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Evaluation of matrix-assisted laser desorption/ionization time of flight mass spectrometry for the identification of ceratopogonid and culicid larvae

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SUMMARY

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) was evaluated for the rapid identification of ceratopogonid larvae. Optimal sample preparation as evaluated with laboratory-reared biting midges *Culicoides nubeculosus* was the homogenization of gut-less larvae in 10% formic acid, and analysis of 0·2 mg/ml crude protein homogenate mixed with SA matrix at a ratio of 1:1·5. Using 5 larvae each of 4 ceratopogonid species (*C. nubeculosus*, *C. obsoletus*, *C. decor*, and *Dasyhelea* sp.) and of 2 culicid species (*Aedes aegypti*, *Ae. japonicus*), biomarker mass sets between 27 and 33 masses were determined. In a validation study, 67 larvae belonging to the target species were correctly identified by automated database-based identification (91%) or manual full comparison (9%). Four specimens of non-target species did not yield identification. As anticipated for holometabolous insects, the biomarker mass sets of adults cannot be used for the identification of larvae, and vice versa, because they share only very few similar masses as shown for *C. nubeculosus*, *C. obsoletus*, and *Ae. japonicus*. Thus, protein profiling by MALDI-TOF as a quick, inexpensive and accurate alternative tool is applicable to identify insect larvae of vector species collected in the field.

Key words: *Culicoides*, Ceratopogonidae, Culicidae, MALDI-TOF MS, identification, insect, larvae, vector.

INTRODUCTION

Culicoides biting midges (Diptera: Ceratopogonidae) are tiny haematophagous insects that can be a nuisance to humans and animals (Mellor *et al*. 2000). More importantly, they may cause chronic insect bite hypersensitivity in equines (Hellberg *et al*. 2009; Sloet van Oldruitenborgh-Oosterbaan *et al*. 2009) and they are incriminated or suspected as vectors of a wide variety of pathogens, including nematodes and protozoa, but mainly of viruses such as bluetongue virus, African horse sickness virus, epizootic haemorrhagic disease virus (Mellor *et al*. 2000), the Toggenburg orbivirus (Planzer *et al*. 2011) and the Orthobunya virus ('Schmallenberg virus') that very recently emerged in Europe (Hoffmann *et al*. 2011; Rasmussen *et al*. 2012). Options to control biting midges focus on insecticide treatments of host animals but the effectiveness of such interventions is controversial (Carpenter *et al*. 2008; Bauer *et al*. 2009; Venail *et al*. 2011). Alternatively, the midges' breeding sites can be targeted by applying chemical or biological agents to kill the larvae or by removing these habitats (Carpenter *et al*. 2008; Ansari *et al*. 2011). *Culicoides* larvae are in general reported to dwell in aquatic and semi-aquatic habitats or are soil-living (Kettle and Lawson, 1952). Specific breeding sites described in Europe are e.g. salt marshes, moorlands, livestock dung piles and silages (Kettle and Lawson, 1952; Blackwell and King, 1997; Uslu and Dik, 2007; Zimmer *et al*. 2008). *Culicoides* larvae are recognized by their lashing or eel-like swimming movements (Kettle and Lawson, 1952; Mellor *et al*. 2000) but their morphological identification, mainly based on features of the head and on the pigmentation (Kettle and Lawson, 1952) is difficult. As an alternative, larvae can be reared to adults, whose morphological identification is better established, but this is a laborious and rather inefficient approach. Genetic identification by PCR has been described for many *Culicoides* species (summarized by Wenk *et al*. 2012) but only a single or few (in multiplexed assays) species can be identified in a test and, thus, several tests may be required until a specimen, particularly of an unexpected or rare species, is identified.

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) is a well-established technique for high throughput, accurate and reproducible identification of clinically relevant micro-organisms (bacteria, yeasts, filamentous fungi) at low cost and with minimal sample preparation (Mellmann *et al*. 2009; Santos *et al*. 2010;

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Sauer and Kliem, 2010; Stevenson *et al*. 2010; Van Veen *et al*. 2010). Organisms are rapidly identified by comparing their MALDI-TOF spectra with all the biomarker mass sets of reference specimens available in databases. This proteomic approach has also been applied in a few studies to identify metazoans, namely fish species (Mazzeo *et al*. 2008), plants (lentil varieties; Caprioli *et al*. 2010), ticks (Karger *et al*. 2012), and insects [*Drosophila* spp. (Campbell, 2005; Feltens *et al*. 2010) and aphid species (Perera *et al*. 2005)]. Recently, we have demonstrated the suitability of MALDI-TOF MS to characterize *Culicoides* flies (Kaufmann *et al*. 2011, 2012). Whereas the different developmental life stages (nymphs, adults) of a hemimetabolous aphid yielded similar MALDI-TOF MS protein profiles (Perera *et al*. 2005), no corresponding data are as yet available for the juvenile stages (larvae, pupae) of holometabolous insects.

