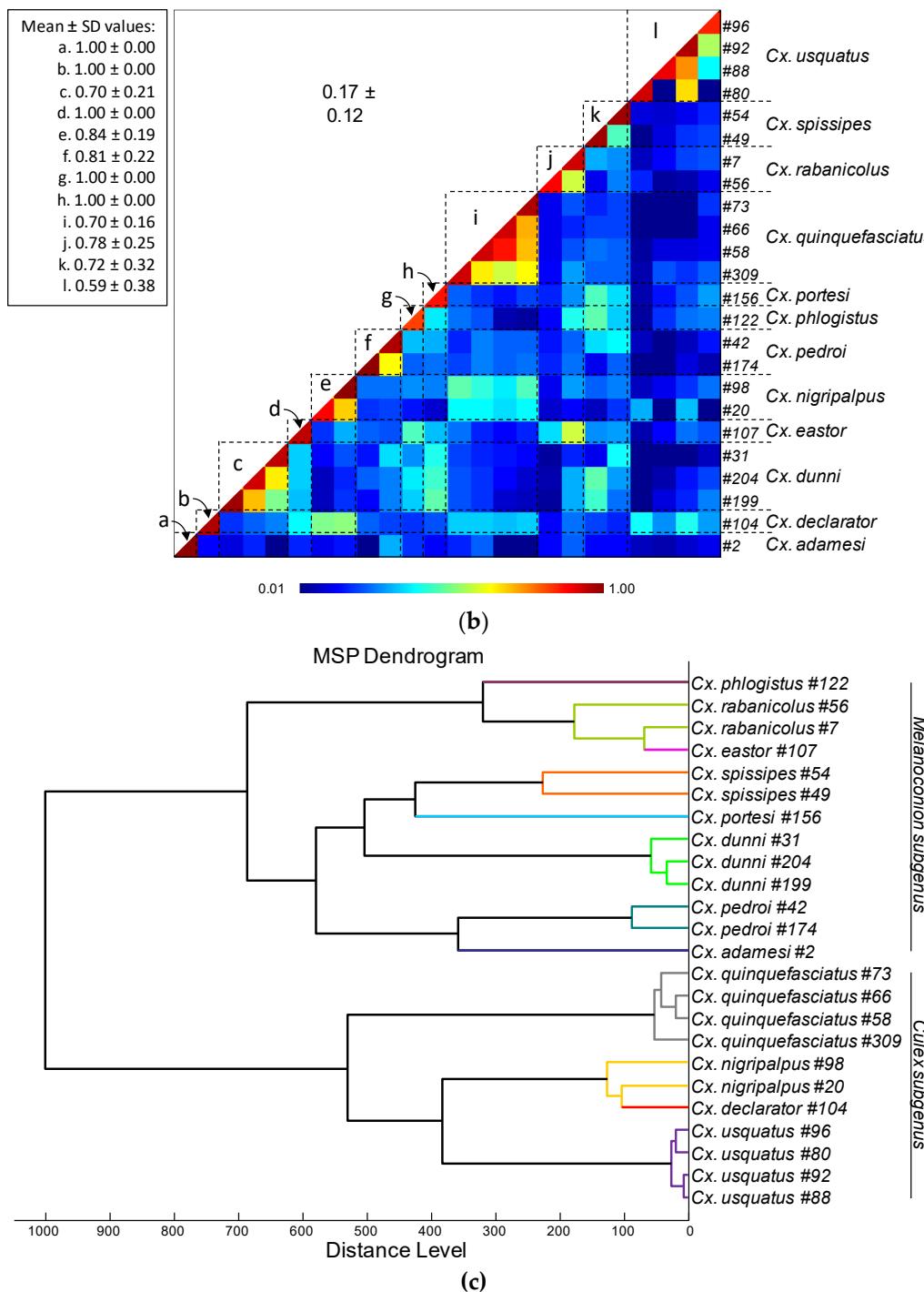
### 3. Reproducible and specific MS spectra from two *Culex* body parts

