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Evaluation of matrix-assisted laser desorption/ionization time of flight mass spectrometry for characterization of *Culicoides nubeculosus* biting midges

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Abstract. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) has shown promise in species identification of insect species. We evaluated its potential to consistently characterize laboratory-reared biting midges of the species *Culicoides nubeculosus* (Meigen) (Diptera: Ceratopogonidae). Twenty-one reproducible potential biomarker masses for *C. nubeculosus* were identified under different experimental treatments. These treatments included the homogenization of insects in either water or known concentrations of formic acid. The biomarker masses were present independent of age, gender and different periods of storage of individuals in 70% ethanol (a standard preservation method). It was found that the presence of blood in females reduced the intensity of the MALDI-TOF pattern, necessitating the removal of the abdomen before analysis. The protein profiles of a related non-biting midge, *Forcipomyia* sp. (Diptera: Ceratopogonidae), and of *Aedes japonicus japonicus* (Theobald) (Diptera: Culicidae) mosquitoes were also examined and were distinctly different. These findings provide preliminary data to optimize future studies in differentiation of species within the *Culicoides* genus using MALDI-TOF MS which is a rapid, simple, reliable and cost-effective technique.

Key words. *Culicoides*, biting midge, MALDI-TOF MS, mosquito.

Introduction

Biting midges of the genus *Culicoides* are among the smallest haematophagous insects known and are of significant importance as vectors of orbiviruses such as bluetongue virus (BTV) and African horse sickness virus. They are additionally the causative agents of chronic insect bite hypersensitivity (known colloquially as 'sweet itch'), particularly in equids. Currently, over 1400 species of these tiny (1–3 mm) insects have been described (Mellor *et al*., 2000), although the lack of detailed investigation of taxonomy means that this probably represents an underestimation of their true numbers. Identification at present is primarily carried out using morphological features, particularly wing patterns, but is very difficult in many cases (Meiswinkel *et al*., 2008). During the large-scale entomological surveys carried out after the spread of BTV serotype 8 across northern Europe, trapped midges were commonly grossly separated into Obsoletus and Pulicaris groups and other *Culicoides* spp. (Goffredo & Meiswinkel, 2004). For a more accurate identification of a few species, several polymerase chain reaction (PCR)-based tests have been developed, single- or multiplexed assays both in conventional and in real-time PCR formats (Cetre-Sossah *et al*., 2004, 2008; Pages *et al*., 2005; Mathieu *et al*., 2007; Nolan *et al*., 2007;

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Balczun *et al*., 2009; Schwenkenbecher *et al*., 2009; Stephan *et al*., 2009).

Protein profiling using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) has emerged as a technique to characterize bacteria. Fenselau & Demirev (2001) have summarized the commonly used instrumentation for this application, the protocols for collection and preparation of samples, as well as the algorithms for data analyses. In the last decade, this technique was further developed to become a routine method for the identification of these microorganisms in diagnostic laboratories (Sauer & Kliem, 2010). In two pioneering works, this technique has also been evaluated for its capability to identify insects. Campbell (2005) demonstrated that MALDI-TOF MS could distinguish between sibling species of the fruit fly *Drosophila melanogaster*. Similarly, unique species-specific protein profiles were identified among three species from three genera of plant-sucking aphids (Perera *et al*., 2005). In a very recent study, Feltens *et al*. (2010) used this proteomic approach to establish phylogenetic relationships among 13 species of *Drosophila* flies. Finally, MALDI-TOF MS has proven its value for authentication of different fish species (Mazzeo *et al*., 2008).

The aim of the present study was to investigate MALDI-TOF MS to consistently characterize biting midges of the species *Culicoides nubeculosus*, a model species, under a series of conditions as a precursor to use in identification of *Culicoides* species. These conditions include age, gender, storage in 70% ethanol and feeding status (blood fed or unfed), some of which have not been addressed before.