The aim of this study was to develop the first reference database of MALDI-TOF biomarker mass sets to identify larval stages of holometabolous Ceratopogonidae, including the larvae of mosquitoes (*Aedes aegypti, Ae. japonicus*) as an outgroup.

MATERIALS AND METHODS

Insect colonies

Culicoides nubeculosus biting midges (initially kindly provided by the IAH, Pirbright, UK) and mosquitoes (*Aedes aegypti* [Rockefeller], kindly provided by the Swiss TPH, Basel, Switzerland); *Ae. japonicus*, field collected) were maintained at 24±0·5 °C, 85±5% relative humidity under long-day conditions (14L, 10D). Larvae were fed with pulverized fish food (TetraMin®) and adults with 10% glucose solution. For egg production, bloodmeals were given through a Nescofilm®-membrane. Colonies of *C. nubeculosus* and *Ae. aegypti* were kept as described (Boorman, 1974; Timmermann and Briegel, 1993). *Aedes japonicus* were reared from field-collected eggs from Switzerland.

Collection and isolation of ceratopogonid larvae

Larvae of *Culicoides* and *Forcipomyia* were obtained from the uppermost few centimetres of soil collected from putative breeding sites (e.g. humid, shaded, around decomposing plant material) in the Zürich region, either on a farm with dairy cows and pigs or around the Institute of Parasitology with cows and sheep in the near vicinity. Prior studies at these sites had shown that mainly *C. obsoletus* was present (as determined by genetic analyses; C. Kaufmann, *unpublished observations*). In addition, larvae of *C. decor* from Guadeloupe (Caribbean), collected at 1250 metres above sea level in water retained in leaf axils of bromeliads plants, were available. Larvae of midges belonging to the genus *Dasyhelea* were obtained from vases from a cemetery in Zürich. For morphological identification (see below), some of the field-collected larvae of *C. decor*, *Dasyhelea* sp. and *Forcipomyia* sp. were reared to adults either in water in a mix of soil and ground TetraMin® as a food source (*C. decor* and *Dasyhelea* sp.) or in moist soil (*Forcipomyia* sp.).

Larvae were separated from soil samples by a combination of sequential sieving and flotation adapted from the literature (Kettle and Lawson, 1952; Khamala, 1975; Kline *et al*. 1975; Hribar, 1990). Briefly, the soil samples were washed with water through stacked analytical sieves (Retsch®, Haan, Germany) with mesh sizes of 2 mm, 600 μm, 400 μm, 200 μm and 150 μm. The material retained in the 2 mm mesh sieve was discarded, whereas the material from the other sieves was pooled in the 150 μm sieve and subjected to flotation in a 30% (w/v) sugar solution (Kidder, 1997). Floating *Culicoides* biting midge larvae were identified by their typical eel-like swimming movements (Mellor *et al*. 2000), collected by pipette and washed with distilled water to remove remnants of the sugar solution. Larvae were kept overnight at 4 °C in distilled water.

Preparation of larvae

Larvae were rinsed 3 times with distilled water and carefully immobilized on a glass slide under a cover slip. Head length and width were recorded using a stereomicroscope (Olympus SZX10) and CellSens software (Olympus Europa Holding GmbH, Hamburg, Germany) in order classify the specimens into similar larval stages.

The larval gut with its content was removed using forceps (Regine Nr. 5, Morbio Inferiore, Switzerland) which were rinsed with distilled H_2O and 70% ethanol between each dissection. For genetic identification, the last 2 abdominal segments of all field-caught larvae were stored dry in a 1·5 ml Eppendorf tube at −20 °C. The remaining larval parts as well as unprocessed larvae to be used for MALDI-TOF MS analyses were stored separately in 70% ethanol at 4 °C.

Genetic identification

DNA was extracted from the last 2 abdominal segments of ceratopogonid larvae with the Qiamp DNA mini kit (Qiagen, Hombrechtikon, Switzerland) using the 'tissue protocol'. DNA from abdomens of adults was isolated as described (Wenk *et al*. 2012). The DNA was used in species-specific real-time polymerase chain reactions (PCR) or conventional PCRs (Wenk *et al*. 2012) followed by sequencing of the purified amplicons (MinElute® PCR purification kit, Qiagen) by a private company

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(Synergene Biotech, Schlieren, Switzerland), and the sequences were blasted against GenBank (www.ncbi.nlm.nih.gov). In cases where novel sequences were obtained from field-collected larvae, specimens from the same sample were reared to adults that were morphologically and genetically characterized, allowing assignment of a species to the larval stages. Sequences are deposited in GenBank under Accession numbers JN657064–JN657080.

