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Accurate identification of Australian mosquitoes using protein profiling

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Research Article

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Abstract

Australian mosquito species significantly impact human health through nuisance biting and the transmission of endemic and exotic pathogens. Surveillance programmes designed to provide an early warning of mosquito-borne disease risk require reliable identification of mosquitoes. This study aimed to investigate the viability of Matrix-Assisted Laser Desorption/Ionization–Time-of-Flight Mass Spectrometry (MALDI-TOF MS) as a rapid and inexpensive approach to the identification of Australian mosquitoes and was validated using a three-step taxonomic approach. A total of 300 mosquitoes representing 21 species were collected from south-eastern New South Wales and morphologically identified. The legs from the mosquitoes were removed and subjected to MALDI-TOF MS analysis. Fifty-eight mosquitoes were sequenced at the cytochrome c oxidase subunit I (*cox1*) gene region and genetic relationships were analysed. We create the first MALDI-TOF MS spectra database of Australian mosquito species including 19 species. We clearly demonstrate the accuracy of MALDI-TOF MS for identification of Australian mosquitoes. It is especially useful for assessing gaps in the effectiveness of DNA barcoding by differentiating closely related taxa. Indeed, *cox1* DNA barcoding was not able to differentiate members of the *Culex pipiens* group, *Cx. quinquefasciatus* and *Cx. pipiens molestus*, but these specimens were correctly identified using MALDI-TOF MS.

Introduction

Australia's mosquito fauna consists of over 300 species, many of which transmit pathogens of human and veterinary concern (Webb *et al.*, 2016). Australian mosquito-borne pathogens such as Ross River virus (RRV) and Barmah Forest virus (BFV) can cause chronic debilitation in affected patients, while Murray Valley encephalitis virus (MVEV) can be potentially fatal, and West Nile virus (Kunjin strain) is of both human and veterinary importance (Burrow *et al.*, 1998; Harley *et al.*, 2001; Jacups *et al.*, 2008). Australia is also at risk to exotic pathogens such as Japanese encephalitis virus, which has been identified as a threat in northern Australia where suitable mosquito species and reservoir hosts are present (Mackenzie *et al.*, 2002; Ritchie *et al.*, 2007). Exotic mosquito species have already become established in Australia, including *Aedes aegypti* [the key vector of dengue viruses, chikungunya virus and Zika virus (Jansen and Beebe, 2010; Hall-Mendelin *et al.*, 2016)], *Culex pipiens molestus* (Kassim *et al.*, 2012) and *Cx. gelidus* (Williams *et al.*, 2005). Exotic mosquito incursions are increasingly common at Australia's ports, where international cargo ships and aircraft can provide a source of foreign species – both immature and adult stages – with *Ae. aegypti* and *Ae. albopictus* of particular concern (van den Hurk *et al.*, 2016). The development of strategic response plans for these species requires appropriate surveillance methods (Webb and Doggett, 2016) which rely on accurate and rapid mosquito specimen identification.

Mosquito and arbovirus surveillance in Australia is primarily performed using traditional morphological taxonomy, which can be time consuming, requires specialist skills and is challenging when specimens are immature or damaged. Mosquito and arbovirus surveillance continues to evolve in Australia with new technologies for mosquito collection (Flies *et al.*, 2015) and arbovirus detection (van den Hurk *et al.*, 2014) as well as the development and application of molecular methods for specimen identification (Batovska *et al.*, 2017). New technologies for mosquito and arbovirus surveillance aim to provide rapid and accurate data collection as well as easily translatable results to guide public health interventions.

Matrix-Assisted Laser Desorption/Ionization–Time-of-Flight Mass Spectrometry (MALDI-TOF MS) is an important rapid identification tool in microbiology and is traditionally used for the identification and analysis of large biomolecules including proteins, peptides and nucleic acids (Lin and Cai, 2018). MALDI-TOF MS is based on the ionization of the biomolecules (such as proteins) of an organism of interest, co-crystallized with a chemical acidic matrix. The molecules are accelerated in a flight tube according to their mass-to-charge ratio and

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their time-of-flight is measured by an MS detector. Each detected molecule is associated with a peak, on a global spectrum which will be the specific fingerprint signature of the sample (Yssouf *et al.*, 2016). MALDI-TOF MS is now validated and utilized for identification of mammals (Tran *et al.*, 2011), Archaea (Dridi *et al.*, 2012), giant viruses (La Scola *et al.*, 2010) and a range of arthropods relevant to human and animal health including *Culicoides* biting midges (Kaufmann *et al.*, 2012), phlebotomine sand flies (Dvorak *et al.*, 2014), tsetse flies (Hoppenheit *et al.*, 2013), ticks (Karger *et al.*, 2012; Yssouf *et al.*, 2013*a*), fleas (Yssouf *et al.*, 2014*b*), triatomine bugs (Laroche *et al.*, 2017*b*) and mosquitoes (Müller *et al.*, 2013; Yssouf *et al.*, 2013*b*; Schaffner *et al.*, 2014; Raharimalala *et al.*, 2017; Mewara *et al.*, 2018).

