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Morphological versus molecular identification of *Culex annulirostris* Skuse and *Culex palpalis* Taylor: key members of the *Culex sitiens* (Diptera: Culicidae) subgroup in Australasia

Cassie C Jansen,[1,2]† Stéphane Hemmerter,[3] Andrew F van den Hurk,[4] Peter I Whelan[5] and Nigel W Beebe[1,2]*

[1]*CSIRO Ecosystem Sciences, Brisbane, Qld 4102, Australia.*
[2]*School of Biological Sciences, University of Queensland, Brisbane, Qld 4072, Australia.*
[3]*University of Technology, Sydney, Sydney, NSW 2007, Australia.*
[4]*Public Health Virology Laboratory, Forensic and Scientific Services, Department of Health, Brisbane, Qld 4108, Australia.*
[5]*Centre for Disease Control, Department of Health and Families, Darwin, NT 0811, Australia.*

Abstract Members of the *Culex sitiens* subgroup of mosquitoes are important arbovirus vectors in the Australasian region. However, some species in this group, particularly *Cx. annulirostris* and *Cx. palpalis*, are difficult to distinguish based on morphological characters alone. We evaluated the reliability of morphological characters commonly used in taxonomic keys against a PCR-based diagnostic tool that examined the ribosomal ITS1 gene region. Although reliable morphological characters allow identification, molecular identification remains the most accurate means for distinguishing *Cx. annulirostris* and *Cx. palpalis*.

Key words arbovirus, Australasia, *Culex*, identification.

INTRODUCTION

The *Culex sitiens* subgroup comprises at least eight morphological species (Lee *et al*. 1989), with some of its members involved in the transmission of medically important arboviruses in the Australasian region. Within this subgroup, three species are of particular interest owing to their widespread distribution and/or incrimination in arbovirus transmission. *Cx. annulirostris* Skuse has the widest distribution and is a major vector of arboviruses, including Ross River virus (RRV), Murray Valley encephalitis virus (MVEV) and Kunjin virus (KUNV; a subtype of West Nile virus (WNV)) (Marshall 1988; Russell 2002). This species was implicated also in the emergence of Japanese encephalitis virus (JEV) in south-western Papua New Guinea (PNG) and northern Australia (Hanna *et al*. 1996, 1999; Ritchie *et al*. 1997; Mackenzie *et al*. 2002; van den Hurk *et al*. 2003), and it is capable of transmitting a North American strain of WNV (Jansen *et al*. 2008). Isolates of MVEV have been obtained from *Cx. palpalis* Taylor (Mackenzie *et al*. 1994), and this species comprised the majority of *Cx. sitiens*-subgroup mosquitoes identified from collections that yielded isolates of JEV in PNG (Chapman *et al*. 2000; Johansen *et al*. 2000). Although its role in the transmission of endemic viruses is relatively minor, *Culex sitiens* Weidmann is an efficient laboratory vector of JEV (van den Hurk *et al*. 2003). The *Cx. sitiens* subgroup has a primarily Australasian distribution, but with *Cx. sitiens* present throughout an Afro-Eurasian coastal arc including India, Burma, Bangladesh, Iran, Oman, Saudi Arabia and eastern Africa (Gaffigan *et al*. 2013).

Unfortunately, because of overlapping morphology, *Cx. annulirostris*, *Cx. palpalis* and, to a lesser extent, *Cx. sitiens* can be difficult to distinguish based on morphological criteria (Lee *et al*. 1989; Chapman *et al*. 2000), an issue exacerbated when key diagnostic features such as scales or bristles are damaged during collection (Chapman *et al*. 2000). Uncertainty in morphological identification is exemplified by the duration of synonymy of the names *Cx. palpalis* and *Cx. annulirostris*, beginning with Edwards (1924), with revalidation proposed only in 1989 by EN Marks (Lee *et al*. 1989). Furthermore, *Cx. palpalis* has been referred to by several names, including 'Normanton sp.', 'sp. near *annulirostris*', 'Normanton sp. NT form', and 'Mt Molloy form' (Lee *et al*. 1989).

