Molecular systematics, morphological analysis, and hybrid crossing identify a third taxon, *Aedes* (*Halaedes*) *wardangensis* sp.nov., of the *Aedes* (*Halaedes*) *australis* species-group (Diptera: Culicidae)

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Abstract: Phylogenetic and morphological analyses, male morphology, and hybrid crossing indicate that a population from Wardang Island, South Australia, is distinct from the monophyletic series of populations of *Aedes* (*Halaedes*) *australis* (Erichson) 1842 from Victoria, Tasmania, New South Wales, and New Zealand. The name *Aedes* (*Halaedes*) *wardangensis* has been assigned to the new species. Phylogenetic analysis of DNA sequences from the cytochrome oxidase II and internal transcribed spacer loci support the resurrection of *Aedes* (*Halaedes*) *ashworthi* Edwards, 1921 (Brust and Mahon, 1997). *Aedes ashworthi* is known only from Western Australia and was found to be infertile when crossed with *Ae. wardangensis* from South Australia and *Ae. australis* from New Zealand. The hybrid of *Ae. australis* from New South Wales × *Ae. australis* from New Zealand was fertile for three generations, documenting these as conspecific.

Résumé : Des analyses phylogénétiques et morphologiques, la morphologie des mâles et le croisement d'hybrides indiquent que la population de l'île de Wardang, en Australie méridionale, est distincte de la série monophylétique de populations d'*Aedes* (*Halaedes*) *australis* (Erichson) 1842 de Victoria, de Tasmanie, de Nouvelle-Galles du Sud et de Nouvelle-Zélande. Le nom *Aedes* (*Halaedes*) *wardangensis* a été assigné à la nouvelle espèce. L'analyse phylogénétique des séquences d'ADN de la citychrome oxydase II et de l'espaceur interne transcrit justifient la résurrection du taxon *Aedes* (*Halaedes*) *ashworthi* Edwards, 1921 (Brust et Mahon, 1997). *Aedes ashworthi* n'a été rencontré qu'en Australie occidentale et il est stérile lorsqu'il est croisé à *Ae. wardangensis* d'Australie méridionale ou à *Ae.* de Nouvelle-Zélande. L'hybride obtenu par croisement d'*Ae. australis* de Nouvelle-Galles du Sud et d'*Ae. australis* de Nouvelle-Zélande s'est avéré fertile pour trois générations, ce qui confirme le statut conspécifique de ces insectes.

[Traduit par la Rédaction]

Introduction

The *Aedes* (*Halaedes*) *australis* group is known as a saline rock pool mosquito of Australasia. In Australia, larval populations have been reported from rock-pool habitats along the sea coast from the Queensland (Qld) – New South Wales (NSW) border to Victoria (Vic.), South Australia (SA), islands in Bass Strait, Tasmania (Tas.), and Western Australia (WA). It is also known from Lord Howe Island, Norfolk Island, and New Zealand (NZ) (Belkin 1962, 1968; Dobrotworsky 1966; Lee et al. 1984). Recently, Brust and Mahon (1997) reported that *Ae. australis* sensu auctorum consists of two species. They showed from biological crosses that there was hybrid sterility between a population from Western Australia and one from New South Wales, and they found morphological differences between adults of the two populations. They resurrected *Aedes ashworthi* Edwards, 1921, which was originally collected and named from Western Australia specimens, as a valid species (Brust and Mahon 1997).

The present study reports on (*i*) morphological differences between males of a population of the *Ae. australis* group from Wardang Island, SA, and males of known *Ae. australis* from NSW, Tas., and NZ, (*ii*) genetic incompatibility of adults from WA, SA, NSW, and NZ, demonstrated by infertility of hybrids of three populations of the *Ae. australis* group, (*iii*) conspecificity of a population from NSW and a population from NZ, and (*iv*) phylogenetic analyses of DNA sequence data from the cytochrome oxidase II (COII) and

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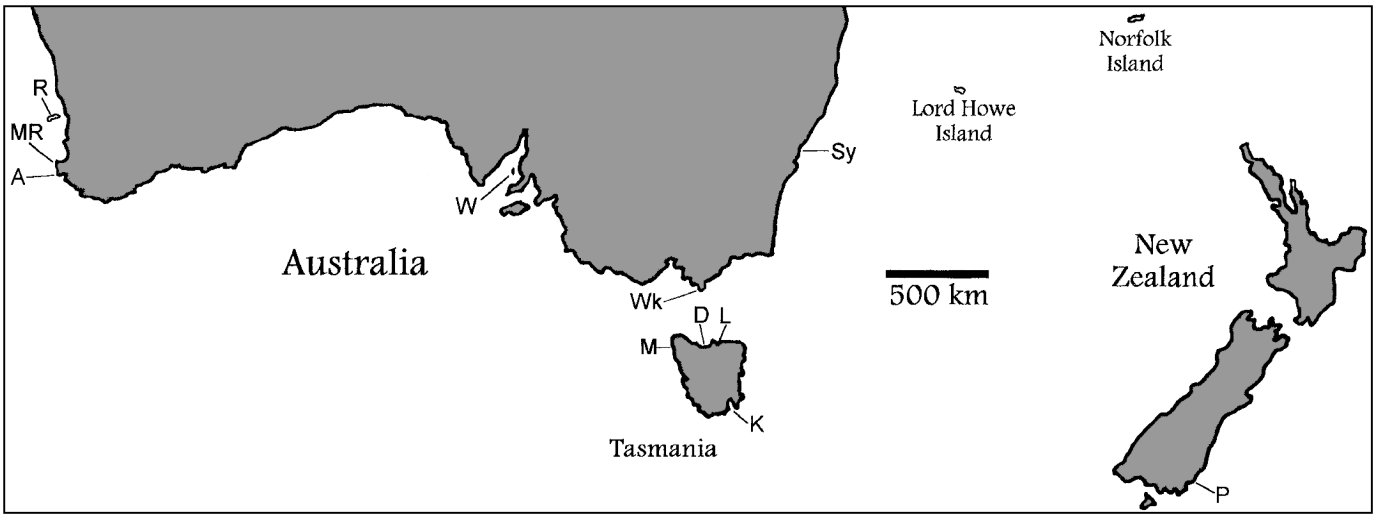
Fig. 1. Map of the sites from which larval populations of the *Ae. australis* group were obtained. D, Devonport, Tas.; L, Low Head, Tas.; K, Kingston, Tas.; M, Marrawah, Tas.; Wk, Walkerville, Vic.; Sy, Sydney, NSW; A, Augusta, WA; MR, Margaret River, WA; R, Rottnest Island, WA; W, Wardang Island, SA; P, Purakanui Bay, NZ.



internal transcribed spacer (ITS) loci of 11 populations of the *Ae. australis* group from Australia and NZ.

Methods

Larval collections were obtained from Sydney, NSW; Walkerville, Vic.; Devonport, Low Head, Kingston, and Marrawah, Tas.; Wardang Island, SA; Augusta, Margaret River, and Rottnest Island, WA; and Purakanui Bay, NZ. (Fig. 1). Larvae were transported by air or by road, and reared under controlled environmental conditions in the laboratory. They were fed liver powder (ICN Biomedicals, Aurora, Ohio, U.S.A.) daily, in excess of consumption, and were reared uncrowded in pans 33 × 33 × 10 cm high. Pupae were removed and placed in clear acrylic cube cages either 32 or 15 cm per side.