Sample preparation for MALDI-TOF MS

Residual ethanol from the larval samples was removed in a Barnstead Genevac miVac concentrator (Ipswich, England). Depending on dimensions determined under the stereomicroscope, single larvae were re-suspended in 15, 40, 60 or 240 μl of homogenization solution (10% formic acid), then homogenized for 1 min using a manual homogenizer (BioVortexer, Fisher Scientific, Wohlen, Switzerland) with disposable pellet pestles. After a short centrifugation (5000 ***g*** for 30 s), the supernatant was transferred into a new 1·5 ml Eppendorf tube. Pools (n = 20) of *C. nubeculosus* were re-suspended in 100 μl of homogenization solution and proceeded as above.

Crude protein concentration was determined using a modified Bio-Rad Protein Assay (Bradford, 1976) by adding NaOH to the diluted dye reagent to a final concentration of 0·13 M. The absorbance was measured with a Multiskan RC (Thermo Labsystems, Zürich, Switzerland) at λ = 595 nm, and bovine serum albumin (Sigma Aldrich, Buchs, Switzerland), dissolved in 10% formic acid, was used as the standard. Adjusted with 10% formic acid to the desired concentration, the supernatant was mixed with SA matrix (saturated solution of sinapic acid in 60% acetonitrile and 0·3% trifluoracetic acid) at a ratio 1:1·5 (homogenate to SA matrix) and incubated for 5 min at room temperature. Then 1 μl aliquots of the mixture were spotted onto a steel target plate and air-dried prior to MALDI-TOF MS analysis.

Pupae of *C. nubeculosus* were processed as described above for larvae with the exception that the gut was not removed (dissection not feasible because of the metamorphosis process). Adults of *C. nubeculosus* were processed according to the protocol of Kaufmann *et al.* (2011).

MALDI-TOF MS analysis

Protein mass fingerprints were obtained using a MALDI-TOF Mass Spectrometry AXIMA™ Confidence machine (Shimadzu-Biotech Corp., Kyoto, Japan) with the specifications described earlier (Kaufmann *et al.* 2012). For the generation of biomarker mass sets, protein mass fingerprints were determined in triplicate from 5 larvae of each of 3 haematophagous and 1 non-biting midge species as well as from 2 culicid species. From the laboratory-reared species, larval instars 3 and 4 were used, and similarly sized instars from the field-caught larvae were selected (instar not determined, but most probably instar 3 or 4). All spectra had at least 30 peaks. The peak lists were imported into SARAMIS™ (AnagnosTec, Potsdam-Golm, Germany), trimmed to a mass range of 2 to 20 kDa, and peaks with a relative intensity below 1% were removed. Peak lists were binned and average biomarker masses were calculated using the SARAMIS™ SuperSpectrum tool with an error of 800 ppm. The specificity of these potential biomarker masses was determined by comparison against the whole SARAMIS spectral archive and additional Mabritec-owned spectral data sets including more than 90 000 spectra covering >2700 different species of various taxa (mostly bacteria, but also fungi, eukaryotic cell lines, and few insects, see Kaufmann *et al.* 2011, 2012). In accordance with the SARAMIS user guidelines, the threshold for identification was set at 75% biomarker matches based on the reference data set. Peak matrix generation for unsupervised cluster analysis was done as described (Kaufmann *et al.* 2011).

Validation study

For validation of the biomarker mass sets, 71 ceratopogonid (n = 59) and culicid (n = 12) larvae were analysed in duplicate. These included laboratory-reared (n = 22) and field-collected (n = 49) larvae belonging to one of the species for which a biomarker mass set was generated. As non-target species, field-collected larvae of *C. pulicaris* (n = 1) and *Forcipomyia* sp. (n = 3) were used. Generated mass fingerprints were imported into SARAMIS software for automated identification against >3400 biomarker mass sets, including insect species-specific ones, and, if required, for manual full spectra comparison against the insect reference library.

RESULTS

In the first step, MALDI-TOF profiles of a total of 228 *C. nubeculosus* larvae were analysed to evaluate the influence of parameters of sample preparation (e.g. parts of larvae used, protein concentration) to establish the proof-of-principle using protein profiling for holometabolous insect larvae.

The presence of the larvae's gut strongly impaired the protein profile, as shown in Fig. 1. The profile obtained from an entire *C. nubeculosus* larva is depicted in Fig. 1A, showing a high background noise and a suppression effect in the lower mass range as revealed by the baseline shift which is caused