Because of the difficulty of distinguishing these species, researchers often group them under the name '*Cx. sitiens* subgroup', particularly in cases where they occur sympatrically, such as in Queensland and the Northern Territory (Johansen *et al*. 2001, 2004; van den Hurk *et al*. 2006). In some instances, retrospective molecular analysis (Beebe *et al*. 2002) of such virus-positive pools has confirmed the presence of several *Cx. sitiens*-subgroup species (van den Hurk *et al*. 2002, 2006). However, because of the lack of initial

*n.beebe@uq.edu.au

†Current address: Metro North Public Health Unit, Queensland Health, Windsor, Qld 4030, Australia.

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morphological classification, the true species identity of the virus-infected mosquito or mosquitoes within these pools could not be obtained. Clearly, the uncertainty surrounding morphological taxonomy has implications for our understanding of transmission cycles.

Chapman *et al*. (2000) examined the taxonomy of the *Cx. sitiens* subgroup in Australia and PNG using non-morphological tools, but the allozyme electrophoretic method employed left 6.9% of samples unidentified. *Cx. sitiens* could be distinguished clearly from *Cx. annulirostris* based on allozymes, but *Cx. palpalis* could not, and the authors reported that allozymes could not further resolve this problem. Furthermore, the use of allozyme analysis in the field is problematic because the protein enzymes can degrade rapidly and the samples need to be stored at −50°C. In addition, the quantities and forms of enzymes differ between life stages (Beebe *et al*. 2002). To complement the allozyme diagnostic, Beebe *et al*. developed a DNA-based PCR method to identify these three species genetically (Beebe *et al*. 2002). This relatively simple diagnostic assay, which clearly resolves the three species, is based on the ribosomal DNA internal spacer 1 (ITS1), which is PCR-amplified and cleaved with the restriction enzyme Rsa I. The ITS1 region is frequently used in insect systematics owing to its variability and ease of amplification (Caterino *et al*. 2000).

Nonetheless, in many instances it is impractical to use molecular tools for mosquito identification because of resource or technical limitations, because of cost, or when identification of thousands of mosquito specimens is required (Johansen *et al*. 2000). Thus, morphological identification remains the primary method employed, particularly in field-based studies, and consequently, it is important that field researchers be aware of the accuracy of identifications made with morphology. Herein, we utilised the PCR-RFLP procedure of (Beebe *et al*. 2002) to assess the accuracy of existing morphological keys (Marks 1982a; Lee *et al*. 1989; Liehne 1991; Russell 1993; Chapman *et al*. 2000) for the identification of adult female *Cx. annulirostris* and *Cx. palpalis* from Australia, PNG, the Solomon Islands and Timor-Leste. We identify a combination of morphological features to assist in the separation of specimens of these two species.

MATERIALS AND METHODS

Mosquito collection

Adult mosquitoes were collected between 1996 and 2007 from 53 sites in 33 locations throughout Australia, PNG, Timor-Leste and the Solomon Islands (Fig. 1). Mosquitoes were collected using either CO_2-baited encephalitis virus surveillance traps or Centers for Disease Control light traps baited with CO_2 and 1-octen-3-ol (octenol). Mosquitoes were stored in liquid nitrogen, dry ice, silica gel or 70% ethanol prior to further analysis. A colony of *Cx. annulirostris* from Brisbane established in 1998 by the Army Malaria Institute (Brisbane) was used as reference material.