Morphological analyses

Twenty males were randomly selected from each of five populations: Rottnest Island, WA; Wardang Island, SA; Sydney, NSW; Marrawah, Tas.; and Purakanui Bay, NZ. The genitalia of these males were cleared in cold 10% KOH and mounted in glycerin. The illustration was prepared with the aid of a compound microscope tracing device (a type of camera lucida). The terms used to describe the anatomical structures are those used by Harbach and Knight (1980). Morphometric analyses were conducted on the gonocoxite length and width (Fig. 2) and on the gonostylus length of the genitalia of males from each population.

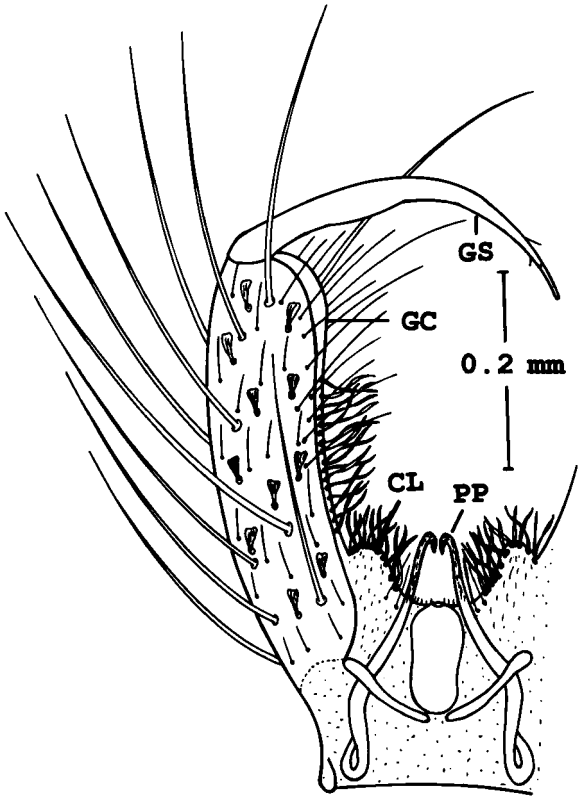
Deposition of study material

Male and female specimens of *Ae. australis*, *Ae. ashworthi*, and *Aedes wardangensis* have been deposited in the Australian National Insect Collection, CSIRO, Canberra, ACT 2601, Australia. The larvae, and the remainder of the adults of the above species, have been deposited in the J.B. Wallis Museum, Department of Entomology, University of Manitoba, Winnipeg, MB R3T 2N2, Canada.

Biological crosses

Autogenous populations were stenogamous and mated readily in either the 32 cm/side or the 15 cm/side cube cages. Populations that were partially or fully anautogenous mated readily when reared and maintained at 16–18°C, and when more males (10×) than females were available in either size of cage. Biological crosses were conducted as described in Brust and Mahon (1997). Adults were offered 10% sucrose and anautogenous females were fed on a human host.

Fig. 2. Diagram of the dorsal aspect of the male genitalia of *Ae. wardangensis*. CL, claspette; GC, gonocoxite; GS, gonostylus; PP, paraproct.



DNA extraction

DNA from individual mosquitoes was extracted using a chelex extraction modified from the method of Walsh et al. (1991).

Briefly, pestles were prepared by sealing P1000 pipette tips and molding them into a microcentrifuge tube. Individual mosquitoes were placed in a microfuge tube, cooled under liquid nitrogen, and ground using the molded pipette tip. Five hundred microlitres of 5% Chelex (Bio-Rad Cat No. 143–2832; the chelex was kept on a magnetic stirrer to keep the "beads" in suspension) was added before the specimen thawed. The sample was mixed using the pestle and 2.5 µL of proteinase K (10 mg/mL) and 9 µL of 1M DTT was added. The macerated mosquito was vortexed and incubated at 65°C for 30 min. The sample was then vortexed again for 5 s, spun briefly at 13 000 rpm in a microfuge and boiled for 7 min. The sample was vortexed once again, then spun for 3 min in a microfuge. The supernatant was transferred to a clean tube and stored at –20°C until use.

COII

Amplification

The mitochondrial COII gene was amplified using the conserved TL2-J-3037 (A-tLEU) and TK-N-3785 (B-tLYS) primers (Simon et al. 1994). The polymerase chain reaction (PCR) was done in a Corbett thermal cycler with an initial denaturation at 95°C for 5 min followed by addition of Taq polymerase, then 32 cycles for 1 min at 94°C, 1 min 15 s at 52°C, and 1 min 30 s at 72°C.

The PCR products were purified and prepared for sequencing by electrophoresis in 0.8% TAE agarose gels containing 10 $\mu g \cdot mL^{-1}$ ethidium bromide. Fragments were cut out and transferred to a microfuge tube. The agarose slices were mashed with a toothpick in 30 µL sterile distilled water and incubated at 50°C for 1 h. Samples were left at room temperature overnight to allow the DNA to elute from the gel. The samples were then stored at –20°C.

Sequencing

Sequencing reactions were done in a total volume of 10 µL. Each reaction contained 5 µL of the eluted PCR fragment, 1.6 pM of either t-LEU, t-LYS, or the internal primers C2-J-3400 and C2-N-3661 (Simon et al. 1994), and 4 µL Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Mix from Applied Biosystems Inc. (ABI), Sydney, Australia. Sequencing reactions were done in a Corbett Research Thermocycler using the following program: cycle 1 at 96°C for 3 min, then 30 cycles at 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min. The sequencing reactions were purified according to the manufacturer's instructions and loaded onto an ABI Model 373A Sequencer. Base calling was checked visually.

ITS-2

Amplification

The ITS region of the rDNA was amplified using primers Aed5.8F-5′-TGTGAACTGCAGGACACATGAAC-3′ and AedAB28–5′-TATGCTTAAATTTAGGGGGTAGTC-3′ (Kjer et al. 1994). These primers amplify the ITS-2 and regions of 5.8S and 28S rDNA. The PCR amplification followed the protocol outlined for COII above, but 32 cycles of amplification were employed, not 30.

Cloning

The PCR products were made blunt-ended by the addition of 1 U (unit) of T4 DNA polymerase to the PCR reaction for 30 min at 37°C, followed by heat denaturation of the enzyme for 10 min at 65°C. The PCR product was purified by extraction with an equal volume of phenol:chloroform (1:1), followed by ethanol precipitation and resuspension in 10 µL of distilled water.

A total of 5 µL of the resuspended PCR product was kinased in a 10-µL reaction volume consisting of 1× reaction buffer, 1 mM ATP, and PNK (10 U) incubated for 30 min at 37°C, followed by heat denaturation of the enzyme for 10 min at 75°C. Five microlitres of the kinased PCR product was then ligated into pUC18 in a 20-µL reaction volume consisting of 100 ng pUC18 (SmaI/BAP from Pharmacia Biotech Catalogue No. 27–4860–01), 1× reaction buffer, and 1 U T4 DNA Ligase (Boehringer, Mannheim). The ligation reaction was incubated at room temperature overnight. From 2.5 to 5.0 µL of the reaction volume was used to transform DH5 alpha cells.

Sequencing

Three clones from each individual were manually sequenced using the Pharmacia LKB Sequencer and Pharmacia T7 Sequence Kit in both directions according to the manufacturer's instructions. Autoradiographs were interpreted by eye.

Outgroup designation and sequence alignment

Aedes aegypti was selected as the outgroup for the phylogenetic analysis. It is in the same genus as, but a different subgenus from, *Ae. australis* and *Ae. ashworthi*. The COII sequence data were obtained from Ho et al.(1995) and the ITS-2 data from Wesson et al. (1992).