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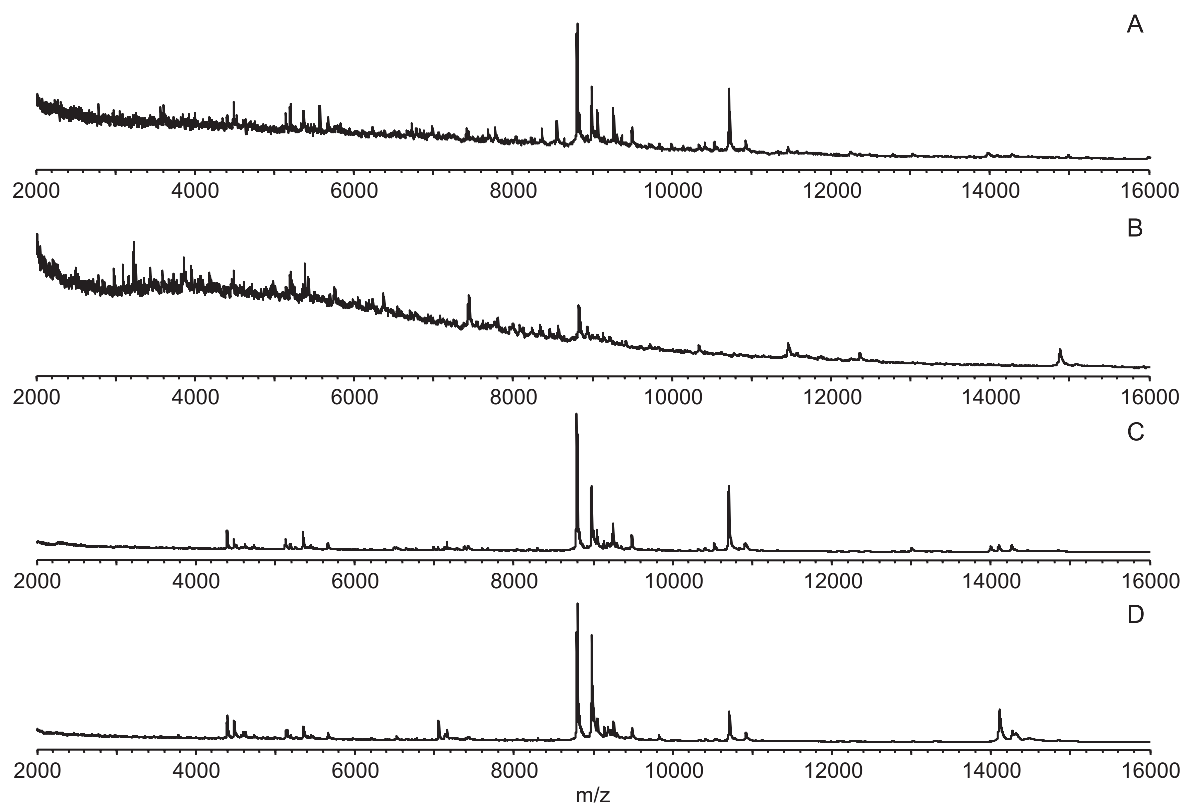

Fig. 1. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra of *Culicoides nubeculosus* larvae in the range of 2 to 16 kDa. (A) entire larva; (B) dissected larval gut; (C) larva without gut and last 2 segments; (D) larva without gut, last 2 segments and head.