MALDI-TOF MS has been shown to be highly effective for the rapid identification of endemic (Yssouf *et al.*, 2014*a*) and invasive (Schaffner *et al.*, 2014) mosquito species in Europe, North India (Mewara *et al.*, 2018) and Madagascar (Raharimalala *et al.*, 2017). However, there has been no research into the creation and use of MALDI-TOF MS protein spectra for the identification of Australian mosquitoes. Currently, MALDI-TOF MS is primarily used for microbiology diagnostics in Australia and expansion of MALDI-TOF MS utilization to arthropods would be beneficial for vector surveillance. This study aimed to investigate the viability of using MALDI-TOF MS for rapid mosquito identification for vector surveillance and research purposes in Australia. To address this, we used a three-part approach to taxonomic classification that allowed a performance comparison between morphological identification, DNA barcoding and MALDI-TOF MS. We not only benchmark the use of MALDI-TOF MS for identification of Australian mosquitoes but also show the utility of using a range of taxonomic tools for mosquito classification.

Materials and methods

Sample collection

Adult mosquitoes were collected using battery-powered, CO_2-baited light traps [Encephalitis Virus Surveillance (EVS) traps (Australian Entomological Supplies, NSW] from six locations in south-eastern Australia from January to October 2016 (Table 1). Mosquito larvae and pupae were collected from two of the six locations (Berringer and Audley, NSW) and reared to adulthood in the laboratory. A single mosquito sample (92-B103189; *Cx. australicus* from Homebush, NSW) collected in December 2014 was used in this study. After collection or emergence, mosquitoes were immediately stored at −20 °C in petri dishes until morphological identification was performed on a cold bench. Mosquitoes were identified to species level with the aid of various locally relevant keys and descriptions (Dobrotworsky, 1965; Lee *et al.*, 1989; Russell, 1993, 1996). It is important to note that in many parts of the world, there is great difficulty in differentiating *Cx. p. molestus* from other mosquitoes in the *C. pipiens* group based on morphological characteristics. However, in Australia where *Cx. p. pipiens* is not present, *Cx. p. molestus* can readily be differentiated from other mosquitoes in the *C. pipiens* group (e.g. *Cx. quinquefasciatus* and *Cx. australicus*) based on morphological characteristics. Mosquito specimens were placed in individual tubes for storage at −80 °C. Only female mosquitoes were included in this study.

MALDI-TOF MS sample preparation and mass spectrometer parameters

A total of two to 18 specimens were subjected to MALDI-TOF MS for each species depending on sample availability (Table 1). For species that were collected in more than one location, multiple specimens from each of the collection locations were tested to evaluate any populational variations within the species due to geographical dissociation. Prior to MALDI-TOF MS testing, mosquito samples were thawed and the legs were removed using forceps and placed into individual 1.5 mL Eppendorf tubes. The remaining body of the mosquito was placed in 70% ethanol for DNA isolation and molecular analysis. A solution containing 15 μL of 50% acetonitrile, 15 μL of 70% formic acid and a small amount (roughly half the liquid volume) of glass beads ($\leqslant$106 μm; Sigma-Aldrich, St Louis, Missouri, USA) was added to each tube containing mosquito legs. Mosquito legs were crushed as previously described (Yssouf *et al.*, 2013*b*) using the TissueLyser II (Qiagen, Hilden, Germany) with three cycles of 1 min at a frequency of 30 Hz. The homogenates were centrifuged for 30 s at 20 784 × ***g*** after which 1 μL of each sample solution was spotted onto a steel target plate (Bruker Daltonics, Germany) in quadruplicate. Where possible, each plate contained two to four phylogenetically similar species (when known) to minimize between run bias and ensure that any variation exhibited was natural and not a result of variation between runs. Additionally, variation between runs was assessed by appraising the reproducibility of spectra for the same species run on different plates using the gel view tool of the ClinProTools software. One microliter of matrix overlay containing saturated α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich), 100% acetonitrile, 100% trifluoroacetic acid and high-performance liquid chromatography-grade water was then spotted directly onto the dried sample plate. The matrix solution was allowed to dry completely at room temperature before launching the plate with the MALDI-TOF Mass Spectrometer (Microflex LT, Bruker Daltonics, Germany) to obtain protein mass profiles. Step-by-step instructions are available at Protocols.io (doi: dx.doi.org/10.17504/protocols.io.md5c286). Parameters were set according to a previous study (Laroche *et al.*, 2017*b*). Spectrum profiles were obtained then visualized using Flex analysis v.3.3 software (Fig. 1). The profiles were exported to ClinProTools software v.2.2 and MALDI-Biotyper v.3.0. (Bruker Daltonics) for data processing (smoothing, baseline subtraction and peak picking) as previously described (Yssouf *et al.*, 2013*b*).

MALDI-TOF MS spectra analysis and reference database creation

Initially, all spectra were tested against the in-house database containing reference spectra for 31 species of European and African mosquitoes to observe whether any cross-matching could occur between the mosquitoes in the database and the test spectra [the most recent list of specimens comprising this database can be found in Boucheikhchoukh *et al.* (2018)]. Spectra from two cosmopolitan species (*Mansonia uniformis* and *Cx. quinquefasciatus*) from Europe and Africa were already present in the database. Additionally, spectra for *Cx. pipiens pipiens* – a closely related taxon to *Cx. p. molestus* – was also present in the database. Spectra reproducibility was tested through visual inspection of gels, dendrograms and comparison of mean spectra for each sample using ClinProTools 2.2 and Flex analysis v.3.3 software (Bruker Daltonics). Virtual gel views are a representation of an alignment of spectra generated by ClinProTools 2.2 that allow a visual assessment of reproducibility for a group of spectra. Dendrograms are based on the results of Composite Correlation Index matrix (CCI), a parameter that defines the distance between spectra. CCIs are calculated by dividing spectra into intervals and comparing the correlation of these intervals across a dataset. A CCI match value of 1 represents complete correlation, whereas a CCI match value of 0 represents an absence of correlation (Laroche *et al.*, 2017*b*).