Sequences derived from the COII locus were aligned unambiguously without insertions or deletions using ClustalW (Higgins and Sharp 1988). ITS-2 sequences were not easily alignable. First, a consensus of the cloned ITS-2 sequences for each individual was constructed using the IUPAC (International Union of Pure and Applied Chemistry) code. Second, the consensus sequences were iteratively aligned using ClustalW (Higgins and Sharp 1988). In this latter procedure unambigious insertion or deletion events were constrained by inserting an "X" into each gap. ClustalW was then rerun six times with increasing constraints to obtain the best estimate of the alignment. The inserted Xs were then removed and each unequivocally defined insertion or deletion was scored as a single event. For phylogenetic analyses, sites where the alignment was ambiguous were excluded and, unless otherwise shown (see the Appendix), gaps were treated as "missing."

Phylogenetic analyses

All phylogenetic analyses were completed in PAUP* d61 (Swofford).[2] The permutation probability (PTP) test was employed to investigate whether the observed tree length could have been obtained by "chance alone" (Archie 1989; Faith and Cranston 1991). Ninety-nine randomizations were employed to determine significance at the 0.05 level. (We are aware that the utility of this method has been vigorously debated by Carpenter 1992 and Faith 1992.)

The homogeneity test of Farris et al. (1995) was employed to investigate whether partitions are evolving under distinct biological processes (Bull et al. 1993). This random partitioning test is an extension of a measure originally reported by Mickevich and Farris (1981) and is based on the null hypothesis of congruence. The a priori defined process partitions investigated were COII first, second, and third positions and the ITS 5.8S, ITS-2, and 28S regions.

Monophyly of a clade may be tested by a variety of techniques including bootstrapping (Efron 1982; Felsenstein 1985), jackknifing (Lanyon 1985), the decay or support index (Bremer 1988; Donoghue et al. 1992), and the topology dependent permutation (T-PTP) test (Faith 1991). We used bootstrapping and decay indices as measures of support for a particular clade. Hillis and Bull (1993) show that for their particular suite of data matrices a bootstrap value greater than or equal to 70% usually corresponds to a

[2] D.L Swofford. PAUP*: phylogenetic analysis using parsimony, version 4.0d54. Sinauer Associates, Sunderland, Mass. In preparation.

Table 1. Mean measurements (μm) of selected characters on the male genitalia of members of the *Ae. australis* group. $N = 20$.

Character	*ashw.*	*ward.*	*aust.*1	*aust.*2	*aust.*3
GXL	536 (7.7)*a*	547 (3.4)*a*	564 (4.7)*b*	565 (4.3)*b*	568 (3.4)*b*
GXW	91 (2.1)*a*	104 (1.8)*b*	134 (1.4)*d*	120 (2.2)*c*	120 (1.6)*c*
GSL	254 (4.4)*a*	286 (2.6)*b*	311 (3.1)*c*	312 (3.5)*c*	313 (3.1)*c*

Note: Values are given as the mean, with SEM in parentheses. Within a row, values followed by a different letter are significantly different at the 95% confidence interval. Species are designated as follows: *ashw*, *Ae. ashworthi* from Rottnest Island, WA; *ward*, *Ae. wardangensis* from Wardang Island, SA; *aust.1*, *Ae. australis* from Sydney, NSW; *aust.2*, *Ae. australis* from Marrawah, Tas.; *aust.3*, *Ae. australis* from Purakanui Bay, NZ. GXL, gonocoxite length; GXW, gonocoxite width; GSL, gonostylus length.

probability of at least 95% that the corresponding clade is real. Decay indices were obtained by calculating the difference in tree length between the shortest trees that contained a group versus those that lacked that same group.

Mapping characters

We mapped the morphological characters onto the tree generated by the molecular characters, using MacClade (Maddison and Maddison 1992). However, only a few morphometric characters of adults of the *Ae. australis* group were significantly different. Further, *Ae. aegypti* was not included in any mating experiments. As a result it was considered likely that the signal from the morphometric characters would be swamped by the molecular characters. To test this assumption, five hypothetical characters that exactly contradicted the molecular data were posthumously included in the data matrix (two more than were obtained in this study). Addition of these characters did not change the topology of the minimum-length tree.

Results

Morphological analyses

Adult male

Genitalia: There is significant heterogeneity in the morphometric measurements of male genitalia (Table 1). The gonocoxite lengths for *Ae. ashworthi* and *Ae. wardangensis* are similar. However, there is a significant difference between the gonocoxite widths and gonostylus lengths in these species. *Aedes australis* 1 (NSW), *Ae. australis* 2 (Tas.) and *Ae. australis* 3 (NZ) have similar gonocoxite and gonostylus lengths, but *Ae. australis* 1 (NSW) has a significantly wider gonocoxite.

Biological crosses

When the parents of populations of *Ae. australis* from Sydney, NSW, and Purakanui, NZ, were crossed, viable F_1 eggs, larvae, and adults were obtained from the cross. The F_1 and the F_2 adults were fertile, indicating genetic affinity (Table 2). The females were all autogenous, like the NSW parent females, and the F_1 developed a mean of 164 and the F_2 142 eggs per female ($N = 30$ for each generation). The F_3 eggs were viable, but the hybrid was not colonized beyond this stage.

When SA *Ae. wardangensis* females were crossed with NSW *Ae. australis* males, the F_1 adults all appeared to be fertile. Some of the F_1 females were mated, they were anautogenous like the SA parents, and all blood-fed females developed a full complement of eggs. The F_1 male gonads were normal in size and appearance, and were filled with motile spermatozoa. The reciprocal cross, NSW *Ae. australis* females × SA *Ae. wardangensis* males, yielded F_1 females that appeared normal. A few of these females were mated, they were anautogenous like the SA *Ae. wardangensis* parents, and all blood-fed females developed a full complement of eggs. Ninety-two percent of the males had normal-size testes with motile spermatozoa; 8% of the males had half-size testes that lacked normal germ cells or primary spermatogonia, and were sterile (Table 2).

Table 2. The results of biological crosses of different parents of *Ae. australis* from Australia and NZ, with *Ae. wardangensis* from SA and *Ae. ashworthi* from WA.

	Condition of gonads of F_1 adults	
Females × males	% female sterility	% male sterility
NZ *australis* × NSW *australis*[a]	0	0
SA *wardangensis* × NSW *australis*	0	0
NSW *australis* × SA *wardangensis*	0	8
NZ *australis* × WA *ashworthi*	73	100
WA *ashworthi* × NZ *australis*	100	100
WA *ashworthi* × SA *wardangensis*	100	100
SA *wardangensis* × WA *ashworthi*	90	100

Note: Rearing conditions were 16°C, 16 h light : 8 h dark. NZ, Purakanui Bay, New Zealand; NSW, Maroubra, Sydney, New South Wales; SA, Wardang Island, South Australia; WA, Rottnest Island, Western Australia.

[a] F_2 adults and F_3 eggs of this cross were fertile as well.

When NZ *australis* females were crossed with WA *ashworthi* males, 73% of the F_1 females had sterile ovaries and 100% of the males had half-size, sterile testes. The F_1 females took a full blood meal, but sterile females developed no eggs. The blood was defecated by postfeeding day 4, and only a few follicles (<10 per female) developed to Christophers (1911) stage III. The remainder of each ovary consisted of undifferentiated cells. Twenty-seven percent of the females developed a normal complement of eggs. The reciprocal cross, WA *ashworthi* females × NZ *australis* males, gave rise to F_1 progeny that consisted of 100% sterile females and 100% sterile males (Table 2).