Morphological identification

Specimens were identified from morphology by experienced entomologists familiar with the mosquito fauna of each region using keys that include either all mosquitoes from Australasia (Lee *et al*. 1989; Chapman *et al*. 2000) or those from a more restricted region (Marks 1970a, 1982a; Liehne 1991; Russell 1993; Nguyen & Whelan 2009). Each of these keys employs different characters depending on the region for which it was designed. Some regionally specific keys, for example that concerning mosquitoes from southern Australia (Russell 1993), did not include *Cx. palpalis*, as it is uncommon or rare in the regions in question. Of note, the keys of Marks (1982a) were modified recently by Chapman *et al*. (2000) to include *Cx. palpalis*. A summary of the previously identified key features used in these keys to distinguish the three key members of the *Cx. sitiens* subgroup are presented in Table 1. Some features commonly used include basal banding on abdominal tergites, size of the pale ring on the proboscis and the pattern of the scaling of the tibia. Certain features, such as the size of the specimens, were included only by Chapman *et al*. (2000) and Liehne (1991).

A subset of specimens from Queensland and the Northern Territory was used to evaluate three morphological characters common to keys that describe both *Cx. annulirostris* and *Cx. palpalis* from these regions. These specimens were evaluated for the width of the ring on the proboscis, the shape of the basal tergal bands and the appearance of pale scaling on the anterior hind tibia. Following morphological assessment, DNA was extracted and amplified as described below.

Molecular identification

All mosquitoes were identified using the PCR-RFLP procedure of Beebe *et al*. (2002), which discriminates between three Australasian members of the *Cx. sitiens* subgroup (*Cx. sitiens*, *Cx. annulirostris* and *Cx. palpalis*). Total DNA was extracted from mosquitoes using a salt extraction and ethanol procedure. Mosquitoes (either partial or whole) were ground thoroughly in a 1.5 ml microfuge tube containing 50 μL of lysis buffer (0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris-HCl at pH 9.0, 0.05 M EDTA and 0.5% sodium dodecyl sulfate (SDS) using a 1.5 ml plastic grinding pestle (Kontes Glass, Vineland, NJ, USA). Tubes were briefly centrifuged to concentrate the homogenate at the bottom of the tube before incubation at 65°C for 30 min. While tubes were still warm, 7 μL of 8 M potassium acetate was added and mixed in. The tubes were incubated in the freezer for at least 40 min to precipitate SDS and centrifuged for 15 min at 13 200 g. Supernatants were transferred to a new tube, to which 100 μL of 100% ethanol was added. Tubes were incubated for 15 min at room temperature to precipitate the nucleic acid and centrifuged for 15 min at 13 200 g, and supernatants were removed. Ethanol (100 μL, 70%) was added, and tubes were centrifuged again at 13 200 g for 5 min. Supernatants were again removed, and tubes were air-dried and resuspended in 50 μL TE buffer (100 mM Tris/HCl, 10 mM EDTA) containing RNase (5 μg/mL).