Among the 1352 MS spectra (169 specimens  $\times$  two body parts  $\times$  four replicates) acquired, MS profiles of high intensity ( $>3000$  a.u.) were reached for the vast majority of the samples ( $>95\%$ ). The samples presenting MS spectra with low intensity were not selected for their introduction as reference spectra in the MS DB. For example, MS spectra of legs from the two *Cx. idottus* specimens were excluded of the analysis due to the low intensity and inter-sample heterogeneity of MS profiles (**Supplementary file S3**). Then, in total, MS spectra of 12 and 13 *Culex* species were analyzed for legs and thoraxes, respectively. The visual comparison of MS profiles acquired for legs (**Figure 1A**) and thoraxes (**Figure 2A**) differed importantly among the *Culex* species. To assess the reproducibility and specificity of MS spectra per body part according to species, CCI and cluster analyses were carried out. MS spectra of high intensity ( $>3000$  a.u.) from paired legs ( $n=24$ ) and thorax ( $n=34$ ) of one to four specimens per species were selected for these analyses. The mean CCI-values obtained among MS spectra per body part and per species were elevated, ranging from 0.59 to 0.84 for legs (**Figure 1B**) and from 0.66 to 0.93 for thoraxes (**Figure 2B**). Conversely, the mean CCI-values obtained between species for legs and thoraxes were very low ( $<0.20$ ), supporting the high MS spectra species-specificity. Nevertheless, it is interesting to notice, that elevate CCI-values were observed for thoraxes MS spectra between *Cx. idottus* and *Cx. rabaniculus* (mean  $\pm$  SD:  $0.77 \pm 0.01$ ).

The MSP dendograms created per body part with the same MS spectra, revealed the clustering of specimens from the same species on the same branch for each *Culex* species for legs (**Figure 1C**) and thoraxes (**Figure 2C**). The proximity and low distance of branches between *Cx. idottus* and *Cx. rabaniculus* for thorax body part, confirmed the proximity of their MS spectra. However, the absence of species interlacing emphasized the reproducibility and specificity of the protein profiles for both body parts. Although, a clustering of *Culex* species from the same subgenus was obtained on both MSP dendograms, the separation was clearer for MS spectra from legs than from thoraxes. Interestingly, the ordination of the species on MSP dendrogram was similar to those obtained in the phylogenetic tree from COI gene sequences of the 34 *Culex* specimens (**Supplementary file S2**).