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Table 1. Australian mosquitoes used for MALDI-TOF analysis including reference spectra creation and blind test specimens

Species	Collection location(s) in NSW, Australia	Lat-lon of collection points	Number of specimens added to database	Number of specimens used for blind testing	ID Log Score Values: mean (range)
Aedes alternans	Hexham	32.8475′S 151.6902′E	2	10	2.426 (2.065–2.83)
	Homebush	33.8641′S 151.0823′E	1	5	
Aedes australis	Audley	34.1093′S 151.1520′E	2	7	2.616 (2.306–2.898)
Aedes flavifrons	Bendalong	35.2191′S 150.4864′E	3	15	2.512 (2.232–2.888)
Aedes mallochi	Hexham	32.8475′S 151.6902′E	0	2	NA
Aedes multiplex	Ballina	28.8380′S 153.5629′E	3	15	2.568 (2.404–2.86)
Aedes notoscriptus	Ballina	28.8380′S 153.5629′E	0	3	2.392 (1.816–2.841)
	Berringer	35.2498′S 150.5052′E	0	1	
	Hexham	32.8475′S 151.6902′E	1	6	
	Homebush	33.8641′S 151.0823′E	2	5	
Aedes procax	Hexham	32.8475′S 151.6902′E	2	14	2.447 (2.142–2.847)
Aedes rubrithorax	Berringer	35.2498′S 150.5052′E	0	2	NA
Aedes vigilax	Hexham	32.8475′S 151.6902′E	1	8	2.495 (2.27–2.942)
	Homebush	33.8641′S 151.0823′E	2	7	
Aedes vittiger	Hexham	32.8475′S 151.6902′E	1	3	2.559 (2.425–2.824)
Anopheles annulipes	Hexham	32.8475′S 151.6902′E	1	8	2.413 (2.174–2.842)
	Homebush	33.8641′S 151.0823′E	2	7	
Coquillettidia linealis	Hexham	32.8475′S 151.6902′E	2	7	2.547 (2.333–2.541)
	Homebush	33.8641′S 151.0823′E	1	8	
Coquillettidia xanthogaster	Ballina	28.8380′S 153.5629′E	2	4	2.457 (1.486–2.839)
	Hexham	32.8475′S 151.6902′E	0	6	
	Homebush	33.8641′S 151.0823′E	1	5	
Culex annulirostris	Hexham	32.8475′S 151.6902′E	2	7	2.408 (2.145–2.853)
	Homebush	33.8641′S 151.0823′E	1	8	
Culex australicus	Homebush	33.8641′S 151.0823′E	2	7	2.347 (1.889–2.527)
Culex pipiens molestus	Homebush	33.8641′S 151.0823′E	3	15	2.561 (2.326–2.88)
Culex orbostiensis	Ballina	28.8380′S 153.5629′E	2	7	2.426 (2.19–2.882)
	Hexham	32.8475′S 151.6902′E	1	8	
Culex quinquefasciatus	Hexham	32.8475′S 151.6902′E	1	8	2.483 (2.238–2.955)
	Homebush	33.8641′S 151.0823′E	2	7	
Culex sitiens	Ballina	28.8380′S 153.5629′E	1	8	2.481 (2.279–2.845)
	Homebush	33.8641′S 151.0823′E	2	7	
Mansonia uniformis	Ballina	28.8380′S 153.5629′E	1	5	2.55 (2.283–2.845)
	Hexham	32.8475′S 151.6902′E	1	5	
	Homebush	33.8641′S 151.0823′E	1	5	
Verralina funerea	Hexham	32.8475′S 151.6902′E	2	4	2.541 (2.29–2.818)
	Total		51	249	

NB, Log Score Values >1.9 were considered adequate for MS identification; NA, not submitted to blind test analysis.

MALDI-Biotyper software v3.0 (Bruker Daltonics) was used to create reference spectra (MSP, Main Spectrum Profile) for each sample to capture biological variation by taking into account the peak intensity, positioning and frequency in an automated algorithm. Spectral dendrograms were created to aid in selecting representative spectra from the dendrogram clusters. Two to three spectra were added to the reference database where possible, depending upon sample availability and spectra reproducibility (Supplementary Dataset S1). For samples with the highest reproducibility, only two reference spectra were selected. Only one reference spectrum was added for the species *Ae. vittiger* due to low sample number ($n = 4$). Following the addition of the reference spectra to the reference database for each species, the remaining samples were screened against the database containing the new reference spectra to test identification power. A further six specimens for each species (excluding four species with low sample number – Table 1) were subjected to MALDI-TOF MS and screened against the upgraded reference