Materials and methods

Insects

The studies were carried out with laboratory-reared *C. nubeculosus* biting midges (long-run laboratory colonies from IAH, U.K.) and *Aedes japonicus japonicus* mosquitoes (strain collected in the field in Switzerland in 2009) which were reared as described previously (Boorman, 1974; Williges *et al*., 2008). The insects were kept at 24 ± 0.5 °C, 85 ± 5% relative humidity, long-day conditions (LD 14 ; 10 h). Adults had access to 10% sucrose solution or distilled water. Blood feeding was achieved through a Nescofilm®-membrane (fresh heparinized sheep blood for the midges) or by offering a human arm for the mosquitoes. Non-biting midges of another ceratopogonid genus, *Forcipomyia*, were caught at a farm site (Dittingen/BL; Kaufmann *et al*., 2009) using Onderstepoort UV-light suction traps as described previously (Venter & Meiswinkel, 1994). Individuals of the predominant morphotype of this genus were used for the study. Insects were stored in 70% ethanol at room temperature.

Preparation of samples for MALDI-TOF MS

Whole insects or body sections (after dissection using a stereomicroscope) were mechanically ground in one of two ways. The first method involved homogenization in 1.5-mL microtubes using a manual homogenizer (Fisher Scientific, Wohlen, Switzerland) and disposable pellet pestles. The second method used a Mixer Mill MM 300 device (Retsch GmbH, Haan, Germany) with one steel bead of 3 mm diameter placed in each tube and the mill run for two 1-min intervals at 30 Hz. Additionally, two different homogenization solutions were used; either 10 μL (for thoraxes) or 20 μL (for entire insects) sterile distilled water, or a range of concentrations of formic acid (5, 10, 25 and 50%). Suspensions were mixed directly in the tube with the same volume of either SA (saturated solution of sinapic acid in 60% acetonitrile, 0.3% trifluoroacetic acid) or DHB (saturated solution of 2.5-dihydroxybenzoic acid in 33% ethanol, 33% acetonitrile, 33% H_2O, 0.3% trifluoroacetic acid). One microlitre of matrix suspensions was spotted on steel target plate in duplicates and air dried at room temperature.

MALDI-TOF MS parameters

Protein mass fingerprints were obtained using a MALDI-TOF Mass Spectrometry Axima™ Confidence machine (Shimadzu-Biotech Corp., Kyoto, Japan), with detection in the linear, positive mode at a laser frequency of 50 Hz and within a mass range from 2–30 kDa. Acceleration voltage was 20 kV, and the extraction delay time was 200 ns. A minimum of 10 laser shots per sample was used to generate each ion spectrum. For each sample, a total of 100 protein mass fingerprints were averaged and processed using the Launchpad™ version 2.8 software (Shimadzu-Biotech Corp., Kyoto, Japan). This software was also used for peak processing of all raw spectra with the following settings: the advanced scenario was chosen from the parent peak cleanup menu, peak width was set 80 channels, smoothing filter width 50 channels, baseline filter width 500 channels and the threshold apex was chosen as the peak detection method. The threshold apex peak detection was set as a dynamic type and the offset was set to 0.020 mV with a response factor of 1.2. The processed spectra were exported as peak lists with m/z values for each peak and signal intensity in the ASCII format. Calibration was conducted for each target plate using spectra of the reference strain *Escherichia coli* DH5α.

MALDI–TOF MS spectral analyses

Generated protein mass fingerprints were analysed with SARAMIS™ (spectral archive and microbial identification system, AnagnosTec, Potsdam-Golm, Germany). Potential species-specific biomarker mass patterns, called SuperSpectrum™, were calculated for three genera of Diptera: *C. nubeculosus*, *Ae. japonicus* and *Forcipomyia* sp. using the SARAMIS™ superspectra tool. Therefore peak lists of representative isolates were imported into the SARAMIS™ software. Peak lists were trimmed to a mass range of 2–30 kDa and peaks with a relative intensity below 1% were removed. Peak lists were binned and average masses were calculated using the SARAMIS™ superspectrum tool with an error of 800 p.p.m.