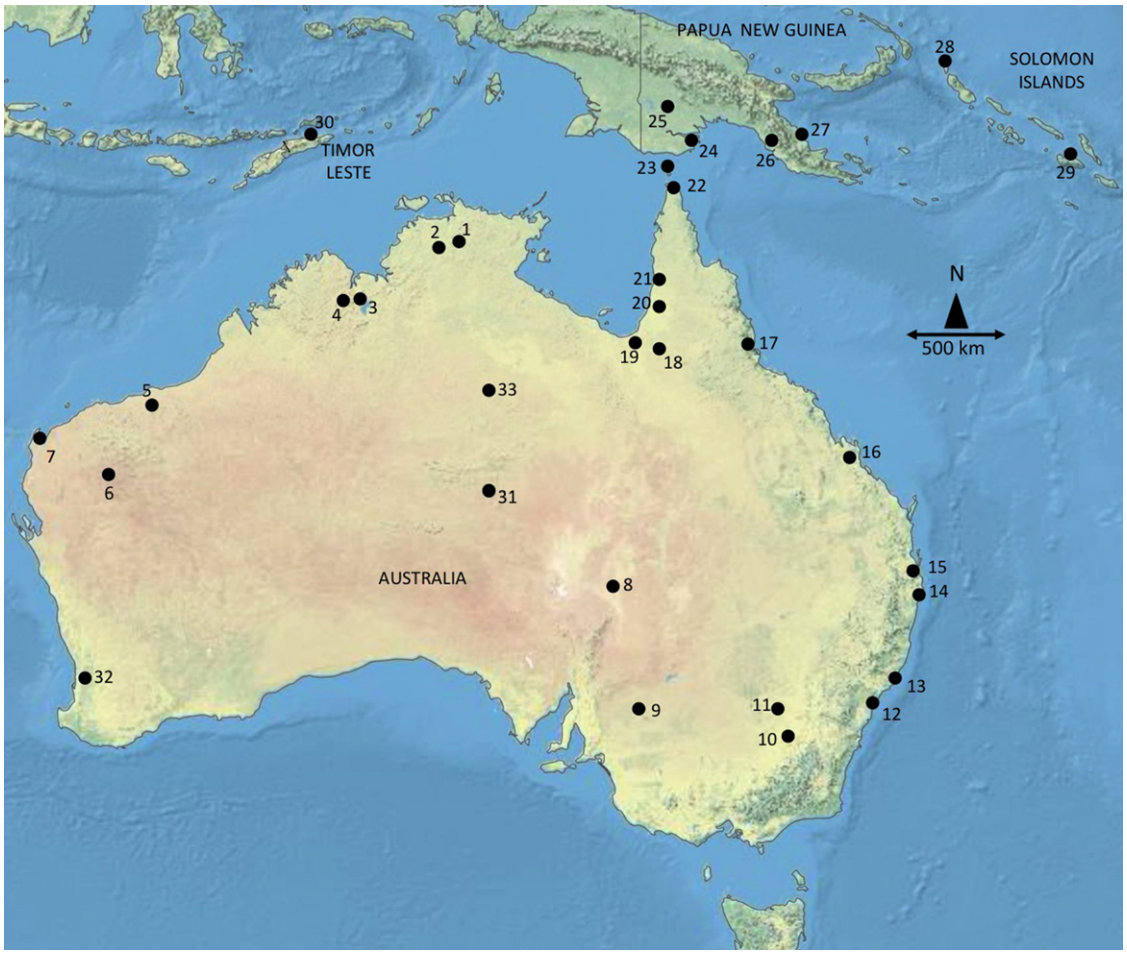

Fig. 1. Mosquito collection sites in Australasia. Site names are listed in Table 2.

Table 1 Summary of key features used to distinguish *Cx. annulirostris*, *Cx. palpalis* and *Cx. sitiens*, compiled from different taxonomic keys

Morphological feature	Taxonomic key					
	Australasia (Lee *et al.* 1989)	Australasia (Chapman *et al.* 2000)	Queensland (Marks 1982a)†	Western Australia (Liehne 1991)	Southern Australia (Russell 1993)†	Northern Territory (Marks 1970; Nguyen & Whelan 2009)
Size of specimen	✗	✓	✗	✓	✗	✗
Femora mottled	✗	✓	✗	✗	✗	✗
Pale patch on hind tibia	✓	✓	✗	✗	✗	✓
Pattern on the scutum (lyre pattern)	✗	✓	✗	✓	✗	✓
Width of pale band on the proboscis	✗	✓	✓	✓	✗	✓
Tergal banding	✓	✓	✓	✓	✓	✓
Sternal banding	✗	✓	✓	✓	✓	✗
Colour of the fore-tibia	✗	✓	✗	✗	✗	✓
Proximity of the base of the cells R_2 and M_1 to the base of the wing	✓	✗	✓	✓	✗	✓

†Does not include *Cx. palpalis*.