When WA *ashworthi* females were crossed with SA *wardangensis* males, one of the crosses yielded 100% sterile F_1 females and 100% sterile F_1 males. The reciprocal cross yielded 90% sterile females; 10% of the females produced a normal complement of eggs after consuming a full blood

Table 3. The results of the parsimony analysis.

Region	Partition	Characters	Alignable characters	Parsimony-informative characters	Steps	No. of trees	CI	PTP
COII	First	229	229	10	28	4	0.93	0.01
	Second	228	228	1	5	1	1	
	Third	229	229	34	106	390	0.89	0.01
	All COII	686	686	45	140	35	0.89	0.01
ITS	5.8S	67	67	1	3	1	1	0.01
	ITS-2	243	142	16	44	132	1	
	28S	16	16	1	3	3	1	
	All ITS	326	225	18	51	198	0.96	0.01
COII + ITS	All	1012	911	63	191	14	0.92	0.01

Note: COII, cytochrome oxidase II locus; ITS, internal transcribed spacer locus; PTP, permutation probability test.

meal. The F_1 males of this cross had half-size testes and 100% were sterile (Table 2).

Phylogenetic analyses

The COII sequences (25) have been deposited in EMBL/GenBank (Accession Nos. AF042682–AF042706). At the COII locus there were 10, 1, and 34 parsimony informative characters at the first, second, and third codon positions, respectively (Table 3). Owing to the small number of parsimony-informative second positions, the first and second positions were pooled for PTP analysis. The combined first and second and the third codon positions have phylogenetic structure ($P < 0.01$ for each; Table 3). The cloned ITS-2 sequences (33) have been deposited in EMBL/GenBank (Accession Nos. AF44507–AF44539). The consensus sequence for each individual and the alignment are presented in the Appendix. For the ITS region, phylogenetically informative positions were pooled for PTP analysis. Parsimony analysis showed that the ITS region had PTP structure ($P < 0.01$; Table 3).

The homogeneity test did not reject the null hypothesis that the three partitions (COII first and second positions, COII third position, and the ITS region) are evolving homogeneously (191 steps, $P = 1.0$). As a result, all the data were pooled. The pooled sample had PTP structure ($P < 0.01$; Table 3).

Parsimony analysis of the 911 base data set generated 14 equally parsimonious trees 191 steps in length (Table 3). A χ^2 test of homogeneity of base frequencies across taxa showed that there is no significant heterogeneity between taxa ($\chi^2 = 3.87$, df = 81, $P = 1.0$), though there are more nucleotide As and Ts (0.343 ± 0.003 and 0.369 ± 0.002, respectively) than Cs and Gs (0.159 ± 0.004 and 0.129 ± 0.003, respectively).

To investigate the robustness of nodes to perturbation, a bootstrap analysis of the 63 parsimony-informative characters was conducted. Three distinct clades are supported by bootstrap analysis (Fig. 3). The basal clade, consisting of *Ae. ashworthi* specimens collected in Western Australia, is supported by a 100% bootstrap and a decay index of 21 steps. The basal position of *Ae. ashworthi* is in conflict with the suggestion of Brust and Mahon (1997) that this species is the most derived member of the *Ae. australis* complex. Brust and Mahon (1997) may be wrong, and *Ae. australis* may be the most derived member. Additional DNA sequence data from individuals in closely related subgenera and from individuals in other distinct populations of *Ae. australis* are required to test this apparent conflict. Within *Ae. ashworthi*, two of the eight individuals form a monophyletic assemblage with a bootstrap of 70% and a decay index of 1 step.

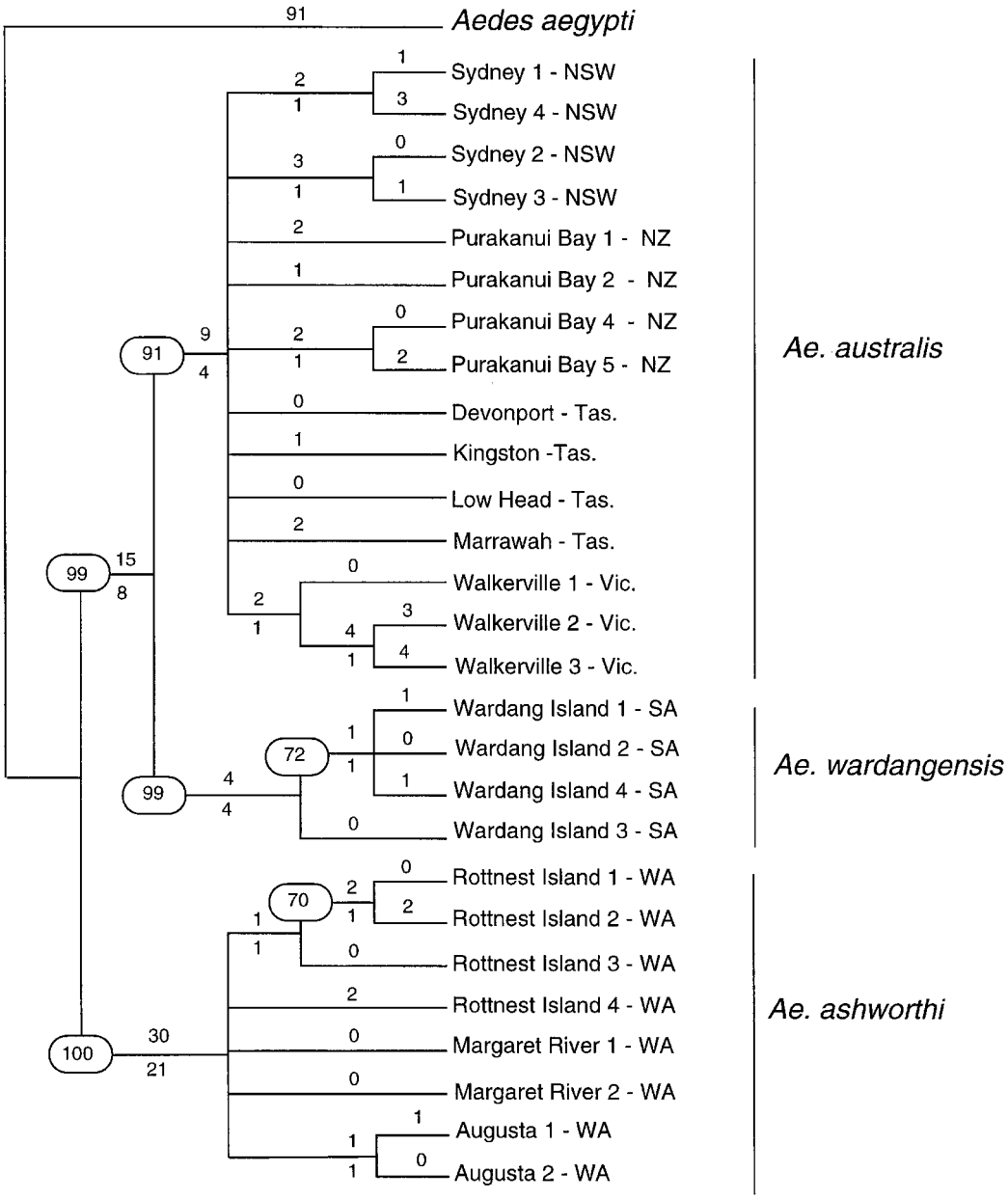
The specimens of the *Ae. australis* species-group from SA, NSW, Tas., and NZ form a monophyletic assemblage with a bootstrap of 99% and a decay index of 8 steps. Within this monophyletic assemblage there are two distinct clades. One clade contains specimens collected from Wardang Island and the other contains specimens collected from Tas., NZ, and NSW. The Wardang Island clade is supported by a 99% bootstrap and a decay index of 4 steps. Three of the four specimens from Wardang Island form a monophyletic assemblage with a bootstrap of 72% and a decay index of 1 step. The clade containing specimens from NSW, NZ, and Tas. is supported by a bootstrap of 91% and has a decay index of 4 steps. There is no evidence of population subdivision in *Ae. australis* at the >70% bootstrap level. However, inclusion of more data would be required to test this hypothesis.