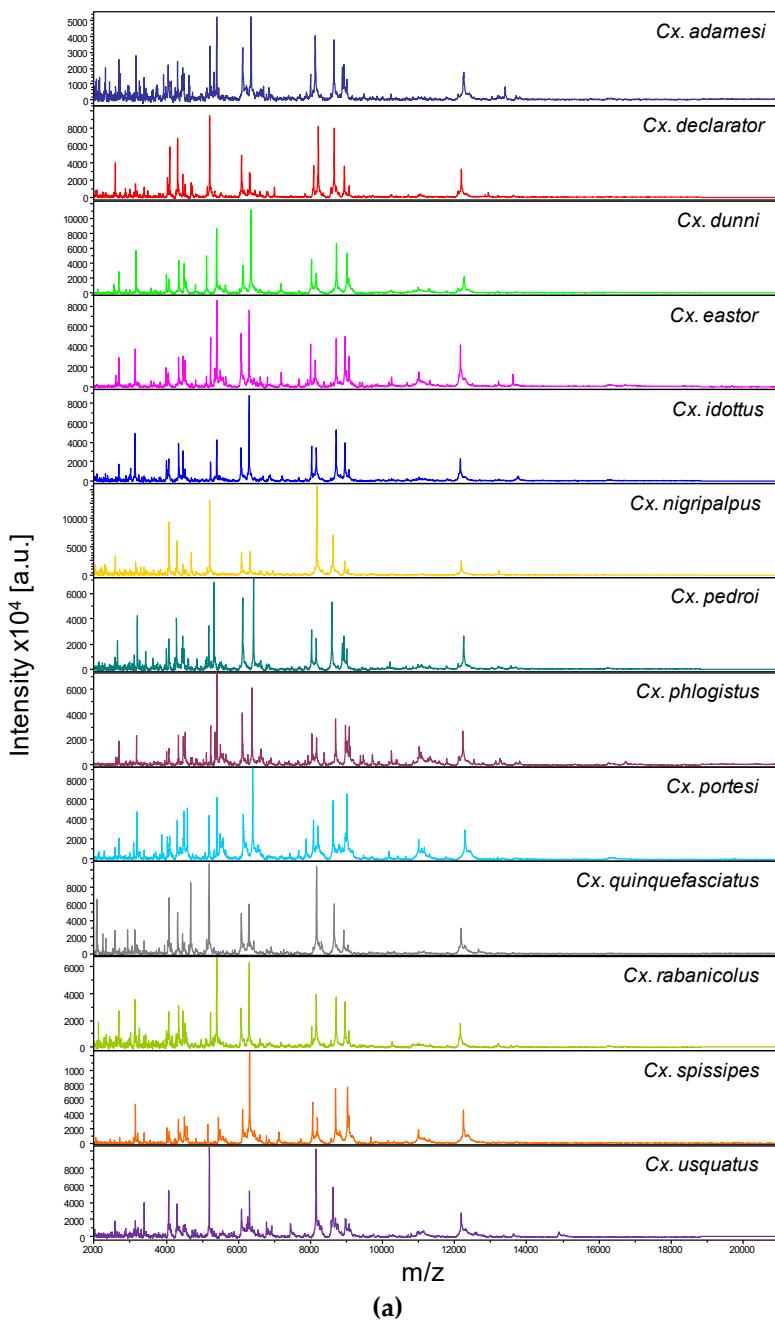


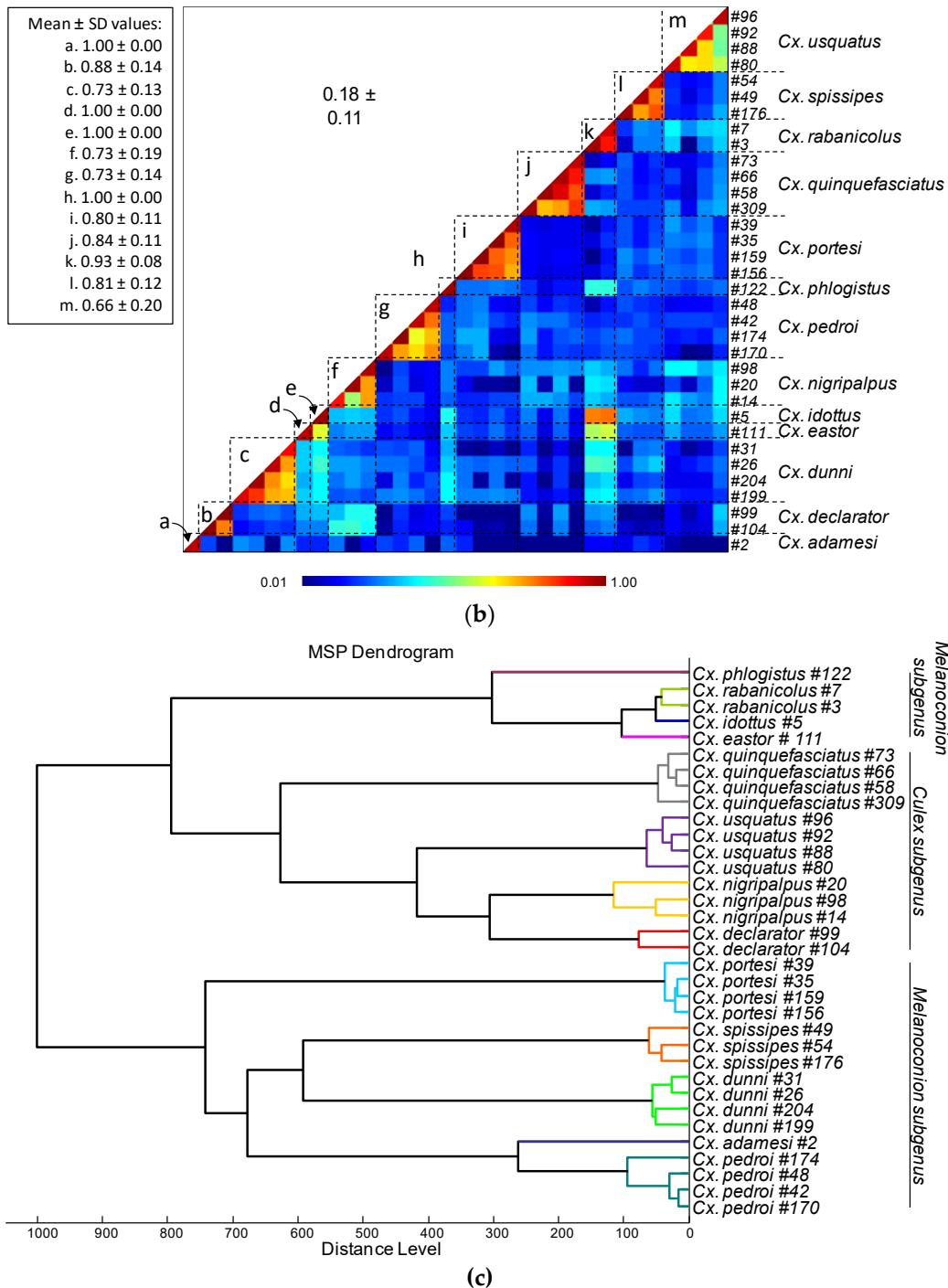
(a)