All PCR reactions were carried out in a 25 μL volume on a MJ-PTC200 Thermocycler (MJ Research, Maltham, MA, USA). The final PCR mixture contained 1 to 10 ng of template DNA, 20 pM of each primer, 1.25 mM $MgCl_2$, 1.5 mM of each dNTP, 1X Taq reaction buffer and 1 U of Taq DNA polymerase. The ITS1A forward primer binds to the flanking segment of the 18S gene (5′-CCT TTG TAC ACA CCG CCC GTC G-3′). The ITS1B primer was designed to bind to the 5.8S gene (5′-ATG TGT CCT GCA GTT CAC A-3′). Cycling involved initial denaturation at 94°C for 4 min, followed by 30 cycles at 94°C for 30 s, 30 cycles at 51°C for 40 s and 30 cycles at 72°C for 30 s. Restriction endonuclease digestion was carried

out in a 0.5 ml microfuge tube containing 3 µL of PCR product, to which was added 3 µL of 2X Rsa I buffer (premade stock) containing 1 U Rsa I enzyme/reaction (NEB II, New England Biolabs, Beverly, MA, USA). The mixture was incubated at 37°C for 1 h and the digest visualised on a 3% agarose gel containing ethidium bromide (0.5 µg/mL) at 100 V for 30 min, viewed on a UV transilluminator at 312 nm.

RESULTS

A total of 702 mosquitoes belonging to the *Cx. sitiens* subgroup and morphologically identified as either *Cx. annulirostris* or *Cx. palpalis* were examined using both morphological criteria and PCR-RFLP. As identified by PCR-RFLP, these mosquitoes comprised 492 (70.1%), 207 (29.5%) and 3 (0.4%) individuals of *Cx. annulirostris*, *Cx. palpalis* and *Cx. sitiens*, respectively. *Cx. annulirostris* was distributed more widely than *Cx. palpalis*, being collected from all locations, whereas the latter was collected from only 8 of the 33 *Cx. annulirostris* collection site locations. Overall, there was 92.2% concordance between morphological and molecular identification of *Cx. sitiens*-subgroup mosquitoes (Table 2). Morphological identification of *Cx. annulirostris* appeared most reliable, with over 96.2% of morphologically identified specimens confirmed by PCR-RFLP. In contrast, 16.3% of the mosquitoes morphologically identified as *Cx. palpalis* were misidentified, with all but two (of 37) misidentified specimens being identified as *Cx. annulirostris* by PCR-RFLP.

We assessed 126 specimens from Queensland and the Northern Territory to determine what morphology listed in Table 1 could most reliably separate *Cx. annulirostris* and *Cx. palpalis* (Fig. 2). This revealed that 100% of *Cx. annulirostris* had a median pale ring on the proboscis covering no more than one-third its length, 93% had basal bands on abdominal tergites produced into a median triangle and 95% had no distinct pale patch or line of scales on the anterior hind tibia despite the presence of pale scales in this region. In contrast, the length of the median pale band on the proboscis of *Cx. palpalis* was quite variable, ranging from a quarter of the length of the proboscis to more than half. The majority of *Cx. palpalis* had basal bands on the abdominal

Table 2 Molecular identification using PCR-RFLP of specimens from the Australasian region previously morphologically identified as *Cx. annulirostris* or *Cx. palpalis*