From the remaining peaks only masses present in at least 60% of the spectra were selected for the *C. nubeculosus* superspectrum. Alternatively, masses present in at least 75% of the spectra were selected for *Ae. japonicus* and *Forcipomyia* sp. superspectra. Specificity of these potential biomarker masses was determined by comparison against the whole SARAMIS™ spectral archive. Twenty-one masses for the genus *Culicoides*, 29 masses for the genus *Aedes* and 30 masses for the genus *Forcipomyia* were weighted and used as SuperSpectrum™ for automated identification in the future.

For dendrogram generation SARAMIS™ Premium software package was used. Dendrogram was based on whole spectra including all signals passing the peak detection criteria of the Launchpad software. A binary mass list was calculated with an error of 800 p.p.m. and the SARAMIS™ single-link clustering algorithm was applied.

Results and Discussion

During the present study, more than 400 individual *C. nubeculosus* were subjected to MALDI-TOF MS analyses. Sample preparation (ways of homogenization and matrix suspensions) was evaluated, and protein profiles were determined from insects of both genders, of different age and duration of storage in 70% ethanol, of whole insects and body sections, of unfed and blood-fed females.

The profiles of entire *C. nubeculosus* insects revealed several peaks within the considered mass range of 2–30 kDa (Fig. 1A). Male and female specimens had very similar spectra with a high count of identical masses which is in agreement with the findings of Campbell (2005) for both sexes of the fruit fly *Drosophila melanogaster*. The presence of blood had a considerable impact on the MALDI-TOF pattern of the haematophagous female midges, reducing the intensity of the midges biomarker masses (Fig. 1B). This effect was particularly obvious with midges measured immediately after the bloodmeal, but was less evident 2 days after the bloodmeal and was undetectable with insects 5 days after the ingestion of blood. This effect was more pronounced in insects kept frozen than in those in 70% ethanol (not shown). Hence, in order to avoid the influence of blood, which might not easily be detectable in the female midges, further studies were done with insects from which the abdomen was removed. The protein profiles of dissected insect thoraxes were very similar from males and females, either unfed or blood-fed (Fig. 2A–D). In addition, the head, wings and legs left attached to the thorax did not alter the spectra as compared with the thorax alone (Fig. 2E) and resulted in the same biomarker masses calculated with the SARAMIS™ superspectrum tool from representative measurements of abdomen-less insects in the present study as described above (Fig. 2F). For comparative purposes, the spectrum obtained with the thorax of a female *Forcipomyia* sp. nonbiting midge and diagram of the relevant biomarker masses are included (Fig. 2G, H). Hence, reliable MALDI-TOF results can be obtained for both male and female *C. nubeculosus* after a simple preparation of the insects by cutting between the thorax and the abdomen. On the other hand, removal of the head, legs and wings (to be used for other purposes, such as morphological identification of midges) did not alter the profile.