**Figure 1.** Comparison of MALDI-TOF MS spectra from legs of *Culex* mosquitoes. **(a)** Representative legs MS spectra of *Culex* species. The name of each species was indicated on the right corner of each spectrum. **(b)** Composite Correlation Index (CCI) matrix value representing the levels of legs MS spectra reproducibility among *Culex* specimens of the same species and between species. The 24 specimens from the 12 *Culex* species, which were selected as reference MS spectra introduced in the MS DB, are shown. 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 software. **(c)** MSP dendrogram of MALDI-TOF MS spectra from legs of *Culex* mosquitoes, from the 24 *Culex* specimens selected as reference MS spectra. The *Culex* and *Melanoconion* subgenus were indicated at the right part. The distance units correspond to the relative similarity of MS spectra. The dendrogram was created by Biotyper v.3.0 software. The

same color code was used for specimens of the same *Culex* species between panels (a) and (c). a.u., arbitrary units; Cx., *Culex*; MSP, Main Spectrum Profile; m/z, mass-to-charge ratio; SD, standard deviation.





**Figure 2.** Comparison of MALDI-TOF MS spectra from thoraxes of *Culex* mosquitoes. **(a)** Representative thoraxes MS spectra of *Culex* species. The name of each species was indicated on the right corner of each spectrum. **(b)** Composite Correlation Index (CCI) matrix value representing the levels of thoraxes MS spectra reproducibility among *Culex* specimens of the same species and between species. The 34 specimens from the 13 *Culex* species, which were selected as reference MS spectra introduced in the MS DB, are shown. 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 software. **(c)** MSP dendrogram of MALDI-TOF MS spectra from thoraxes of *Culex* mosquitoes, from the 34 *Culex* specimens selected as reference MS spectra. The *Culex* and *Melanoconion* subgenus were indicated at the right part. The distance units correspond to the relative similarity of MS spectra. The dendrogram was created by