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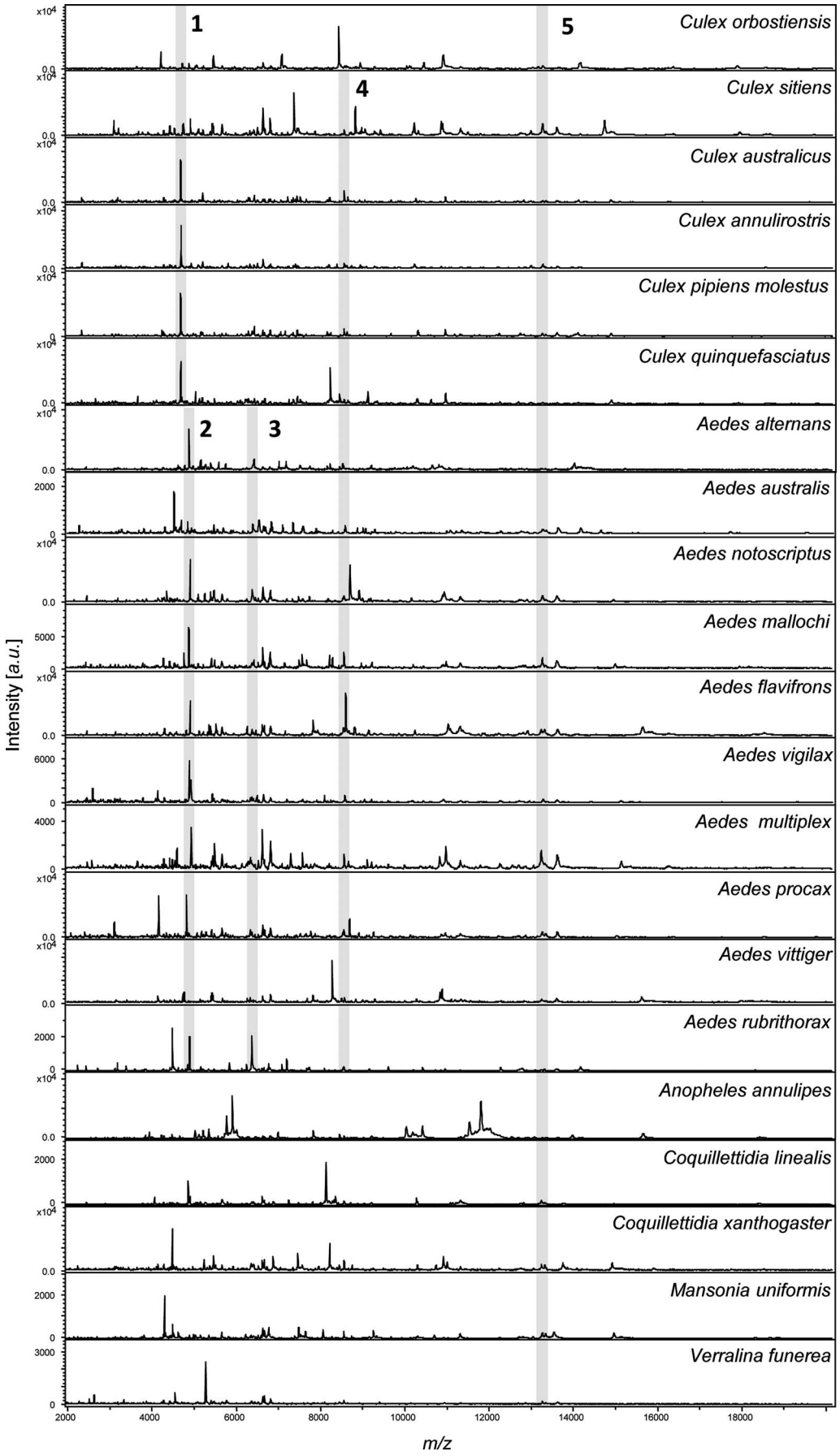

Fig. 1. MALDI-TOF MS spectra for 21 mosquito species. Spectra were obtained from homogenization of the legs of each species. Species name is indicated for each spectrum. Intensity is measured in *a.u.* (arbitrary units) and *m/z* denotes the mass-to-charge ratio. Shaded regions indicate common peaks shared across all *Culex* spp. (1), *Aedes* spp. (2 and 3), both *Culex* spp. and *Aedes* spp. together (4), as well as all species in the analysis (5).

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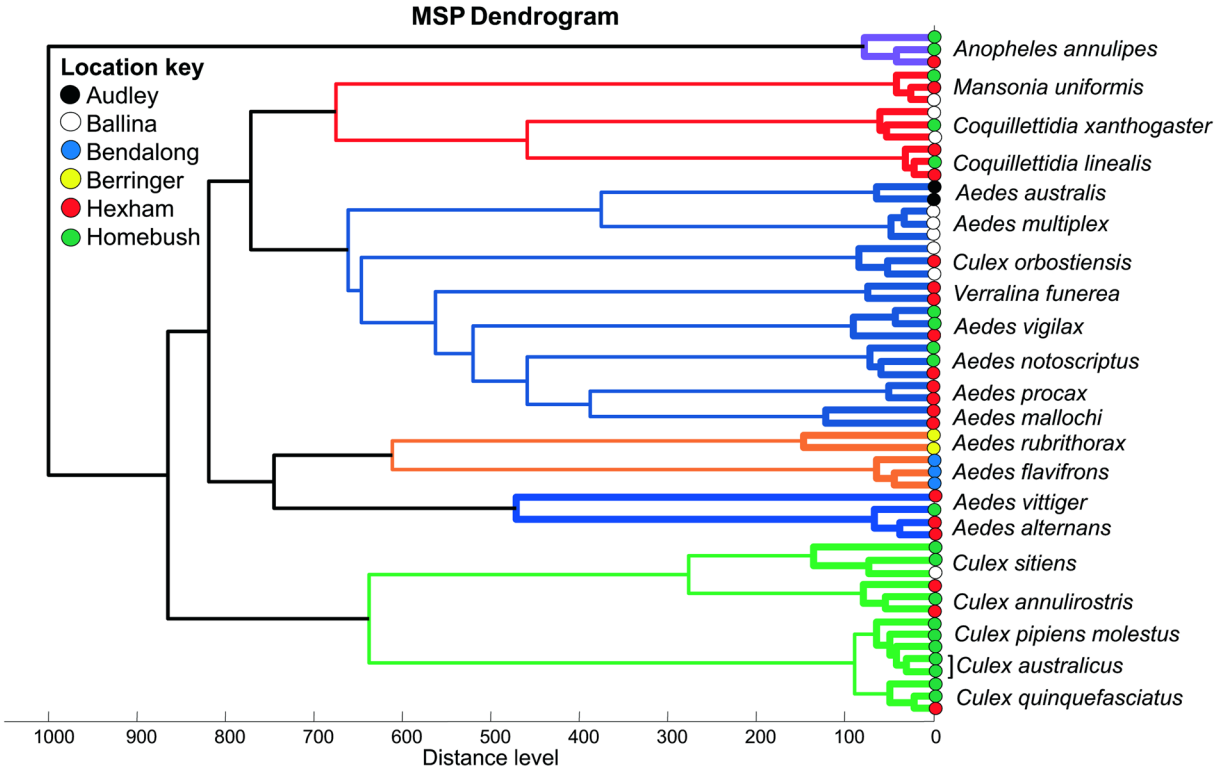