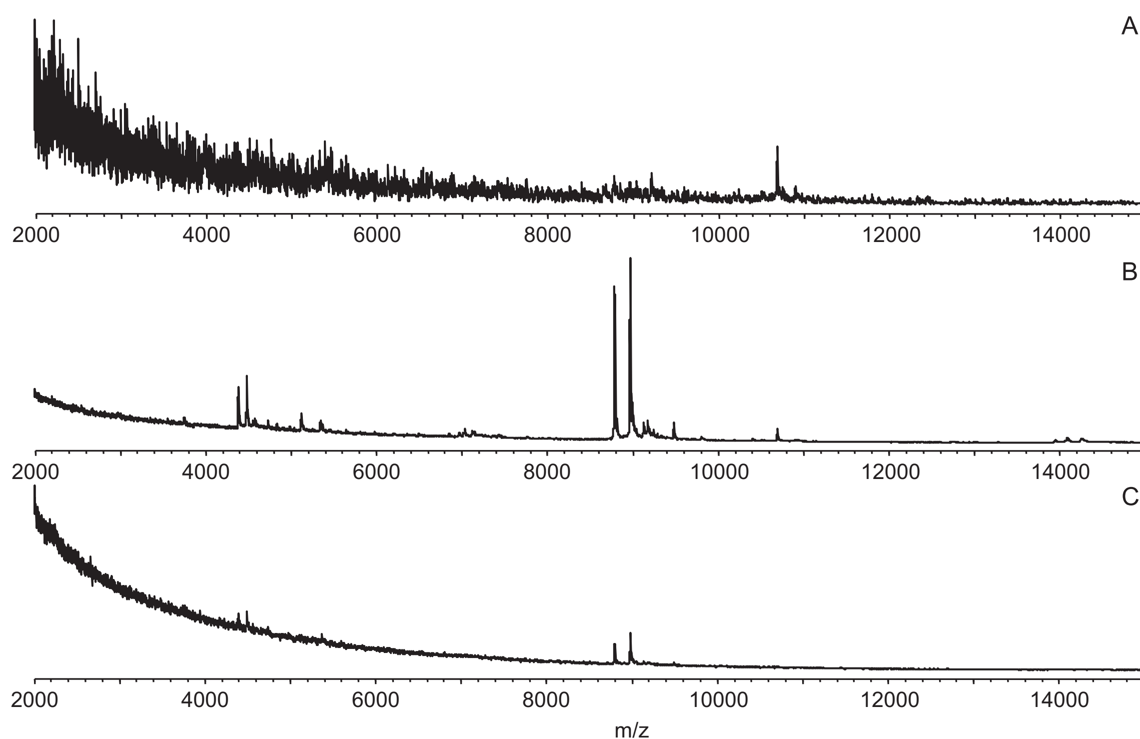

Fig. 2. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra profiles of *Culicoides nubeculosus* larvae (without gut and last 2 abdominal segments) at different raw protein concentrations in the range of 2 to 15 kDa. (A) 1·7 mg/ml, yielding 160 data counts; (B) 0·2 mg/ml, data count of 97 peaks – a qualitatively good spectrum; (C) 0·013 mg/ml, 13 data counts only.

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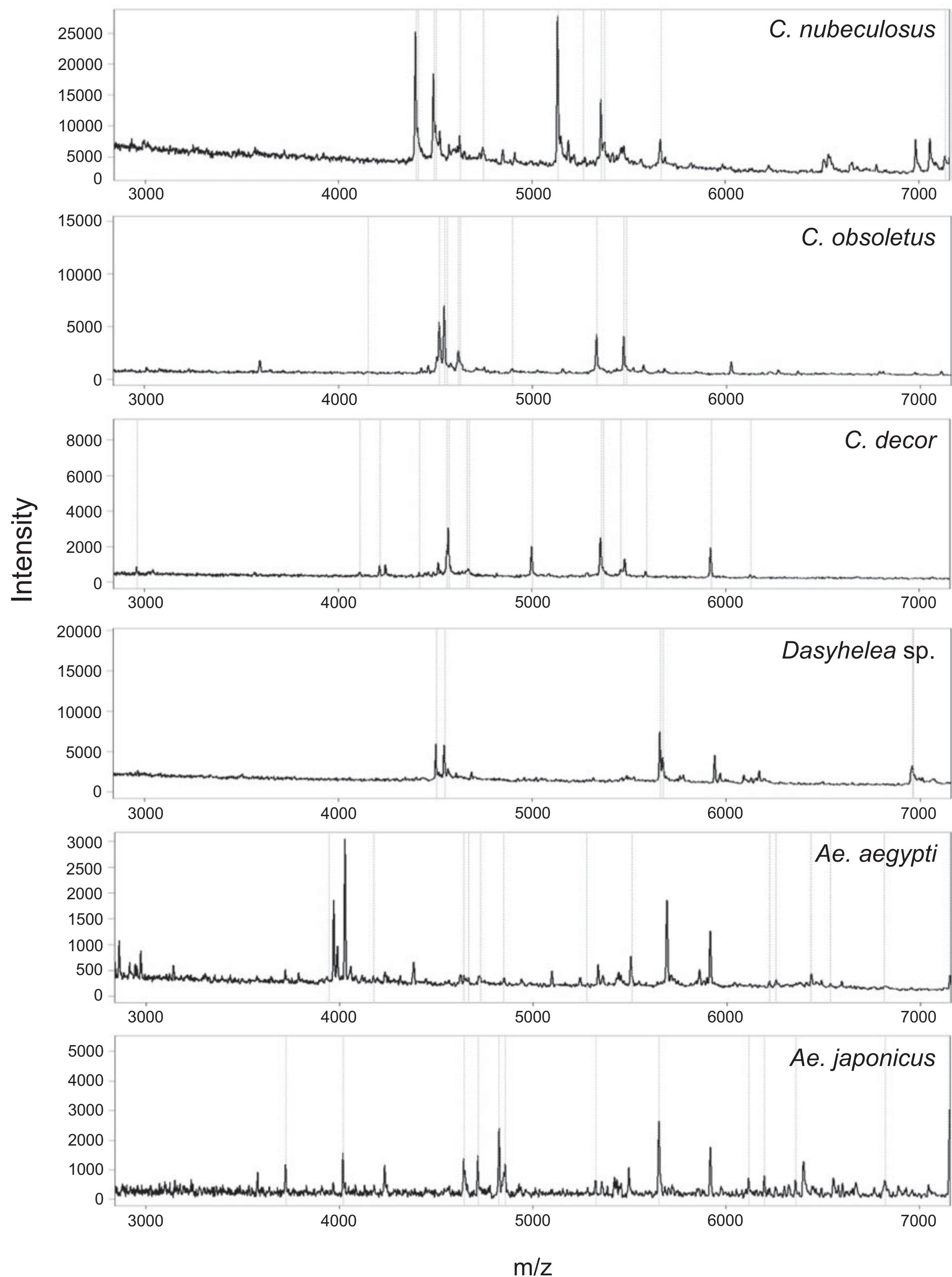

Fig. 3. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra of different larvae (≈ 0·2 mg raw protein/ml). (A) *Culicoides nubeculosus*; (B) *C. obsoletus*; (C) *C. decor*; (D) *Dasyhelea* sp.; (E) *Aedes aegypti*; (F) *Ae. japonicus*. Biomarker masses as eventually determined by SARAMIS are highlighted by dashed lines.

by gut components as revealed in Fig. 1B (profile of the dissected gut). Entire larvae without gut and larvae from which further parts were removed (last 2 segments, Fig. 1C; last 2 segments and head, Fig. 1D) yielded similar spectra of high quality.