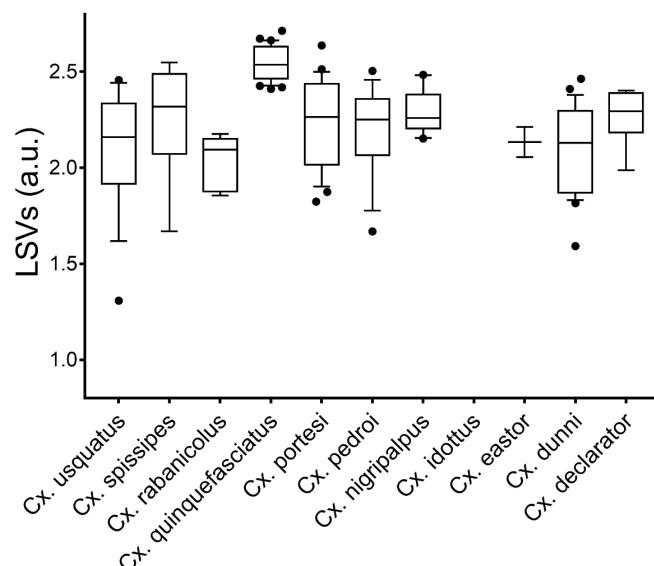
MALDI-Biotyper v.3.0 software. The same color code was used for specimens of the same *Culex* species between panels (a) and (c). a.u., arbitrary units; *Cx.*, *Culex*; MSP, Main Spectrum Profile; m/z, mass-to-charge ratio; SD, standard deviation.

### 3. Leg and Thorax Biomarkers Distinguishing *Culex* Species

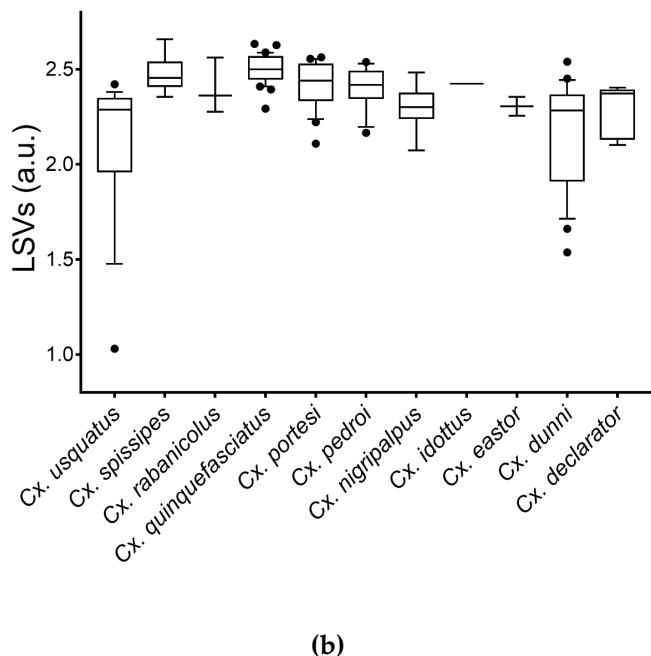
To identify discriminatory MS peaks among the 13 *Culex* species for legs and thoraxes, MS spectra from the 169 specimens were analyzed using the GA tool from ClinProTools 2.2 software. As the accuracy of identification is directly linked to the intensity of MS spectra, the analysis was carried out on the most intense mass peaks from legs and thoraxes per mosquito species. After verification of the peak report in the average spectrum, the selection of the top-five mass peak lists per species conducted to a total of 29 and 26 MS peaks for legs and thoraxes, respectively (**Supplementary files S4 and S5**). The inclusion of these MS peak lists in the GA model displayed respective recognition capability (RC) and cross-validation (CV) values of 93.2% and 91.4% for legs and 94.5% and 86.5% for thoraxes. When the top 10 mass peak lists per species was selected, a total of 37 and 39 MS peaks were found for legs and thoraxes, respectively. The application of the GA model on the top 10 mass peak lists did not improve RC and CV values for legs, 92.8% and 91.4%, respectively, whereas, for thoraxes the CV reached 90.1% and the RC remained at 93.7%. These results underlined that the top five mass peak list per species appeared as the main MS peaks to discriminate these *Culex* species for both body parts. The comparison of the top five mass peak list between legs and thoraxes indicated that three mass-to-charge ratio were similar (at about 5206, 5405 and 6426 m/z), confirming the high specificity of MS spectra per body part (**Supplementary files S4 and S5**).