Fig. 2. Dendrogram of 21 mosquito species based on cluster analysis of MALDI-TOF MS spectra. All specimens selected for reference spectra (MSP) were included with two to three spectra per species. The spectra of two species (*Ae. rubrithorax* and *Ae. mallochi*) where reference spectra were unable to be created due to low sample numbers were included in the cluster analysis. Branch colours highlight the clustering pattern among species.

database. The spectrum for each sample quadruplicate was graded to produce a Log Score Value (LSV). For each spectrum, LSVs correspond to the degree of similarity in mass spectra peaks and signal intensities between the query spectra and the reference spectra. LSVs range from 0.0 to 3.0, and allow the evaluation of reproducibility between a queried spectrum and a reference spectrum, with higher scores representing greater similarity between two spectra (Laroche *et al.*, 2017*b*). Although strict thresholds are established for bacterial identification, no threshold is definitely validated for arthropod identification. Nevertheless, in this study LSVs >1.9 were considered adequate for relevant identification. Correct identification in the blind test was determined by the highest scoring spectrum within the quadruplicates of each sample. A final dendrogram displaying hierarchical clustering of all reference spectra was produced to visualize the distances and grouping between each species (Fig. 2).

DNA isolation and sequencing of cox1

All DNA barcoding analysis was performed using the bodies (without legs) of 58 mosquitoes, including all specimens used to create the MALDI-TOF MS reference spectra. Total DNA was extracted by adding 150 μL of QuickExtract DNA Extraction Solution (Epicentre, USA) and following the manufacturer's instructions. The extracted DNA was quantified using a NandoDrop 1000 spectrophotometer (Thermo Scientific, USA) and diluted to 40 ng μL^{-1} for use in conventional PCR.

A 710 bp region of the *cox1* gene was targeted and amplified using the universal *cox1* barcoding primers LCO1490 5′ GGT CAA CAA ATC ATA AAG ATA TTG G 3′ and HCO2198 5′ TAA ACT TCA GGG TGA CCA AAA AAT CA 3′ (Folmer *et al.*, 1994). The total PCR volume was 25 μL and included 15.8 μL of 0.1 mg mL^{-1} bovine serum albumin; 2.5 μL 10× ThermoPol Reaction Buffer (New England Biolabs, USA); 2 μL 2.5 μM dNTPs; 1.25 μL of each 10 μM L^{-1} primer; 0.2 μL 1.0 U *Taq* DNA polymerase; and 2 μL template DNA. The cycling conditions were as previously described (Batovska *et al.*, 2016) and all PCRs were performed using a Veriti Thermal Cycler (Applied Biosystems, USA). Products were resolved on 2% agarose gel and PCR products showing unambiguous single bands were sequenced bi-directionally on an ABI3730XL (Thermo Fisher Scientific, USA) by Macrogen Inc. (Korea).

DNA sequence analysis

All sequences were trimmed and assembled using Geneious version 8.1 (Kearse *et al.*, 2012). All sequence chromatographs had unambiguous reads. Assembled sequences were first queried against the Barcode of Life Database (BOLD) using the 'Species Level Barcode Records' search (accessed 13 December 2017) to verify molecular identity. MEGA version 6 (40) was then used to align edited sequences (627 bp) with the ClustalW algorithm, calculate sequence divergence using pairwise distance and create a neighbour-joining tree with 1000 bootstrap replicates (Supplementary Dataset S2). All sequences were deposited in GenBank (National Centre for Biotechnology Information, NCBI) under the following accession numbers: MG712511–MG712568, and in BOLD: MONSW001-17–MONSW058-17.

Results

Mosquito samples

A total of 300 female mosquitoes representing 21 species were collected, morphologically identified and analysed in this study including *Ae. flavifrons*, *Ae. multiplex*, *Ae. notoscriptus*, *Ae. vigilax*, *Anopheles annulipes*, *Coquillettidia linealis*, *Cq. xanthogaster*, *Cx. annulirostris*, *Cx. australicus*, *Cx. p. molestus*, *Cx. orbostiensis*, *Cx. quinquefasciatus*, *Cx. sitiens*, *M. uniformis* ($n = 18$ specimens

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for each species); *Ae. procax* ($n = 16$); *Ae. alternans*, *Ae. australis* ($n = 9$ specimens for each species); *Verralina funerea* ($n = 6$); *Ae. vittiger* ($n = 4$); *Ae. mallochi* and *Ae. rubrithorax* ($n = 2$ specimens for each species). Sample collection locations and sample details are displayed in Table 1.