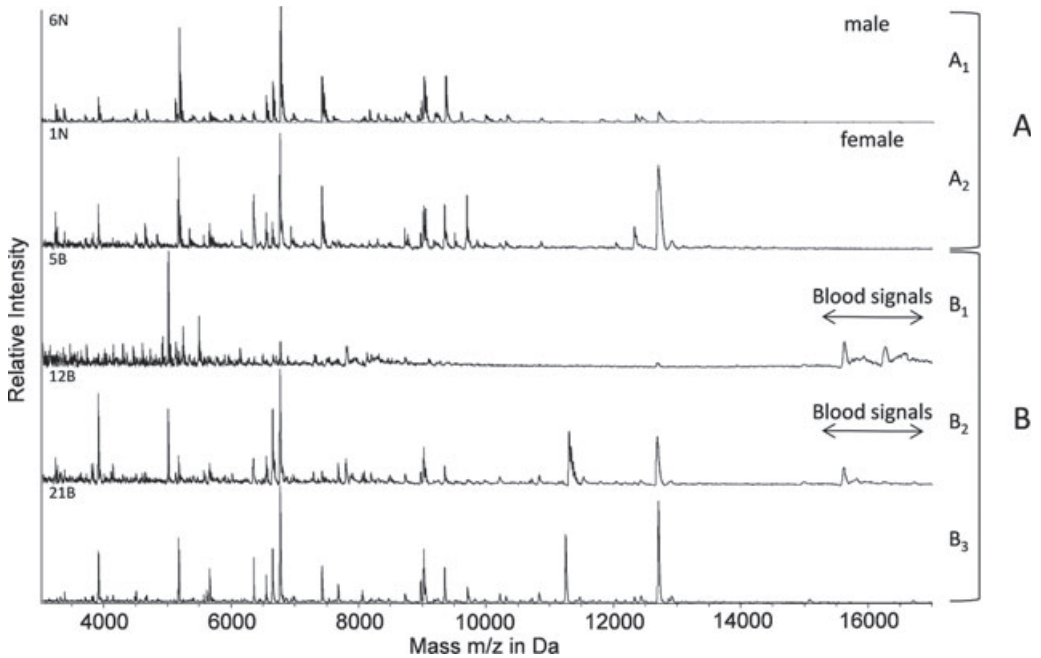

Fig. 1. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra of entire *Culicoides nubeculosus* insects: A, unfed male (A_1) and female (A_2). B, Female insects at different time points after a bloodmeal (B_1: immediately afterwards, B_2: after 2 days, and B_3: after 5 days).