The optimal protein concentration for MALDI-TOF MS analysis was assessed with dilutions of homogenates from 3 pools of 20 *C. nubeculosus* larvae (instar IV, 21 days post-hatching, gut and last 2 segments removed) and later confirmed with

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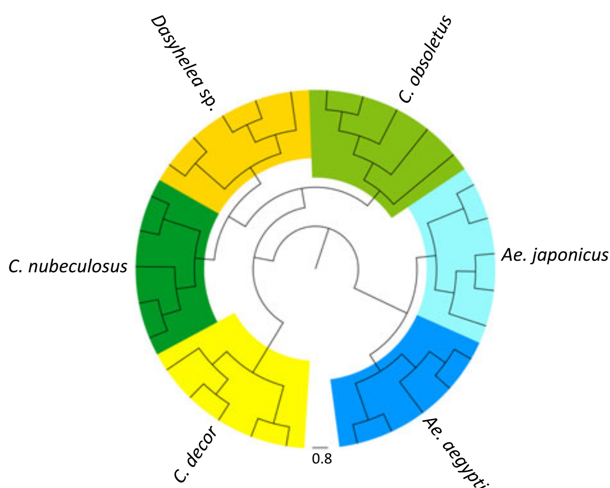

Fig. 4. Dendrogram of matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra of larvae from 5 individuals of 6 insect species. Distance units correspond to the relative similarity calculated from the distance matrix.

corresponding concentrations from single specimens of the same species ($n=9$). Thus, in 7 re-duplicated dilutions, crude protein concentrations between 0·013 mg and 1·7 mg per ml were investigated (Fig. 2) revealing spectra of high quality with regard to low noise, levelled baseline and number of mass data counts (97) for the 0·2 mg/ml concentration (Fig. 2B). Higher protein concentrations yielded generally higher data counts (160) but these originated from the noisiness of the spectrum and the baseline shift in the range between 2 and 6 kDa (Fig. 2A). With decreasing protein concentrations, the number of peaks declined and the spectra appeared noisy with baseline shifts (Fig. 2C).

Taken together, the recommended procedure for MALDI-TOF MS analyses of ceratopogonid larvae is to homogenize the gut-less larvae in 10% formic acid, mixing 0·2 mg/ml crude protein homogenate with SA matrix in a ratio of 1:1·5 and applying 1 μl to mass spectrometry analyses.

Total protein profiles were then generated from 5 larvae each of 3 biting midge species (*C. nubeculosus*, *C. obsoletus*, *C. decor*), 1 non-biting midge species from the genus *Dasyhelea* sp., and 2 mosquito species (*Ae. aegypti* and *Ae. japonicus*). Figure 3 shows 6 individual protein profiles in the range of 3 to 7 kDa of 1 larva from every species. Overall, the data counts included for protein profile generation per spectrum were between 30 and 124 in the mass range of 2 to 20 kDa. The whole protein profiles of 5 individuals per species (data count average of 67) were used to compile the total mass spectra for the 6 species in a dendrogram (Fig. 4), revealing that all larvae of the same species clustered on distinct branches. Based on this clear-cut clustering, species-specific biomarker mass sets could be generated using the SARAMIS™ SuperSpectrum tool (Fig. 3, Table 1). For automated species identification of the different reference larvae, between 27 and 32 biomarker masses were determined (Table 1).

Validation study

The accuracy of the reference database library was validated for all biomarker mass sets in a validation study. To this aim, 67 larvae belonging to the target species were used (laboratory-reared, $n=22$, comprising *C. nubeculosus, n*=10; *Ae. aegypti, n*=9; *Ae. japonicus, n*=3; and field-collected, $n=45$, comprising *C. obsoletus, n*=31; *C. decor, n*=6; *Dasyhelea* sp., $n=8$). In addition, larvae of field-collected *C. pulicaris* ($n=1$) and of a non-biting midge *Forcipomyia* sp. ($n=3$) were included as non-target species. From the investigated 67 specimens of the target species, 61 allowed correct identification by automated biomarker masses set analysis. From 54 of them, both profiles were suitable for automated identification whereas in 7 cases (*C. obsoletus, n*=2; *C. decor, n*=1; *Dasyhelea* sp., $n=2$; *Ae. aegypti, n*=2) only a single profile allowed an automated identification as the second one had fewer than 75% biomarker mass matches, requiring a complete manual full spectra comparison against the whole reference data set library which, in all cases, revealed the correct identification. For 6 larvae (*Dasyhelea* sp., $n=1$; *Ae. aegypti, n*=3; *Ae. japonicus, n*=2) of which neither profile was suitable for automated identification, complete manual full spectra comparison was done, resulting in the correct identification. In addition, none of the 4 specimens of the non-target species (*C. pulicaris, n*=1; *Forcipomyia* sp., $n=3$) yielded a positive identification, neither by automated biomarker identification nor by manual comparison. Taken together, all larvae of the validation study were correctly identified using the protein profiling identification tool.