Evaluation of sample MS spectra

The MS profiles were highly reproducible within each of the 21 mosquito species analysed. All spectra displayed high peak masses ranging from 2000 to 16 000 kDa with a range of 76 to 139 peaks per species (Fig. 1). Visual inspection of the spectra showed little variation between specimens from the same species collected from different locations in Australia. This is evident in Fig. 2, a dendrogram created with the reference spectra, where all specimens within a species clustered together regardless of location.

Initial screening of spectra for the Australian mosquito species that were not already present in the database ($n = 18$) produced incorrect identifications as expected. Due to the genetic similarity between *Cx. p. molestus* and *Cx. p. pipiens*, it was assumed that *Cx. p. molestus* would match to *Cx. p. pipiens* in the initial blind test. This was true for all specimens, but with low LSV ID scores (below 1.7). Likewise, the identification scores for *M. uniformis* already present in the database were below 1.7.

Creation of reference spectra and MALDI-TOF MS blind test validation

Reference spectra were created for 19 mosquito species (Supplementary Dataset S1). To account for within-species spectra diversity, at least four to six specimens are required for creating reference spectra, however less are required if the spectra are highly reproducible. The reference spectra are then used for the identification of the remaining specimens during blind testing. There was an insufficient number of samples for the remaining two species (*Ae. rubrithorax* and *Ae. mallochi*; $n = 2$ for each) to include them in the blind testing, therefore reference spectra could not be created. In the blind test analysis, 100% of the specimens from each species were correctly identified with LSVs >2 for the spot with the highest score and a range of 1.486–2.955 for all spots across all species (Table 1). Despite the spectral similarity between *Cx. australicus* and *Cx. p. molestus* from Homebush, NSW, both species were identified to the correct species, validating the existence of variation in their protein profiles. Similarly, once reference spectra were added for *Cx. p. molestus*, no samples matched to the *Cx. p. pipiens* already in the database and all were 100% correctly identified as *Cx. p. molestus*. Our results demonstrate that MALDI-TOF MS can clearly differentiate these taxa. As shown in the Supplementary Data 3, the MS profiles of these two mosquito species are significantly different with several visible specific peaks, as confirmed by the principal component analysis. All Australian *M. uniformis* and *Cx. quinquefasciatus* were 100% correctly identified to Australian reference spectra only and not to existing European and African spectra for these species. Reference spectra that were representative of the intraspecific variation were selected and a dendrogram was created to demonstrate the clustering of all species based on similarities and differences in their reference spectra. All reference spectra from the same species clustered together, supporting the reproducibility of the spectra and the delineation of each species (Fig. 2).

Molecular identification by DNA barcoding

A *cox1* sequence was generated for 58 mosquito specimens, representing all 21 species (Supplementary Dataset S2). A BOLD search of the *cox1* barcodes was able to correctly identify 15 of the 21 species with >98% match to reference sequences (Supplementary Table 1). Of the six species that were not identified, four were species that have not been previously barcoded (Fig. 3 and Supplementary Table 1) and have now been added to the BOLD and NCBI reference databases. The closest similarity match of these new species with other named species on BOLD was between 90% and 96%. The remaining two species that could not be identified using *cox1* DNA barcoding were *C .p. molestus* and *Cx. quinquefasciatus*. These two species are part of the *Cx. pipiens* complex, and are known to have low genetic diversity (Batovska *et al*., 2016). A further species, *Ae. flavifrons*, now has three publically available barcode sequences as a result of our study, as no public barcodes were available previously for this species.

These results are reflected in the neighbour-joining tree, where the majority of species formed distinct clusters, correlating with morphological identification (Fig. 3). Again, only *Cx. p. molestus* and *Cx. quinquefasciatus* were not distinguishable, with both species generating 100% identical *cox1* sequences and forming a single cluster. The neighbour-joining tree also highlighted the differences in intraspecific diversity between species. For instance, *Ae. rubrithorax*, *Cq. xanthogaster* and *Cx. orbostiensis* all have no intraspecific diversity in this dataset. However, *Ae. notoscriptus* and *An. annulipes* have high intraspecific diversity. These two diverse species groups have been previously documented (Foley *et al*., 2007; Endersby *et al*., 2013) and further investigation would be required to elucidate which specific clades these specimens belong to.

Discussion

We present the first MALDI-TOF MS reference spectra for the identification of a selection of Australian mosquito species. These species are known to be widely distributed, of pest or public health importance, or most commonly collected in mosquito surveillance programmes undertaken by health authorities on the east coast of Australia (Webb *et al*., 2016). Highly accurate differentiation of these species is vital for arbovirus surveillance and vector-borne disease risk management. A number of the species collected in our study (*Ae. notoscriptus*, *Ae. vigilax*, *An. annulipes*, *Cx. annulirostris*, *Cx. p. molestus*, *Cx. quinquefasciatus*, *Cq. linealis*, *Cq. xanthogaster* and *M. uniformis*) are important vectors of mosquito-borne pathogens in Australia such as RRV, BFV, Kunjin virus and MVEV (Mackenzie *et al*., 1994; Russell, 1995; Ryan and Kay, 1999; Selvey *et al*., 2014; Claflin and Webb, 2015). We achieved 100% correct identification of all 19 species that were exposed to the blind testing. Despite the insufficient sample number for the remaining two species (*Ae. rubrithorax* and *Ae. mallochi*), the dendrogram created showed highly reproducible spectra (Fig. 2) that were clearly delineated from the other species; thus, had more samples been available, creation of reference spectra for these species would have undoubtedly been successful.