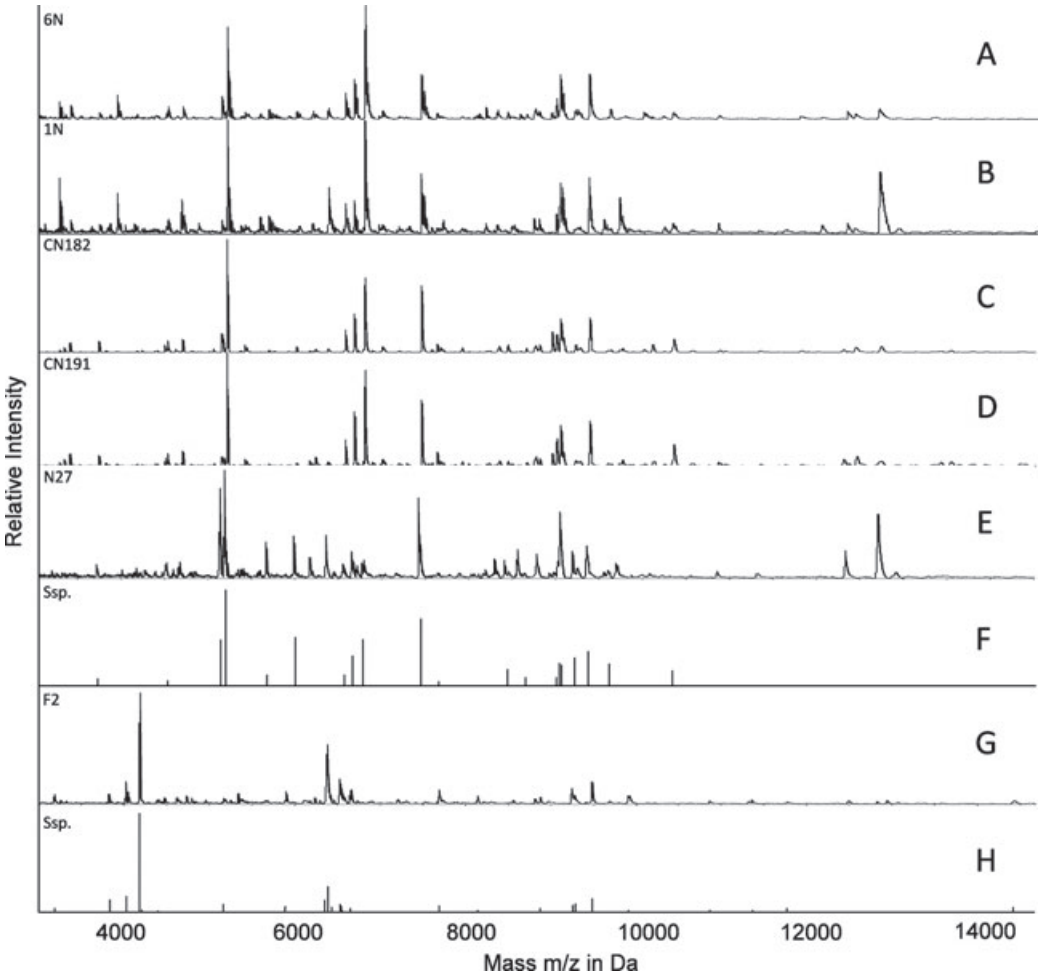

Fig. 2. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra of thoraxes only of *Culicoides nubeculosus* (A–E). A, Male insect; B, female insect (unfed); C, female insect, immediately after blood feeding; D, female insect, 5 days after blood feeding; E, female insect, unfed, with only the abdomen removed (e.g. thorax with head, legs, wings); F, diagram of biomarker masses of *C. nubeculosus* calculated from representative measurements of abdomen-less insects in the present study; G, Mass spectrum of thorax of female *Forcipomyia* sp.; H, diagram of biomarker masses calculated for *Forcipomyia* sp.

Homogenization of insect thoraxes by hand with a pestle or by disruption in a bead beater device yielded spectral profiles of equal quality (not shown), and the former method which is less laborious was used throughout the experiments.

The effect of grinding of thoraxes in different solutions was investigated; hence, the use of water and formic acid (5, 10, 25%) produced similar profiles regarding the absolute number of resolved masses, whereas the quality of the mass spectra was lower when using a higher concentration of formic acid (50%), as a result of inhomogeneous crystallization and therefore a lower chance to hit one of those crystals in the automated spectra acquisition procedure. Hence, whereas homogenization in low concentrated formic acid is the default procedure for MALDI-TOF analyses, water can also be used allowing aliquots to set aside for DNA isolation and analyses (homogenization in formic acid degrades DNA).

Two matrices were compared, revealing the superiority of SA vs. DHB by yielding a larger data count (average of 161 vs. 98 mass peaks), particularly because of the presence of high molecular mass peaks (larger than 10 kDa), and a higher reproducibility as a result of a smaller internal mistake (Fig. 3).

Hence, from this evaluation the following procedure was chosen as standard and applied for the further investigations: homogenization of the abdomen-less insects with pestles in 10 μL 25% formic acid and further processing mixing with the SA matrix in the ratio 1 : 1. The potential biomarker masses of *C. nubeculosus* (Fig. 2F) were calculated with representative measurements after this procedure during the present study.

To investigate the influence of the insect's age on the protein profiles, female midges at 1, 7 and 14 days post-eclosion were investigated, revealing only slight variations (Fig. 4). In addition, midges of these three ages (males and females) were kept for different time periods in 70% ethanol (1–2 h, 14 days, 30 days and 102 days). Midges stored for more than 2 h in 70% ethanol revealed a lower average data count than freshly prepared or only for a short period kept in 70% ethanol samples