For 5 species for which validated biomarker masses of the larvae were determined (*C. nubeculosus*, *C. obsoletus*, *C. pulicaris*, *Forcipomyia* sp. and *Ae. japonicus*), the corresponding masses of the adult stages are also included in the reference database library (Kaufmann *et al*. 2011, 2012). None of the larvae were misidentified as adults. The reference biomarker mass sets of larvae (Table 1) and adults (Kaufmann *et al*. 2011, 2012) of *C. nubeculosus*, *C. obsoletus* and *Ae. japonicus* range between 27 and 33 masses but they are highly distinct as only 3 (*C. nubeculosus*) or 2 (*C. obsoletus* and *Ae. japonicus*) are shared within both sets in the range of ±800 ppm error. In addition, the protein profile changes during the complete metamorphosis as shown in the dendrogram (Fig. 5) displaying the individual spectra of larvae III and IV, early (1 day) and late (3 day) pupae, and adults of both sexes of *C. nubeculosus* (Fig. 5). All larvae III and IV cluster together,

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Table 1. MALDI-TOF MS reference biomarker masses of larvae

Biomarker mass (Da)	*C. nubeculosus*	*C. obsoletus*	*C. decor*	*Dasyhelea* sp.	*Ae. aegypti*	*Ae. japonicus*
2080				x		
2084				x		
2088				x		
2961			x			
3722						x
4019						x
4029					x	
4113			x			
4153		x				
4217			x			
4401	x					
4412	x					
4422			x			
4494	x					
4504	x					
4506				x		
4521		x				
4547		x				
4549				x		
4562			x			
4563		x				
4573			x			
4619		x				
4630	x					
4630		x				
4644						x
4644					x	
4666			x			
4667					x	
4675			x			
4717						x
4728					x	
4749	x					
4827						x
4851					x	
4858						x
4897		x				
5002			x			
5137	x					
5266	x					
5326						x
5333		x				
5358			x			
5360	x					
5370			x			
5377	x					
5461			x			
5473		x				
5484		x				
5509					x	
5592			x			
5653						x
5661				x		
5669	x					
5676				x		
5691					x	
5927			x			
6133			x			
6198						x
6222					x	
6361						x
6436					x	
6960				x		
6964				x		
7138	x					
7153					x	
7157						x
7171	x					
7298		x				
7391				x		
7435					x	
7545					x	
7615		x				
7900					x	
7941						x
8148						x
8159					x	
8361					x	
8363						x
8568					x	
8571						x
8698				x		
8804	x					
8828	x					
8845			x			
8990	x					
9011	x					
9015				x		
9029	x					
9040				x		
9042		x				
9056	x					
9068			x			
9102		x				
9110				x		
9128			x			
9136				x		
9148			x			
9152	x					
9196	x					
9213	x					
9221				x		
9223		x				
9239		x				
9241	x					
9261	x					
9266				x		
9269		x				
9293			x			
9296					x	
9296						x
9316					x	
9334			x			
9342					x	
9353			x			
9371			x			
9381				x		
9426		x				
9435						x
9445		x				
9446					x	
9457						x
9462						x
9464					x	
9469			x			
9499	x					
9606					x	
9655						x
9701						x
9701						x
9708					x	
9717						x
9794		x				
9829	x					
9876					x	
10048					x	
10205			x			
10385		x				
10532	x					
10556	x					
10666		x				
10690		x				
10717			x			
10720	x					
10865					x	
10872		x				
10924			x			
10928	x					
10929				x		
10940			x			
10944		x				
10968		x				
10996						x
11150		x				
11167		x				
11291					x	
11320					x	
11323				x		
11326						x
11529				x		
11706		x				
12199				x		
12772	x					
13633					x	
13643						x
13745			x			
13916				x		
13926				x		
14093						x
14132				x		
14273	x					
14592		x				
14779				x		
14986				x		
15225		x				
15256			x			
18261			x			
18842		x				
19086			x			
19872		x				
total	32	33	32	27	29	27

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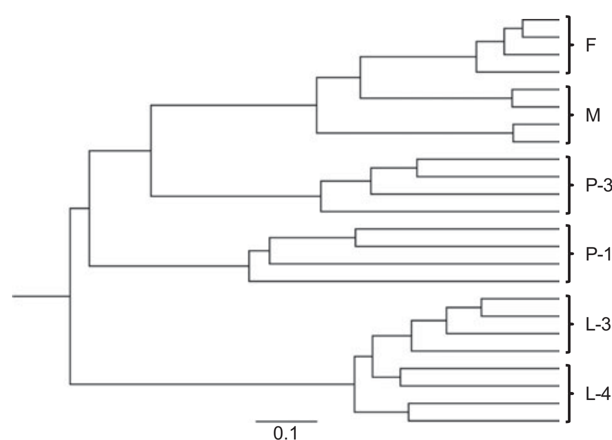