To further investigate the phylogenetic affinities of the populations of the *Ae. australis* species-group from SA, NSW, Tas., and NZ, we excluded *Ae. aegypti* from the analysis and employed *Ae. ashworthi* as the outgroup. This permitted the inclusion of 972 sites (60 more than in the previous analysis). For this analysis a total of 2279 equally parsimonious trees 120 steps in length was found. Bootstrapping these sequence data does not alter the observation that there is strong support for monophyly of the specimens collected from Wardang Island, SA (98% bootstrap), and those collected from NSW, NZ, and Tas. (82% bootstrap). These data also suggest that there is little structure within each of these populations.

Mapping characters

The morphological characters were coded by the significance of means. Thus, gonocoxite length was scored as 0 for *ashworthi*, 0 for *wardangensis*, and 1 for *australis* 1, 2, and 3 (Table 1). Gonostylus length maps exactly to the topology generated by the COII and ITS data and supports the tenet

Fig. 3. Strict consensus maximum parsimony analysis of the 84 equally parsimonious trees 199 steps in length. Numbers in circles represent bootstrap pseudosamples (above 70%) from 500 iterations. The number above each line denotes the branch length, while the number below the line denotes the decay index. See Fig. 1 for site locations.



that *Ae. wardangensis* is biologically distinct. Gonocoxite width is more variable and suggests that the *Ae. australis* (NSW) population is distinct from the Tas. and NZ populations. However, the sequence data do not suggest that there is any population subdivision among the three populations of *Ae. australis*. Gonocoxite length suggests that *Ae. wardangensis* is more closely related to *Ae. ashworthi* than to any of the *Ae. australis* populations (Table 1).

Aedes (Halaedes) wardangensis sp.nov.

Female imago

Head: occiput with broad white scales laterally, and narrow white scales on vertex; scales interspersed with numerous erect, medium to light brown bristles, truncated at the apex; slender, pointed, decumbent, medium-brown bristles anteriorly and laterally. *Thorax*: integument medium brown with narrow scales covering the entire scutum; individual scale colour varies from all golden scales to approximately half golden and half medium-brown scales, fully interspersed. The upper postpronotal, postspiracular, upper and lower meskatepisternal, upper and lower prealar, upper and lower mesepimeral, and paratergal scales mostly white; the postspiracular and paratergal regions have black scales on their upper portions and white scales on their lower portions. *Abdomen*: tergite I without scales or with only a few scattered scales; tergites II–IV with white scales on the basal half and black scales, with a few interspersed white scales,

on the apical half; tergites V–VII with white scales throughout, but with variable numbers of black scales on the apical one-third of each tergite.

Adult male

Head: Palpi 0.8–0.9 proboscis; segment 5 swollen, and 0.9 as long as segment 4; segment 4, 0.5 as long as segments 2 and 3 combined. *Thorax*: as in female. *Abdomen*: tergites as in female. *Genitalia*: gonocoxite 5 times as long as wide; gonocoxite with lanceolate, decumbent, and flattened bristles/scales on the inner edge. The claspette is attached ventrally to the gonocoxite and consists of a lobe with lanceolate bristles/scales (Fig. 2).

Fourth-instar larva

As described in Edwards (1921), and does not differ from *Ae. australis* as described and illustrated in Belkin (1962, Fig. 211). We analyzed 38 larval characters (for a list of the characters and illustrations see Ellis and Brust 1973) of *ashworthi*, *australis*, and *wardangensis* (N = 20 for each species) and found that there were no statistical differences amongst the means for any of the characters (95% confidence interval).

Diagnosis

When populations are reared under identical conditions, males may be separated statistically from *Ae. australis* and *Ae. ashworthi* by the shape and size of the gonocoxite and the gonostylus of the genitalia (Fig. 2, Table 1). These were the only male characters that were found to vary amongst the three species. Females are not separable morphologically from *Ae. ashworthi*. They have a medium-brown integument with mostly golden scales on the scutum, and may be separated from *Ae. australis*, which has a dark brown integument and more brown than golden scales on the scutum. Like those of *Ae. ashworthi*, females are anautogenous, while those of *Ae. australis* are generally autogenous in the first ovarian cycle (Brust 1997).

Holotype

Male

TYPE LOCALITY: Northwest coast of Wardang Island, 137.21°E, 34.30°S, South Australia. Nine paratype males and the holotype are deposited in the Australian National Insect Collection. The associated genitalia of 8 of the paratypes are mounted on slides.

Discussion

Brust and Mahon (1997) resurrected *Ae. ashworthi* as a separate species, based on the results of biological crosses and morphology of populations from Western Australia and New South Wales. The phylogenetic analysis of our data supports this finding and clarifies the status of populations of the *Ae. australis* group from South Australia, New South Wales, Victoria, Tasmania, and New Zealand. It is clear that *Ae. ashworthi* differs genetically from *Ae. australis* collected in south eastern Australia and New Zealand. It also differs biologically, morphologically, and genetically from *Ae. wardangensis* sp.nov. (formerly referred to as SA *Ae. australis* (Brust 1997)) from South Australia.

Prior to the molecular analyses, and additional morphometric analyses of the male genitalia of the *Ae. australis* group, Brust (1997) elected to leave the SA population in the parent group. The F_1 hybrid had 8% sterile males in one cross; however, the reciprocal cross was fertile. Without additional results Brust (1997) felt he had insufficient data to erect a new taxon. Subsequently, the morphometric analyses of the male genitalia and the phylogenetic analyses of DNA sequence data showed that the *Ae. wardangensis* is distinct from both *Ae. ashworthi* and *Ae. australis* (Table 1, Fig. 3). On the basis of both the morphological evidence and the molecular sequence data, it is clear that *Ae. wardangensis* is a new taxon. The addition of DNA sequencing and phylogenetic analyses in the identification of *Ae. wardangensis* as a separate species is another example of the impact of molecular biology and cladistics in mosquito systematics, a subject that was recently reviewed by Munstermann and Conn (1997).

According to the phylogenetic analyses, *Ae. australis* collections from New Zealand, New South Wales, Victoria, and Tasmania, despite their geographical separation, represent a monophyletic series of populations. We hypothesize that there has been a "recent" transfer or, alternatively, multiple transfers of *Ae. australis*, presumably to New Zealand. The possibility of a transfer by ship was raised by Marks (personal communication in Nye 1962) and Pillai and Ramalingan (1984). Brust (1997) found affinities between the NZ population and a population from Kingston, Tas. However, further studies are needed to test this hypothesis, as well as a second hypothesis (Belkin 1968) that the NZ population dispersed from other islands in the Pacific Ocean.