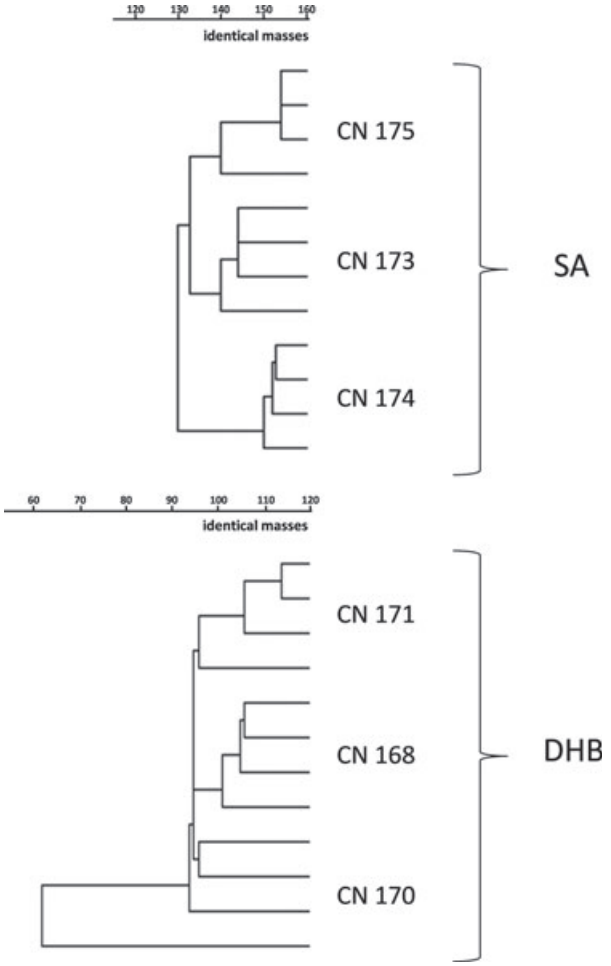

Fig. 3. Cluster analysis of *Culicoides nubeculosus* assessed with sinapic acid (SA) or 2.5-dihydroxybenzoic acid (DHB) matrices. Four replicate readings of thoraxes from six individual female insects are shown.

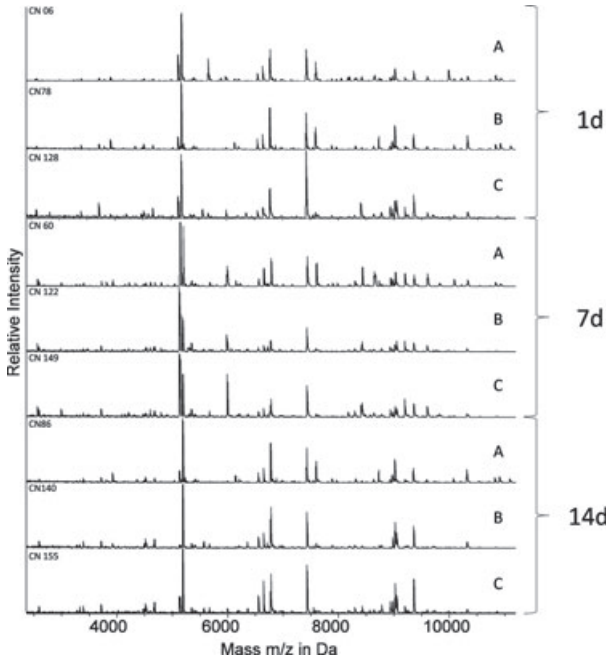

Fig. 4. Protein profiles of thoraxes of female *Culicoides nubeculosus*, aged 1, 7 or 14 days, fed only with 10% sucrose solution, and stored for different periods of time (A, 1–2 h; B, 14 days and C, 30 days) in 70% ethanol at room temperature.

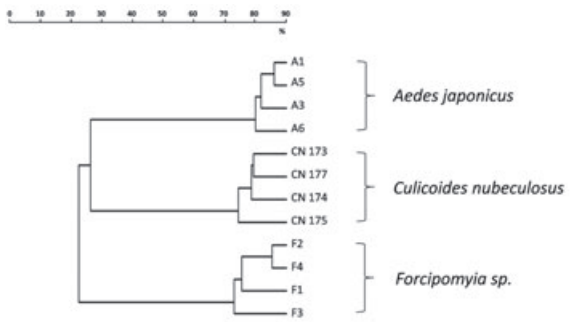

Fig. 5. Dendrogram of matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra of thoraxes from four individual females of *Culicoides nubeculosus*, *Forcipomyia* sp. and *Aedes japonicus*.

with 106 vs. 161 masses. Nevertheless, 21 potential biomarker masses were reproducible identified in all samples independent of storage time in 70% ethanol.