### 3. Creation of the MS reference spectra database and validation steps

MS spectra from 12 and 13 *Culex* species for legs (n=24) and thoraxes (n=34), respectively, including one to four specimens per species, were selected for database creation (**Table 1**). MS spectra from the remaining specimens (legs, n=145; thoraxes, n=135) were queried against the DB. The LSVs ranged from 1.31 to 2.71 for legs (**Figure 3A**) and from 1.03 to 2.66 for thoraxes (**Figure 3B**). For a reliable identification, a LSV upper than the 1.8 threshold is required [42,56]. In these conditions, a correct identification (LSVs>1.8) could be considered for 95.9% (n=139/145) of legs MS spectra and 94.8% (n=128/135) for thoraxes.



(a)



(b)

**Figure 3.** LSVs from query against homemade MS reference database. MS spectra of legs **(a)** and thoraxes **(b)** from *Culex* mosquitoes are presented. Dashed line represents the threshold value (LSV  $\geq$  1.8), for relevant identification. a.u., arbitrary units; LSV, log score value.

Among these MS spectra reaching the LSVs threshold, 100% of the identification results were concordant with the molecular results. Interestingly, if paired samples per specimen were considered ( $n=133$ ), solely two specimens of *Cx. usquatus* failed to obtain a LSV upper than 1.8 for at least one body part (Supplementary file S6). If the LSV cut-off is increased at 2.0 to improve identification confidence, the rate of the specimens which reached this threshold reduces to 78.2% ( $n=104/133$ ) for legs and 90.2% ( $n=120/133$ ) for thoraxes. However, 91.0% ( $n=121/133$ ) of paired samples per specimen achieved this higher threshold in at least one body part.

## Discussion

MALDI-TOF MS is a method routinely used in microbiological diagnostic laboratory for identification of bacteria and archaea [59], but the relevance of the technique remains largely underestimated for identification of multicellular organisms. The success of the MALDI-TOF MS for arthropod identification in this last decade, highlights this emerging tool as a relevant alternative for mosquitoes species identification [43,60–62]. As many mosquitoes species from the *Culex* genus have been proven to be vectors of pathogenic agents of human and veterinary importance [63–65], an accurate classification of the specimens is primordial. The present work demonstrated that MALDI-TOF MS can be used as an alternative to current methods for *Culex* female identification at the adult stage, using legs and thorax.

Prior MS submission, a relevant classification of the *Culex* mosquitoes per species was compulsory. Firstly, the morphological identification was performed allowing to classify mosquitoes from only five distinct species, two from the *Culex* subgenus (*Cx. quinquefasciatus* and *Cx. usquatus*) and three from the *Melanoconion* subgenus (*Cx. portesi*, *Cx. pedroi* and *Cx. spissipes*). The accuracy of this morphological classification was confirmed by molecular DNA barcoding of the COI gene. Morphological method failed to identify at the species level 29% ( $n=60$ ) of *Culex* mosquitoes. The limitation of classification of mosquitoes by morphological approach was also reported for others genera [36,66,67]. For instance, in the *Anopheles* genus, the members of the Gambiae Complex and Funestus Group could not be distinguished uniquely by examining external features of their anatomy

[68,69]. Moreover, the skill and regular training of entomological experts is also another factor playing a role in the capacity of correct specimen identification [70]. The genus *Culex* is recognized as a highly diverse group of mosquitoes for which delimitation and identification of species is particularly difficult. Complementary methods are therefore required for improving species identification.