Brust (1997) suggested that the Marrawah, Tas., collection represents a population separate from the Devonport and Lowhead, Tas., population. The DNA sequences (EMBL/GenBank) and the phylogenetic analysis data (Fig. 3) do not support this suggestion. However, as only single representatives of each population were sampled, it is not possible to unequivocally refute this suggestion with these data.

The *Ae. australis* complex is undoubtedly distributed more widely in the Australasian Region than is currently recorded in the literature. More collections need to be examined to clarify the biosystematic and genetic status of *Ae. australis* in this region. Biological crosses of parents of series of populations are reasonably simple to conduct in the laboratory, and DNA sequences and phylogenetic analyses could be used to genetically characterize these populations. This could be important in understanding the epidemiological significance of this species in the future. Russell (1993) pointed out that blood-feeding *Ae. australis* in eastern Tasmania are occasionally pests of humans. Austin and Maguire (1982) reported that females of *Ae. australis* from New Zealand were able to transmit Ross River Virus, and Kay and Aaskov (1989) reported that females of *Ae. australis* from Tasmania could be experimentally infected with the virus. The biosystematic and genetic characterization of other populations of *Ae. australis* would be helpful, should this species be implicated elsewhere as a potential vector of human or animal viruses.

Acknowledgments

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References

Archie, J.W. 1989. A randomization test for phylogenetic information in systematic data. Syst. Zool. **38**: 219–252.

Austin, F.J., and Maguire, T. 1982. Ross River virus in the southwest Pacific. *In* Viral diseases in south-east Asia and the western Pacific. *Edited by* J.S. Mackenzie. Academic Press, Sydney, NSW, Australia. p. 528.

Belkin, J.N. 1962. The mosquitoes of the South Pacific (Diptera: Culicidae). University of California Press, Berkeley. pp. 328–331 (Vol. 1) and plates 210 and 211 (Vol. 2).

Belkin, J.N. 1968. Mosquito studies (Diptera: Culicidae) VII. The Culicidae of New Zealand. Contrib. Am. Entomol. Inst. No. 3.

Bremer, K. 1988. The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. Evolution, **42**: 795–803.

Brust, R.A. 1997. Ecology of the *Aedes australis* (Erichson) 1842 complex (Diptera: Culicidae) from Australia and New Zealand. Aust. J. Entomol. **36**: 287–292.

Brust, R.A., and Mahon, R.J. 1997. The resurrection of *Aedes* (*Halaedes*) *ashworthi* Edwards 1921: morphological characterization and separation from its sibling *Aedes* (*Halaedes*) *australis* (Erichson) 1842 (Diptera: Culicidae). Aust. J. Entomol. **36**: 129–136.

Bull, J.J., Huelsenbeck, J.P., Cunningham, C.W., Swofford, D.L., and Waddell, P.J. 1993. Partitioning and combining data in phylogenetic analysis. Syst. Biol. **42**: 384–397.

Carpenter, J.M., 1992. Random cladistics. Cladistics, **8**: 147–153.

Christophers, S.R. 1911. The development of the egg follicle in anophelines. Paludism, **2**: 73–88.

Dobrotworsky, N.V. 1966. Mosquitoes of Tasmania and Bass Strait Islands. Proc. Linn. Soc. N.S.W. **91**: 121–146.

Donoghue, M.J., Olmstead, R.G., Smith, J.F., and Palmer, J.D. 1992. Phylogenetic relationships of dipscales based on rbcL sequences. Ann. Mo. Bot. Gard. **79**: 249–265.

Edwards, F.W. 1921. Mosquito notes. II. Bull. Entomol. Res. **12**: 69–80.

Efron, B. 1982. The jackknife, the bootstrap, and other resampling plans. Conf. Board Math. Sci. Soc. Ind. Appl. Math. **38**: 1–92.

Ellis, R.A., and Brust, R.A. 1973. Sibling species delimitation in the *Aedes communis* (Degeer) aggregate (Diptera: Culicidae). Can. J. Zool. **51**: 915–959.

Faith, D.P. 1991. Cladistic permutation tests for monophyly and nonmonophyly. Syst. Zool. **40**: 366–375.

Faith, D.P. 1992. On corroboration: a reply to Carpenter. Cladistics, **8**: 265–273.

Faith, D.P., and Cranston, P. 1991. Could a cladogram this short have arisen by chance alone?: On permutation tests for cladistic structure. Cladistics, **7**: 1–28.

Farris, J. S., Källersjö, M., Kluge, A., and Bult, C. 1995. Testing significance of incongruence. Cladistics, **10**: 315–319.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution, **39**: 783–791.

Harbach, R.E., and Knight, K.L. 1980. Taxonomists' glossary of mosquito anatomy. Plexus Publishing, Inc., Marlton, N.J.

Higgins, D.G., and Sharp, R.M. 1988. CLUSTAL, a package for performing multiple sequence alignment on a microcomputer. Gene, **73**: 237–244.

Hillis, D.M., and Bull, J.J. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst. Biol. **42**: 182–192.

Ho, C.M., Liu, Y.M., Wei, Y.H., and Hu, S.T. 1995. Gene for cytochrome *c* oxidase subunit II in the mitochondrial DNA of *Culex quinquefasciatus* and *Aedes aegypti* (Diptera: Culicidae). J. Med. Entomol. **32**: 174–180.

Kay, B.H., and Aaskov, J.G. 1989. Ross River virus (epidemic polyarthritis). *In* The arboviruses: epidemiology and ecology. *Edited by* T.P. Monath. CRC Press, Baton Rouge, La. pp. 93–112.

Kjer, K.M., Baldridge, G.D., and Fallon, A.M. 1994. Mosquito large sub-unit ribosomal RNA: simultaneous alignment of primary and secondary structure. Biochem. Biophys. Acta, **1217**: 147–155.

Lanyon, S.M. 1985. Detecting internal inconsistencies in distance data. Syst. Zool. **34**: 397–403.

Lee, D.J., Hicks, M.M., Griffiths, M., Russell, R.C., and Marks, E.N. 1984. The Culicidae of the Australasian Region. Entomology Monogr. No. 2, Vol. 3. Australian Government Publishing Service, Canberra. pp. 19–29.

Maddison, W.P., and Maddison, D.R. 1992. MacClade Version 3. Sinauer Associates, Inc., Sunderland, Mass.

Mickevich, M.F., and Farris, J.S. 1981. The implications of congruence in *Menidia*. Syst. Zool. **30**: 351–370.

Munstermann, L.E., and Conn, J.E. 1997. Systematics of mosquito disease vectors (Diptera, Culicidae): impact of molecular biology and cladistic analysis. Annu. Rev. Entomol. **42**: 351–369.

Nye, E.R. 1962. *Aedes* (*Pseudoskusea*) *australis* Erichson (Diptera: Culicidae) in New Zealand. Trans. R. Soc. N.Z. Zool. **3**: 33–34.

Pillai, J.S., and Ramalingan, S. 1984. Recent introductions of some medically important Diptera in the Northwest, Central and South Pacific (including New Zealand). *In* Commerce and the spread of pests and disease vectors. *Edited by* M. Laird. Praeger Special Studies, Praeger Scientific, New York. pp. 81–101.

Russell, R.C. 1993. Mosquitoes and mosquito-borne disease in southeastern Australia. A guide to the biology, relation to disease, surveillance, control and the identification of mosquitoes in southeastern Australia. Department of Medical Entomology, Westmead Hospital, Westmead, N.S.W. 2145, and Department of Medicine, University of Sydney, Sydney, N.S.W.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., and Flook, P. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. **87**: 651–701.