Current mosquito and arbovirus surveillance programmes in Australia rely on morphological identification of mosquito specimens, despite limitations in identifying immature and damaged specimens. Alternative methods are required to complement morphological taxonomy and overcome its limitations in existing programmes. Rapid identification of immature mosquito stages has been developed through real-time reverse transcription-polymerase chain reaction assays (Montgomery *et al*., 2017) but this is currently only being incorporated into limited surveillance programmes. MALDI-TOF MS running costs are extremely economical and could represent a suitable alternative given that successful identification of immature specimens has been demonstrated (Dieme *et al*., 2014; Schaffner *et al*., 2014).

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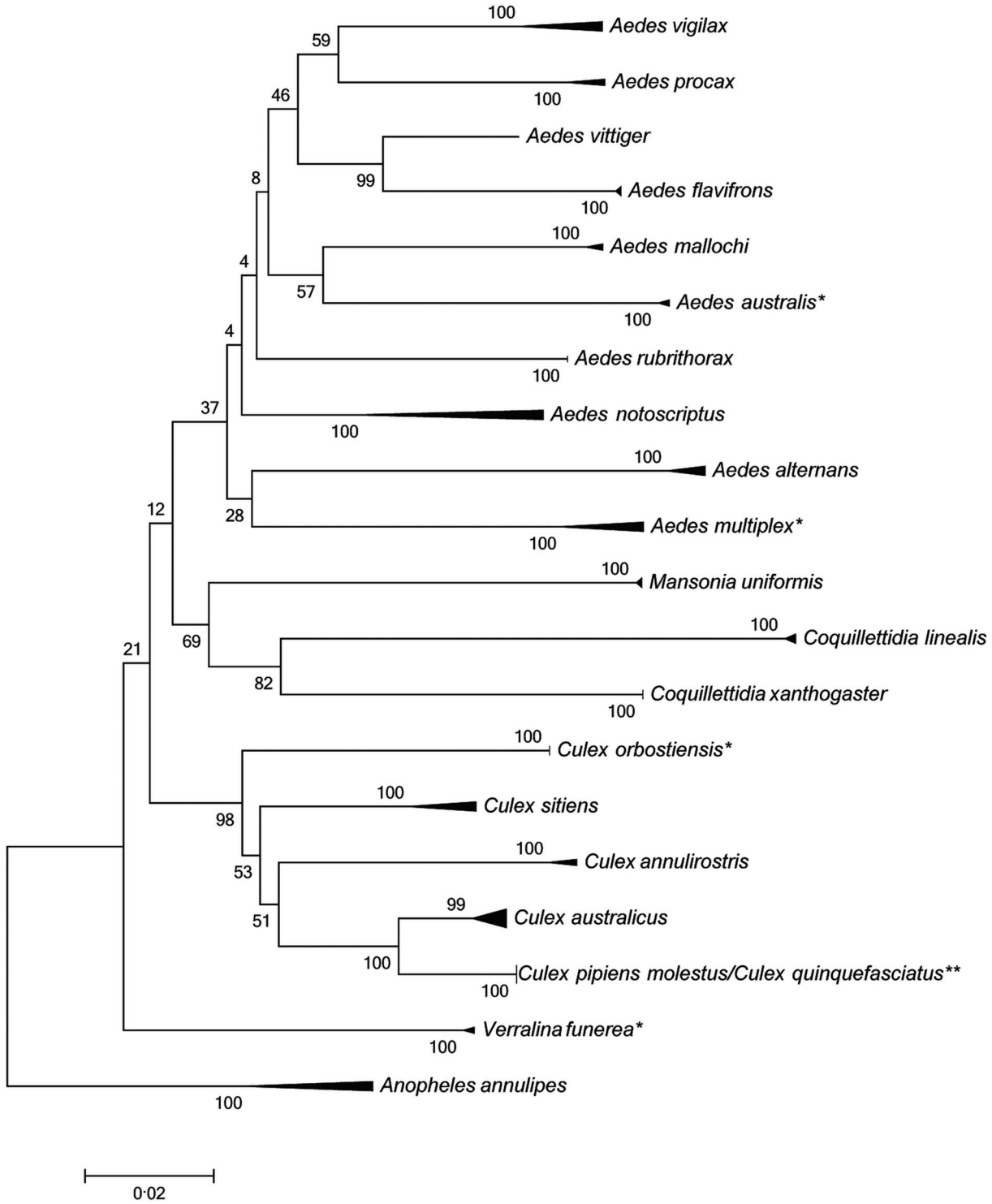

Fig. 3. Neighbour-joining tree of genetic relationships obtained from cox1 DNA sequences from 21 mosquito species. A summarized neighbour-joining tree based on *p*-distance comparisons between 58 mosquito *cox1* sequences (627 bp in length) with bootstrap support values shown (%). The * symbol indicates new DNA barcode reference species; the ** symbol indicates a species complex which could not be resolved using the *cox1* DNA barcode.