DNA barcoding using the mitochondrial COI gene is widely used for species identification in molecular taxonomy [71]. A first COI barcoding database, available in the BOLD system, established based on morphological classification of male *Culex* specimens of FG [3] was used to validate morphological identification and to classify the unidentified females of this study. The COI gene has been recorded as an effective and accessible DNA barcode that provides a high performance in delimiting species within the subgenus *Melanoconion* of *Culex* species, a subgenus displaying the highest species diversity in tropical regions [66]. However, others studies also applied COI barcode fragment for identification of species within the subgenus *Culex* and concluded that this barcode do not contain enough information to distinguish species within this subgenus [36]. Based on COI sequences from Brazil and Argentina, Laurito et al. [36] highlighted that *Cx. declarator* cannot be differentiated from *Cx. bidens* Dyar and *Cx. tatoi* Casal & García. However, these last two species have never been detected in FG [3], and only *Cx. declarator* was considered here. Similarly, COI sequences of *Cx. usquatus* can be confused with *Cx. camposi* Dyar, *Cx. coronator*, *Cx. maxi* Dyar and *Cx. surinamensis* [36]. In FG, only *Cx. coronator*, *Cx. surinamensis* and *Cx. usquatus* have already been reported. Nevertheless, a recent review of *Culex* species from FG revealed that specimens identified as *Cx. coronator* in historical literature were closer to the lectotype of *Cx. usquatus* described from Suriname [3]. *Culex usquatus* is morphologically distinguishable from *Cx. surinamensis*, thus confirming that COI sequences classified as *Cx. usquatus* could be attributed to this species in this study.

To improve the identification, molecular tools often develop new algorithms of analysis or add others molecular markers. For *Anopheles* species, COI is not enough to discriminate species of the *Gambiae* Complex. The addition of ITS2 analysis increases greatly the potential of identification of these species [72]. Indeed, the understanding lack on COI sequences analysis highlight the need to explore complementary or innovative tools.

The proteomic MALDI-TOF MS approach is increasingly used for rapid arthropod identification [42,43,60,61,73]. Indeed, to improve the intra-species reproducibility of MS spectra and to share reference protein profiles, protocols were standardized [56,74]. In order to develop an accessible mosquito spectra database of *Culex* species, the present study employed MALDI-TOF MS double spectra biotyping strategy. Here, for each specimen tested and for DB creation, legs and thorax from the same specimen were independently submitted to MS analysis, whenever possible. Specific MS spectra from the 13 *Culex* species per body part were obtained. The cluster analysis on MSP dendrogram confirmed this species specificity. Besides, similar ordination of the specimens for both body parts with the COI phylogenetic tree, revealed a relationship between molecular taxonomic classification and MS spectra profiles. However, as MS spectra from closely-related species were grouped in the same branch of MS dendrogram, a mismatching could occur. To prevent misidentification, the legs and thorax were independently query against the DB. This double query allowed to corroborate identification of each specimen using both body parts, enhancing the identification confidence [55]. In this study, 100% of the samples were correctly identified at the species level on both body parts and the rate of relevant identification (LSVs>1.8) was about 95% for each ones. Interestingly, a higher reproducibility of MS spectra per species was obtained for thorax compared to legs. The lower reproducibility of legs could be attributed to the number of legs available for each specimen. Effectively, legs are breakable and during the catching or storing period some could be lost, which could induce heterogeneity of MS profile intensities among specimens from the same species [75]. In the cases of all the legs were lost, the specimen could be always identified using thorax compartment. Then, the thorax appears as the more appropriate compartment for mosquito identification, followed by legs accordingly to previous work [44]. Furthermore, these two body parts does not prevent screening for viruses, parasites, or source of blood feeding which can be researched

in the head or abdomen by molecular [76,77] or MALDI-TOF MS methods [78,79]. These complementary data are primordial in the frame of surveillance programs.