Walsh, S.P., Metzger, D.A., and Higuchi, R. 1991. Chelex 100© as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques, **10**: 506–513.

Wesson, D.M., Porter, C.H., and Collins, F.H. 1992. Sequence and secondary structure comparisons of ITS rDNA in mosquitoes (Diptera: Culicidae). Mol. Phylogenet. Evol. **1**: 253–269.

Appendix

The alignment of the internal transcribed spacer locus. Two or three clones were sequenced for each individual and a consensus was constructed using the IUPAC (International Union of Pure and Applied Chemistry) code. Insertion/deletion events were scored as an additional character by the placement of a “1” in the matrix. The regions given in lower case letters were excluded from the analysis because the alignment was ambiguous. The first 67 bases constitute the 5.8S region, the second 243 bases the ITS-2 region, and the last 16 the 28S region.

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Aedes aegypti        CCGACACGTTGAACGCATATTGCACATCGTACTACCAGTACGATGTACACATTTTTGAGTGCCTATATTTAT
Sydney 1-NSW         CCGACAAGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Sydney 2-NSW         CCGACAAGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Sydney 3-NSW         CCGACAAGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Sydney 4-NSW         CCGACAAGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Purakanui Bay 1-NZ   CCGACAAGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Purakanui Bay 2-NZ   CCGACAAGTTGAACGCATATTGCACATCGTACAAC-AGTACGAYGTACACATTTTTGAGTGCCTATCTTTAT
Purakanui Bay 4-NZ   CCGACAAGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Purakanui Bay 5-NZ   CCGACAAGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Devonport Tas.       CCGACAAGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Kingston Tas.        CCGACAAGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Low Head Tas.        CCGACAAGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Marrawah Tas.        CCGACAAGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Walkerville 1-Vic.   CCGACAAGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Walkerville 2-Vic.   CCGACAAGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Walkerville 3-Vic.   CCGACAAGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTWGAGTGCCTATYTTTAT
Wardang Island 1-SA  CCGACACGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGYGCCTATCTTTAT
Wardang Island 2-SA  CCGACACGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Wardang Island 3-SA  CCGACACGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Wardang Island 4-SA  CCGACACGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Rottnest Island 1-WA CCGACACGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Rottnest Island 1-WA CCGACACGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Rottnest Island 1-WA CCGACACGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Rottnest Island 1-WA CCGACACGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Margaret River 1-WA  CCGACACGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Margaret River 2-WA  CCGACACGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Augusta 1-WA         CCGACAAGTTGAACGCATATTGCACATCGYACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT
Augusta 2-WA         CCGACAAGTTGAACGCATATTGCACATCGTACAAC-AGTACGATGTACACATTTTTGAGTGCCTATCTTTAT

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Aedes aegypti        CCATTCAACTATAc--------gcgcc-----------------gcccgcgcgTATGCGTAGTGATGTTTTC
Sydney 1-NSW         CCATTCAACTGTAgctctgga-gagcccggccatacccggcttggctctcggcTACGCGTAGTGGCGTTTTC
Sydney 2-NSW         CCATTCAACTGTAgctctgga-gagcccggccatacccggcttggctctckgcTACGCGTAGTGGCGTTTTC
Sydney 3-NSW         CCATTCAACTGTAgctctgga-gagcccggccatacccggcttggctctctgcTACGCGTAGTGGCGTTTTC
Sydney 4-NSW         CCATTCAACTGTAgctctgga-gagcccgrccatacccggcttggctctcggcTACGCGTAGTGGCGTTTTC
Purakanui Bay 1-NZ   CCATTCAACTGTAgctctgga-gctcc-ggccatacccggcttggctctctgcTACGCGTAGTGGCGTTTTC
Purakanui Bay 2-NZ   CCATTCAACTGTAgctctgga-gctcc-ggccatacccggcttggctctctgcTACGCGTAGTGGCGTTTTC
Purakanui Bay 4-NZ   CCATTCAACTGTAgctctgga-gctcc-ggycatacccggcttggctctctgcTACGCGTAGTGGCGTTTTC
Purakanui Bay 5-NZ   CCATTCAACTGTAgctctgga-gctcc-ggccatacccggcttgrctctctgcTACGCGTAGTGGSGTTTTC
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Devonport Tas.        CCATTCAACTGTAgctctgga-gctcc-ggccatacccggcttggctctctgcTACGCGTAGTGGCGTTTTC
Kingston Tas.         CCATTCAACTGTAgctctgga-gctcc-ggccatacccggcttggctctctgcTACGCGTAGTGGCGTTTTC
Low Head Tas.         CCATTCAACTGTAgctctggaagctcc-ggccatacccggcttggctctctgcTACGCGTAGTGGCGTTTTC
Marrawah Tas.         CCATTCAACTGTAgctctggaggctcc-ggccatacccggcttggctctctgcTACGCGTAGTGGCGTTTTC
Walkerville 1-Vic.    CCATTCAACTGTAgctctssa-gctcc----------------ggctctctgcTACGCGTARTGGCGTTTTC
Walkerville 2-Vic.    CCATTCAACTGTAgctctgga-gctcc-ggccatacccggttgagctctctgcTACCCGTTATGGGGTTTTC
Walkerville 3-Vic.    CCATTCAACTGTAgctctgga-gctcc-ggccatacccggyttggctstctgcTACGCGTACTGGCGTTTTC
Wardang Island 1-SA   CCATTCAACTGTAgctctrgr-gctcc-ggccatacccggcttggctctstgcTACGCGTAGTGGCGTTTTC
Wardang Island 2-SA   CCATTCAACTGTAgctctgga-gctcc-ggccatacccggcttggctctctgcTACGCGTAGTGGCGTTTTC
Wardang Island 3-SA   CCATTCAACTGTAgctctgga-gctcc----------------ggctctctgcTACGCGTAGTGGCGTTTTC
Wardang Island 4-SA   CCATTCAACTGTAgctctgga-gctcc-ggccatacccggcttggctctstgsTACGCGTAGTGGCGTTTTC
Rottnest Island 1-WA  CCATTCAACTGTAgct--------gcc----------------------ctgcTACGCGTAGTGGCGTTTTC
Rottnest Island 1-WA  CCATTCAACTGTAgct--------gcc----------------------ctgcTACGCGTAGTGGCGTTTTC
Rottnest Island 1-WA  CCATTCAACTGTAgct--------gcc----------------------ctgcTACGCGTAGTGGCGTTTTC
Rottnest Island 1-WA  CCATTCAACTGTAgct--------gcc----------------------ctgcTACGCGTAGTGGCGTTTTC
Margaret River 1-WA   CCATTCAACTGTAgct--------gcc----------------------ctgcTACGCGTAGTGGCGTTTTC
Margaret River 2-WA   CCATTCAACTGTAgct--------gcc----------------------ctgcTACGCGTAGTSGSGTTTTC
Augusta 1-WA          CCATTCAACTGTAgct--------gcc----------------------ctgcTACGCGTAGTGGCGTTTTC
Augusta 2-WA          CCATTMAACTGTAgct--------gcc----------------------ctgcTACGCGTAGTGGCGTTTTC