Fig. 5. Dendrogram of matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra of different life stages of *Culicoides nubeculosus*. Each life stage (L-3, larva III; L-4, larva IV; P-1, pupa at 1 day, P-3, pupa at 3 days; F, female; M, male) is represented by 4 different individuals. Distance units correspond to the relative similarity calculated from the distance matrix.

separated from the early pupae, whereas late pupae share the same main branch with the adults of the midges.

DISCUSSION

Insect identification by protein profiling is still a rather novel entomological tool having been utilized in only a few studies (Campbell, 2005; Feltens *et al*. 2010; Kaufmann *et al*. 2011; Perera *et al*. 2005), including the very recent application for the identification of field-collected haematophagous biting midges (Kaufmann *et al*. 2012). In the present study, the application of MALDI-TOF MS for the identification of larvae of holometabolous insects was evaluated for the first time. Biomarker mass sets could be established for 6 species, and all specimens investigated in the validation study were correctly identified.

Similar to the situation with adult midges (Kaufmann *et al*. 2011), it was found that the gut content of the larvae strongly impaired the profile quality. Thus, the gut must be removed by dissection which is a more tedious task with larvae as compared to the situation with adults where simply cutting off the abdomen suffices. For plant vectors, like aphids, it was shown that the gut content, containing plant molecules of low molecular size (e.g. sugars and amino acids; Dinant *et al*. 2010), does not impair the MALDI-TOF MS analysis (Perera *et al*. 2005).

The gut-less larvae were manually homogenized in formic acid, and a raw protein concentration of approximately 0·2 mg/ml was determined as optimal for the mass spectrometry analyses. As a rule of thumb, this concentration can be obtained by homogenizing the ceratopogonid larvae in volumes of 15–40 μl (depending on size) and the larger culicid larvae in 60 μl (*Ae. aegypti*) or 240 μl (*Ae. japonicus*), respectively.

Crude protein purification (precipitation using trichloroacetic acid; metabolite segregation with acetone or methanol/chloroform) was applied in preliminary experiments but did not yield superior profiles (data not shown). As already discussed in a previous study by Kaufmann *et al*. (2011), a more elaborate purification of proteins/peptides including chromatography, as shown by Feltens and co-workers (2010) with *Drosophila* spp., provides high-quality profiles with higher data counts but, due to spectral heterogeneity within species, this approach does not necessarily result in a more 'species-specific' biomarker mass set.

Adult midges stored in 70% ethanol for up to 2 years (Kaufmann *et al*. 2011, 2012) were reported to be suitable for mass spectrometry analyses albeit freshly collected specimens provided slightly better results (Kaufmann *et al*. 2012). For larvae, similar observations were made, and it is therefore recommended to use fresh specimens for the generation of the biomarker mass sets. Nevertheless, as shown in the validation study, correct identification was achieved with larvae stored up to 4 months.

As expected, the generated biomarker mass sets for larvae strongly differed from those of the adults (and pupae), with only very few shared masses, and thus they cannot be used for the identification of the adults (and vice versa). In contrast, in hemimetabolous insects, Perera and co-workers (2005) when investigating the life cycle of the cowpea aphid *Aphis craccivora* found that most of the major biomarker masses of juvenile stages (nymph stages I–IV) were also present in adults and thus a single species-specific biomarker set can be determined. For holometabolous insects, clear-cut biomarker sets were identified for larvae (this paper) and adults (Kaufmann *et al*. 2012). The pupal stages, however, seem not be suitable for species identification by MALDI-TOF MS as their profiles rapidly change during metamorphosis as shown in Fig. 5 for early (1 day) and late (3 days old) specimens. Thus, the identification of pupae preferably is done by genetic or morphological analyses or by analysing adults which emerged in the lab (Rieb and Kremer, 1981; Uslu and Dik, 2007), e.g. by MALDI-TOF MS.

MALDI-TOF MS identification was straightforward with the ceratopogonid larvae but less reliable with the larger culicid larvae using the same raw protein concentration as 42% of the investigated mosquito larvae could not be identified through automated identification but required manual comparison. Thus, improvements in sample preparation are required for mosquitoes that should, for example, include analysis of the head and thorax only, which in culicid larvae easily can be separated from the

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gut-containing abdomen because of their 3 tagmata system.

The applicability of MALDI-TOF MS for the identification of organisms was extended to include larvae of holometabolous insects. In the case of biting midges, this fast, cheap, and reliable diagnostic method might be of value to identify breeding habitats that can be targeted in control programmes.

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