Quality of MS spectra can be altered by various factors such as sample homogenization, quantity of mix buffer, engorgement status, storage conditions or even geographical origin of the collection [42,55,75]. The storage is too critical for field caught mosquitoes. The best method for long storage of arthropods for MALDI-TOF MS analysis is frozen or to maintain them at RT with silica gel [80], when immediate freezing is not possible. In this study, all the specimens were field-derived, dried and stored frozen at -20°C in tubes or plates from one to three years. The elevate intra-species reproducibility and inter-species specificity of MS spectra for both body parts, suggested that specimens were appropriately preserved. Among the 169 mosquitoes tested, only two *Cx. usquatus* failed, on both body parts, to reach the relevant threshold value (LSV>1.8). These low LSVs could be attributed to improper storing of the mosquitoes. It is possible that protein degradation occurred in these specimens conducting to lower quality of MS spectra, as reported in previous studies [58,81,82].

It is noteworthy that all the *Culex* species selected in this study were correctly classified using MALDI-TOF MS profiling, with concordant results between thorax and legs, and in agreement with molecular identification. Three species from the *Melanoconion* subgenus, *Cx. idottus*, *Cx. phlogistus* and *Cx. rabaniculus*, presenting isomorphic traits among females and which could not be accurately distinguished morphologically, were unambiguously identified by this proteomic tool.

The present spectral database, created using thoraxes and legs from females of 13 *Culex* species from FG, represents the first attempt to create a MALDI-TOF MS database for identification of neotropical *Culex* species. However, the database encompass only 12.5% (n=13/104) of the total number of *Culex* species known in FG and solely two out of the eight subgenera from the *Culex* genus of the mosquitoes actually inventoried in the territory [3]. The widening of this MALDI-TOF MS spectra reference DB with missing *Culex* species, become compulsory for application of this tool in the monitoring of *Culex* genus vectors in FG. An upgrading of this DB including others mosquito species occurring in FG (n=242) will improve its usage and may be helpful to discriminate cryptic or close-related species. MS spectra for the identification of eight distinct *Anopheles* species from FG, among which four are malaria vectors, are already available [54].

Several works reported the efficiency of the MALDI-TOF MS for identification of mosquitoes at immature stages [83,84] and demonstrated its interest for management of Culicidae larval habitats [85]. As larva can be confusing to distinguish morphologically, it could be interesting to assess MALDI-TOF MS for identification of immature stages. MALDI-TOF MS appears then highly promising for discriminating mosquito fauna [41,56,82].

The present work demonstrated that MALDI-TOF MS could be an alternative to current methods for identification of *Culex* female, using dissected legs and thorax. The double body part protein signature of each specimen improved the identification quality. Raw MS spectra from legs and thorax of these 13 *Culex* species included in the DB are freely available (**Supplementary file S1**). The sharing of reference MS spectra is essential in the frame of the creation of an international DB. Except for the expensive cost of the MALDI-TOF MS instrument, this approach is highly competitive economically compared to current molecular methods. It does not require particular skills and could be used for "live" monitoring of vectors due to its short time of handling and obtaining results. This approach appears suitable for the identification of mosquitoes from the *Culex* genus, for which morphological and molecular methods remains time consuming and with a potential risk of misidentification. In the near future, the widening of this reference MS spectra DB with specimens from others *Culex* species, at both adult and immature stages, could improve knowledge of these species for adapted surveillance and control measures to reduce the risks of pathogen transmission not exclusively to French Guiana.

**Supplementary Materials:** The following supporting information is available and can be downloaded: Supplementary File S1; Supplementary File S2; Supplementary File S3; Supplementary File S4; Supplementary File S5 and Supplementary File S6.

**Author Contributions:** Conceptualization and methodology of the experiments: L.A.; experiments investigation and tools contribution: M.M.C. and A.G.; data analysis: L.A., A.G., M.M.C. and S.B.; field collections preparation: R.C., P.G. and A.G.; field collections dissection: A.G., J.I. and H.N.; writing—original draft preparation: L.A., S.B., M.M.C. and A.G.; funding acquisition: L.A., J.B.D., I.D. and A.L. All authors have read and agreed to the published version of the manuscript.

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