Region	Locality	Map reference (Fig. 1)	*Culex annulirostris*				*Culex palpalis*			
			n	ann	pal	sit	*n*	ann	pal	sit
Northern Territory	Jabiru	1	26	24	2	0	92	2	90	0
	Mary River	2	32	32	0	0	42	6	36	0
	Alice Springs	31	11	11	0	0	0	0	0	0
	Tennant Creek	33	10	10	0	0	0	0	0	0
Western Australia	Kununurra	3	5	5	0	0	0	0	0	0
	Wyndham	4	24	24	0	0	0	0	0	0
	Port Headland	5	5	5	0	0	0	0	0	0
	Paraburdoo	6	5	5	0	0	0	0	0	0
	Exmouth	7	0	0	0	0	0	0	0	0
	Perth	32	11	11	0	0	0	0	0	0
South Australia	Mungeranie	8	22	22	0	0	0	0	0	0
	Mundic Creek	9	7	7	0	0	0	0	0	0
New South Wales	Albury	10	9	3	6	0	0	0	0	0
	Griffith	11	5	5	0	0	0	0	0	0
	Baulkham Hills	12	4	4	0	0	0	0	0	0
	Port Stephens	13	14	14	0	0	0	0	0	0
	Ballina	14	5	5	0	0	0	0	0	0
Queensland	Ipswich	15	5	5	0	0	0	0	0	0
	Shoalwater Bay	16	5	5	0	0	0	0	0	0
	Cairns	17	6	6	0	0	0	0	0	0
	Croydon	18	48	48	0	0	10	2	8	0
	Karumba	19A	32	32	0	0	5	3	2	0
	Normanton	19B	61	60	1	0	54	5	49	0
	Kowanyama	20	6	5	1	0	0	0	0	0
	Pormpuraaw	21	8	8	0	0	0	0	0	0
	Bamaga	22	15	15	0	0	1	1	0	0
	Badu Island	23	13	12	1	0	0	0	0	0
Papua New Guinea	Lower Fly River	24	29	28	1	0	0	0	0	0
	Balimo	25	19	14	5	0	12	7	5	0
	Terapo	26	14	14	0	0	11	9	0	2
	Poppondetta	27	0	0	0	0	0	0	0	0
	Buka	28	13	13	0	0	0	0	0	0
Solomon Islands	Guadalcanal	29	3	3	0	0	0	0	0	0
Timor-Leste	Dili	30	3	2	0	1	0	0	0	0

ann, *Cx. annulirostris*; pal, *Cx. palpalis*.

Species	*n*	Size of ring on proboscis			Basal bands on tergites produced into median triangles		Defined elongate patch of white scales on hind tibia	
		Narrow	Medium	Broad	Yes	No	Present	Absent
Culex annulirostris	42	100	0	0	93	7	5	95
Culex palpalis	84	57	10	33	2	98	98	2