Emerging technologies are being incorporated into surveillance programmes to provide rapid identification of infective mosquitoes (van den Hurk *et al.*, 2012) but these approaches often do not provide similarly rapid and cost-effective identification of mosquito specimens. The role of DNA barcoding and metabarcoding in mosquito surveillance programmes is increasing (Batovska *et al.*, 2017; Lilja *et al.*, 2017) but is not currently employed on a large scale. Internationally, the species and forms within the *Cx. pipiens* group are demonstrably difficult to differentiate using morphological and molecular tools (Fonseca *et al.*, 2004; Batovska *et al.*, 2016; Shaikevich *et al.*, 2016). While *cox1* differentiation of *Cx. quinquefasciatus* and *Cx. p. molestus* has been unsuccessful in the past and in the current study, alternative gene regions, namely the acetylcholinesterase-2 (*ace-2*) gene have been successfully utilized for species differentiation (Smith and Fonseca, 2004). To address and resolve the ongoing taxonomic problems, an integrated approach should be employed to glean as much information on the species identity as possible. Our work demonstrates that MALDI-TOF MS is a valuable method to add to the taxonomic tool kit for mosquitoes since

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differentiation of *Cx. quinquefasciatus* and *Cx. p. molestus* was achieved. Likewise, differentiation of individuals within the *An. gambiae* complex has also been achieved using MALDI-TOF MS protein profiling (Müller *et al.*, 2013).

For the MALDI-TOF MS approach to be successful for mosquito identification in Australia, a rich database of specimens must be gathered and the issues surrounding geographic variability of mosquito species within Australia such as *Ae. notoscriptus* (Endersby *et al.*, 2013) must be considered as well as geographic variability of cosmopolitan species found worldwide. This is of increasing importance where the identification of less commonly encountered exotic specimens is required or where pathway analysis is sought to identify the country of origin. The differentiation between specimens of the same species sourced from different countries in this study (*M. uniformis* and *Cx. quinquefasciatus*) could have important implications for detecting incursions of cosmopolitan species from foreign countries to Australia and *vice versa*. This is significant since these specimens may carry exotic pathogens into a naïve region that has existing competent hosts (Whiteman Noah *et al.*, 2005; Tompkins and Gleeson, 2006; Nett *et al.*, 2008). However, it should be mentioned that the MS spectra produced by MALDI-TOF MS are highly dependent on the specimen preparation techniques used prior to testing and the variation between the existing and the new spectra may be an artefact of the differing preparation techniques (Nebbak *et al.*, 2016). Despite this, the presence of distinct spectra across two countries within the same species has been observed previously, supporting our results (Raharimalala *et al.*, 2017). Additionally, the differentiation of closely related taxa *Cx. p. pipiens* (already existent in the database) and *Cx. p. molestus* (new spectra) was not influenced by any preparation bias since were collected, stored and prepared using the methods described in this study.

The development of an Australian MALDI-TOF MS spectra database could enable the detection of incursions of Australian mosquito species into new regions (Williams Craig *et al.*, 2012). Moreover, as more and more countries such as India and Madagascar create and utilize MALDI-TOF MS spectra for arthropod identification, data sharing among laboratories could enable the development of comprehensive databases for invasive species monitoring on a global scale (Raharimalala *et al.*, 2017; Mewara *et al.*, 2018). Recent studies have shown MALDI-TOF MS has been utilized to identify mosquito blood meals *in vitro* (Niare *et al.*, 2016), and to identify *Plasmodium* infection within mosquito vectors (Laroche *et al.*, 2017*a*), demonstrating the future scope of MALDI-TOF for advanced vector screening worldwide. Furthermore, MALDI-TOF MS is able to differentiate between naïve, truly infected and exposed but uninfected mosquitoes for malaria; a potentially useful tool for the detection of exotic malaria threats, in Australia and other countries where malaria is not endemic (Laroche *et al.*, 2017*a*).

There are numerous factors to consider before MALDI-TOF MS can be incorporated into existing mosquito and arbovirus surveillance programmes in Australia, including the initial cost of the machine. However, due to the wide scope of MALDI-TOF MS for diagnostic identification across many different organisms, one machine can be used to service a variety of laboratories within an institution and initial costs can be shared as a worthwhile investment. Since MALDI-TOF MS is already utilized in Australia for microbiology diagnostics, the initial cost of the machine may be negated by collaborations between the microbiology laboratories with existing machines and entomology or arbovirus laboratories. Currently, three different MALDI-TOF mass spectrometer machines are used in microbiology diagnostics including the MALDI BioTyper™ (Bruker Daltonics, Germany) used in this study, the MALDI micro MX™ (Waters Corporation, Massachusetts, USA) and SARAMIS™ (Shimadzu & Anagnostec, Kyoto, Japan) (Seng *et al.*, 2010). This may pose a challenge for the direct transfer of reference spectra data between laboratories (between France and Australia, e.g.), since the utilization of these data for organism identification is dependent on the machine and software used (Flaudrops *et al.*, 2017). Thus, it is critical to standardize sample preparation as well as data processing to facilitate data exchange between laboratories (Nebbak *et al.*, 2016). It was recently demonstrated that spectra obtained from different instruments can be analysed together using a centralized database, enhancing the potential for global databases in the future (Mathis *et al.*, 2015).

Rapid and cost-effective species identification of mosquito specimens is essential for the surveillance of mosquito vectors and vector-borne pathogens and our results show that the MALDI-TOF MS tool has significant potential in aiding Australian mosquito identification. The reference spectra produced in our study could act as a base for the creation of a rich database for the identification of Australian and exotic mosquito species. We have demonstrated the methodological pipeline required to build a database of reference spectra that may represent the foundation for utilizing this approach for endemic and exotic mosquito surveillance in Australia and worldwide.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182018001658.

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Ethical standards. Not applicable.

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