These findings are of utmost importance for a conceivable application of MALDI-TOF in field investigations, analysing midges of unknown age and which, as a result of the sometimes large number of insects being caught in such studies, cannot be processed immediately.

Taken together, by analysing more than 400 individual *C. nubeculosus*, we showed that MALDI-TOF spectra were consistent when the abdomen is removed, independent of sex, age and duration of storage of the insects in 70% ethanol. Using the standard procedure (see above), 21 reproducible potential biomarker masses for *C. nubeculosus* (Fig. 2F, Table 1) were identified.

The protein profile of *C. nubeculosus* was distinctly different from the ones of wild caught *Forcipomyia* sp., non-biting midges of the family Ceratopogonidae, and for laboratory-reared mosquitoes of the species *Ae. japonicus* (Fig. 5). The individual specimens of all three insect species displayed homogenous clusters. For the *Forcipomyia* sp. investigated, 30 potential biomarker masses were found whereas for all *Ae. japonicus* mosquitoes analysed 28 such masses could be identified (Table 1). These findings endorse the potential of whole cell MALDI-TOF MS for rapid, simple, reliable and cost-effective species identification of insects. It seems promising to evaluate this technique on the tiny biting midges of the genus *Culicoides*. MALDI-TOF-based identification of these important insect vectors would greatly facilitate field studies addressing a range of basic questions which were hampered by the difficulties of accurate and efficient identification (Carpenter *et al*., 2008; Meiswinkel *et al*., 2008).

Table 1. Potential reference biomarker masses *for Culicoides nubeculosus* (1), *Forcipomyia* sp. (2) and *Aedes japonicus* (3).

m/z	1	2	3	m/z	1	2	3	m/z	1	2	3
2089.7	No	yes	No	5669.0	yes	No	No	8882.6	No	yes	No
2674.9	No	No	yes	5903.5	No	yes	No	8906.1	No	No	yes
2843.9	No	yes	No	5966.3	No	No	yes	8948.6	No	No	yes
2979.5	No	No	yes	5997.3	yes	No	No	8990.7	yes	No	No
3191.0	No	yes	No	6222.7	No	No	yes	9030.4	yes	No	No
3285.7	No	No	yes	6389.2	No	yes	No	9055.0	yes	No	No
3715.6	yes	No	No	6418.9	No	yes	No	9206.5	yes	No	No
3830.6	No	yes	No	6534.5	No	yes	No	9256.0	No	yes	No
3950.9	No	No	yes	6550.2	yes	yes	No	9287.9	No	yes	No
4031.1	No	yes	No	6565.8	No	yes	No	9302.4	No	No	yes
4187.4	No	yes	No	6654.0	yes	yes	No	9361.3	yes	No	No
4204.1	No	yes	No	6670.3	No	yes	No	9466.4	No	No	yes
4394.1	No	yes	No	6780.3	yes	No	No	9495.1	No	yes	No
4472.1	No	No	yes	7435.2	yes	No	No	9605.1	yes	No	No
4513.9	yes	No	No	7472.0	No	yes	No	9751.9	No	No	yes
4649.3	No	No	yes	7643.4	yes	No	No	9920.4	No	yes	No
4731.3	No	No	yes	7706.1	No	yes	No	10 171	No	No	yes
5083.5	No	No	yes	7765.2	No	No	yes	10 327	yes	No	No
5130.8	yes	No	No	7907.1	No	No	yes	10 874	No	yes	No
5141.4	yes	No	No	8047.1	No	No	yes	11 368	No	yes	No
5188.7	yes	No	No	8091.2	No	No	yes	11 779	No	yes	No
5198.8	No	No	yes	8145.3	No	yes	No	12 447	No	No	yes
5346.1	No	yes	No	8197.9	No	No	yes	14 436	No	yes	No
5356.3	No	No	yes	8216.6	No	No	yes	17 773	No	yes	No
5383.3	No	No	yes	8428.7	yes	No	No	19 501	No	No	yes
5563.0	No	No	yes	8641.3	yes	No	yes	—	—	—	—

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