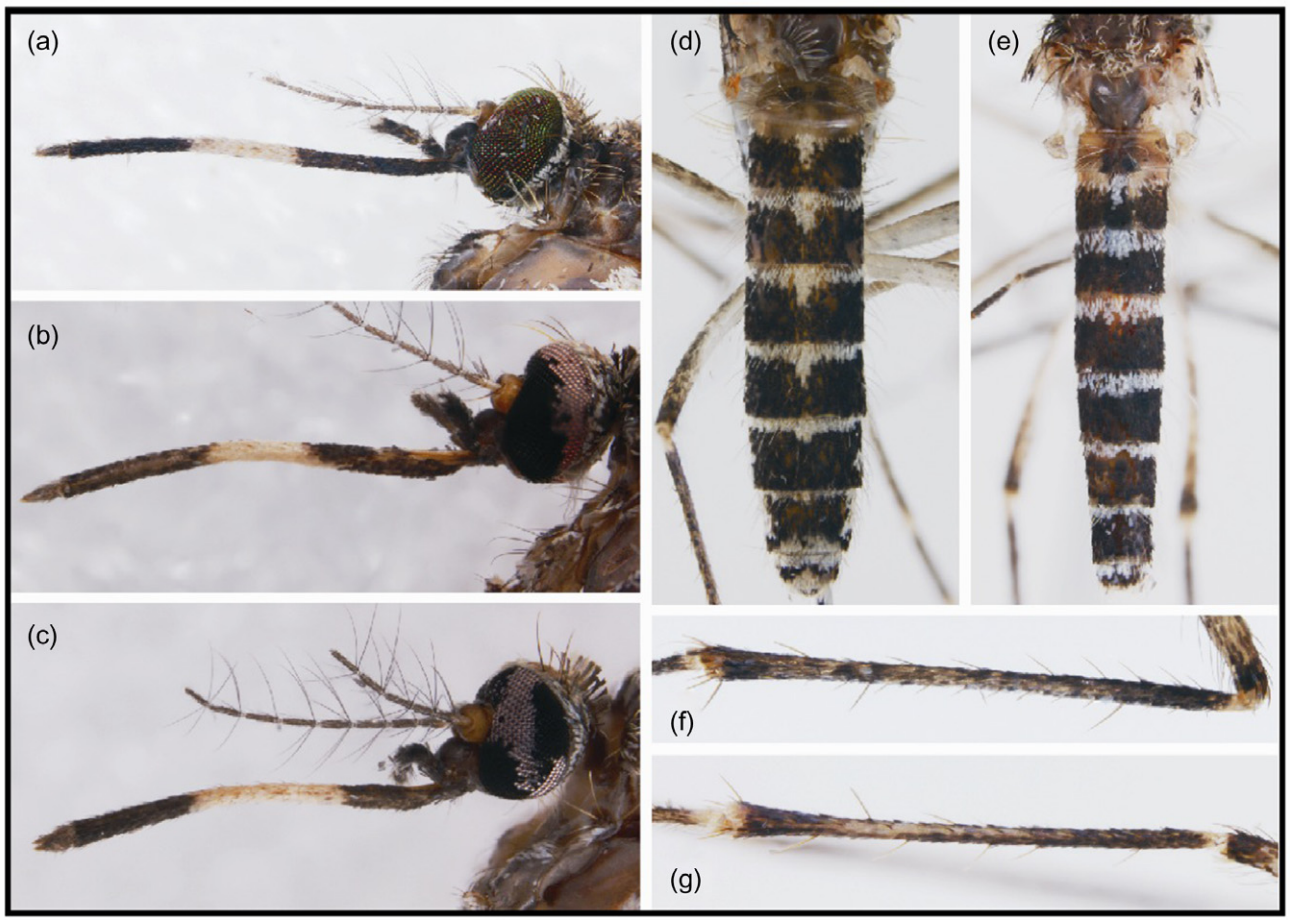

Fig. 2. Percentages of *Culex annulirostris* and *Cx. palpalis* samples from Queensland and Northern Territory possessing commonly used descriptive morphological characters. Probosces of (a) *Cx. annulirostris* and (b) *Cx. palpalis* showing 'narrow' median white ring on the proboscis that is between one-quarter to one-third of the length of the proboscis; (c) proboscis of *Cx palpalis* showing 'medium' ring, from one-third to half of the length of the proboscis (although not shown, a 'broad' ring indicates a band of more than the half of the length of the proboscis); (d) abdomen of *Cx. annulirostris* with basal bands on tergites produced into median triangles; (e) abdomen of *Cx. palpalis* with basal bands on tergites not produced into median triangles but sometimes with widening at the midline; (f) hind tibia of *Cx. annulirostris* without distinct patch of white scales; (g) distinct patch of white scales on hind tibia of *Cx. palpalis*.

tergites that were widened in the midline but produced into observable triangles in only 2% of specimens. The majority (98%) of *Cx. palpalis* had a large pale elongate patch on the anterior surface of the hind tibia.

DISCUSSION

Morphological identification of mosquito species using taxonomic keys can be performed in the field and is inexpensive (Beebe & Cooper 2000). An expert entomologist familiar with the mosquito biodiversity of a given region requires only a few seconds to identify an individual to species level, allowing for the rapid sorting of specimens. However, using morphological criteria alone, it can be difficult to separate *Cx. annulirostris* from *Cx. palpalis*, particularly when the specimen has been damaged during collection (Chapman *et al*. 2000). This problem is exacerbated when species exist sympatrically, as was the case in all locations from which *Cx. palpalis* was

collected during the current study. Indeed, the difficulty in separating these two species is acknowledged explicitly in Lee *et al*. (1989).

The PCR-RFLP developed by Beebe *et al*. (2002) is the only tool used currently to distinguish accurately between members of the *Cx. sitiens* subgroup and facilitated the assessment of current taxonomic keys for separating species in our study. Furthermore, this molecular identification tool is supported completely by phylogeny studies at the mitochondrial DNA level using the cytochrome oxidase I gene and at the nuclear DNA level using the acetylcholinesterase 2 gene (Hemmerter *et al*. 2007; 2009). Thus, when accuracy of identification is critical, such as in the incrimination of vectors responsible for pathogen transmission, the use of PCR-RFLP is necessary.

Although morphological identification of adult females is challenging, identification of larvae is relatively straightforward from morphology alone. Clear and unambiguous keys and descriptions of the larvae of *Cx. annulirostris*, *Cx. palpalis* and *Cx. sitiens* are provided by Marks (1970b, 1982b), allowing determination of the presence of any of the three species. Furthermore, *Cx. palpalis* shows distinctive larval habitat preferences that are restricted to clear, non-polluted fresh water with emergent vegetation but without saline influence. These habitats, typically including freshwater lagoons and swamps with reeds (*Eleocharis* spp. and *Typha* spp.), can be targeted seasonally to seek *Cx. palpalis*.

However, if morphological identification of adult females is required, careful consideration of particular characters is recommended. *Cx. annulirostris* consistently possessed a narrow band on the proboscis, coupled with tergal basal bands produced into median triangles. These distinctive features probably explain the correct morphological identification of *Cx. annulirostris* in most (96%) of the cases. Conversely, *Cx. palpalis* displayed variation in the length of the ring on the proboscis, and while median triangular peaks were absent in most cases, the basal tergal bands sometimes displayed widening at the midline (Fig. 2e). The ambiguity of these features explains the lower accuracy in morphological identification of *Cx. palpalis*, and variation may be attributable to the presence of further mtDNA and nuclear lineages within *Cx. palpalis* (Hemmerter *et al*. 2007; 2009). Although the presence of an elongate patch on scales on the anterior tibia appears reliable (Fig. 2), we recommend caution, as pale scaling was clearly identifiable on the anterior hind tibia in *Cx. annulirostris* under high magnification, although producing a less distinct patch than in *Cx. palpalis*.

Keys for the identification of adult mosquitoes in the Australasian region utilise several different criteria to distinguish *Cx. annulirostris* from *Cx. palpalis*. Based on the results of our comparison, careful consideration of the presence/absence of triangular peaks on basal tergal bands and of hind tibia marking, as in the couplet separating *Cx. annulirostris* and *Cx. palpalis* in the dichotomous key of Lee *et al*. (1989), appears to separate adult females most reliably. Further, this study highlights the need to exercise caution when interpreting keys and to consider extralimital species. In particular, this study confirmed the identity of *Cx. palpalis* from Albury in New South Wales, despite its absence from the regional key of Russell (1993).

The *Cx. sitiens* subgroup is of paramount importance in transmission of arboviruses in the Australasian region. Thus, the need for accurate identification of constituent species cannot be overemphasised. For example, there has recently been suggestion that the existence of further cryptic species within *Cx. annulirostris*, identified using both the *CO1* and *ace-2* genes, which may help explain why there is a latitudinal limit of JEV transmission in Australasia just 70 km north of the Australian mainland despite the abundance of capable hosts further south (Hemmerter *et al*. 2009). Although morphological identification of the *Cx. sitiens* subgroup may be unreliable, it remains an essential. Herein, we have highlighted the most reliable morphological characters for separation of *Cx. annulirostris* and *Cx. palpalis*. Nevertheless, identification based on morphology alone will be difficult or impossible for some specimens, especially when diagnostic features, such as scales, are obscured or lost. In these cases, molecular identification using the ITS1 gene region is currently the best available alternative.

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