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Aedes aegypti         CCGCCTTCAGTGCGCGGTAAAACATTGAAGATAGTCAGACGTGGTGTGG------TGA1C-ACACCGCGGTT
Sydney 1-NSW          CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACGTG-TGC1--------GA1CTAGCGCGCGGCT
Sydney 2-NSW          CTGCCTTCAGTG-GTGGTAAAACGTTGARGATAGTCAGACGTG-TGC1--------GA1CTAGCGCGCGGCT
Sydney 3-NSW          CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACGTG-TGC1--------GA1CTAGCGCGCGGCT
Sydney 4-NSW          CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACGTG-TGC1--------GA1CTAGCGCGCGGCT
Purakanui Bay 1-NZ    CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACGTG-TGC1--------GA1CTAGCGCGCGGCT
Purakanui Bay 2-NZ    CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACGTG-TGC1--------GA1CTAGCGCGCGGCT
Purakanui Bay 4-NZ    CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACGTG-TGC1--------GA1CTAGCGYGCGGCT
Purakanui Bay 5-NZ    CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACGTG-TGC1--------GA1CTAGCGCGCGGCT
Devonport Tas.        CTGCCTTCAGTG-GTGGTAARACGTTGAAGATAGTCAGACGTG-TGC1--------GA1CTAGCGCGCGGCT
Kingston Tas.         CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACGTG-TGC1--------GA1CTAGCGCGCGGCT
Low Head Tas.         CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACGTG-TGC1--------GA1CTAGCGCGCGGCT
Marrawah Tas.         CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACGTG-TGC1--------GA1CTAGCGCGCGGCT
Walkerville 1-Vic.    CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACGTG-TGM1--------GA1CTAGCGCGCGGCT
Walkerville 2-Vic.    CTGCCTTCAGTG-GTGGTAAAACTTTGAAGATAGTCAGACGTG-TGC1--------GA1CTAGCGCGCGGCT
Walkerville 3-Vic.    CTGCCTTCAGTG-GTGGTAAAACKTTGAAGATAGTCRGACGTG-TGC1--------GA1CTAGCGCGCGGCT
Wardang Island 1-SA   CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACGTG-TGC1--------GAACAAGCGCGCGGCT
Wardang Island 2-SA   CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACGTG-TGC1--------GAACWAGCGCGCGGCT
Wardang Island 3-SA   CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACGTG-TGC1--------GAACWAGCGCGCGGCT
Wardang Island 4-SA   CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACGTG-TGC1--------GAACAAGCGCGCGGCT
Rottnest Island 1-WA  CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACTGA-TGCCCCCCCCCTGA1CGGGTATCAGGCT
Rottnest Island 1-WA  CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACTGA-TGCCCCCCCCCTGA1CGGGTATCAGGCT
Rottnest Island 1-WA  CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACTGA-TGYCCCCCCCCTGA1CGGGTATCAGGCT
Rottnest Island 1-WA  CTGCCTTCAGTG-GTGGTAGAACGTTGAAGATAGTCAGACTGA-TGCCCCCCC--TGA1CGGGTATCAGGCT
Margaret River 1-WA   CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACTGA-TGCCCCCCCCCTGA1CGGGTATCAGGCT
Margaret River 2-WA   CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACTGA-TGCCCCCCC--TGA1CGGGTATCAGGCT
Augusta 1-WA          CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACTGA-TGCCCCCCC----A1CGGGTATCAGGCT
Augusta 2-WA          CTGCCTTCAGTG-GTGGTAAAACGTTGAAGATAGTCAGACTGA-TGCCCCCCCCCTGA1CGGGTATCAGGCT

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                      11122222222223333333333444444444455555555556666666666777777777888888888
                      78901234567890123456789012345678901234567890123456789012345678901234567

Aedes aegypti         GATGAATACATCCCACTATggcgcgctcg---ctcgccttgt---------GTTGTATTCCATCAttca---
Sydney 1-NSW          GATGAATACATCCCA1-----agc-agag---ctcgcctcyc---------1ATGTATTCCATCGgcaatcc
Sydney 2-NSW          GATGAATACATCCCA1-----agc-agag---ctcgtctctc---------1ATGTATTCCATCGgcaatcc
Sydney 3-NSW          GATGAATACATCCCA1-----agc-agag---ctcgtctctc---------1ATGTATTCCATCGgcaatcc
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Sydney 4-NSW          GATGAATACATCCCA1-----agc-agag---ctcgtctctc---------1ATGTATTCCATCGgcaatcc
Purakanui Bay 1-NZ    GATGAATACATCCCA1-----agc-agag---ctcgtctctc---------1ATGTATTCCATCGgcaa---
Purakanui Bay 2-NZ    GATGAATACATCCCA1-----agc-agag---ctcgtctctc---------1ATGTATTCCATCGgcaa---
Purakanui Bay 4-NZ    GATGAATACATCCCA1-----agc-agag---ctcgtctctc---------1ATGTATTCCAYCGgcaa---
Purakanui Bay 5-NZ    GATGAATACATCCCA1-----agc-agag---ctcgtctctc---------1ATGTATTCCATCGgcaa---
Devonport Tas.        GATGAATACATCCCA1-----agc-agag---ckcgtctctc---------1ATGTATTCCATCGgcaaa--
Kingston Tas.         GATGAATACATCCCA1-----agc-agag---ctcgtctctc---------1ATGTATTCCATCGgcaaa--
Low Head Tas.         GATGAATACATCCCA1-----agccagag---ctcgtctctc---------1ATGTATTCCATCGgcaatcc
Marrawah Tas.         GATGAATACATCCCA1-----agc-agag---ctcgtctctc---------1ATGTATTCCATCGgcaay--
Walkerville 1-Vic.    GATGAATACATCCCA1-----agc-agag---ctcgtctctc---------1ATGTATTCCATCGgcaatcc
Walkerville 2-Vic.    GATGAATACCTCCCA1-----agc-aaag---ctcgtctctc---------1ATGTATTCCACCGgcaatcc
Walkerville 3-Vic.    GATGAATACMTCCCA1-----agc-agag---ctcgtctctc---------1ATGTATTCCAYCGgcaatcc
Wardang Island 1-SA   GATGAATACATCCCA1-----agc-agag---ckcgtctctc---------1ATGTATTCCATCGgcaatcc
Wardang Island 2-SA   GATGAATACATCCCA1-----agc-agag---ckcgtctctc---------1ATGTATTCCATCGgcaatcc
Wardang Island 3-SA   GATGAATACATCCCA1-----agc-agag---ckcgtctctc---------1ATGTATTCCATCGgcaa---
Wardang Island 4-SA   GATGAGTACATCCCA1-----agc-agag---ckcgtctctc---------1ATGTATTCCATCGgcaatcc
Rottnest Island 1-WA  GATGAATACATCCCACTAGtgggttggggtgatccatctccccctaatccaAGTGTATTCCATCAgcaaag-
Rottnest Island 1-WA  GATGAATACATCCCACTAGtgggttggggtgatccayctccccctaatccaAGTGTATTCCATCAgcaaag-
Rottnest Island 1-WA  GATGRATACATCCCACTAGtgggttggggtgatccayctccccctaatccaAGTGTATTCCATCAgcaaag-
Rottnest Island 1-WA  GATGAATACATCCCACTAGtgggttggggtgatccatctccccctaatccaAGTGTATTCCATCAgcaaag-
Margaret River 1-WA   GATGAATACATCCCACTAGtgggttggggtgatccatctccccctaatccaAGTGTATTCCATCAgcaaag-
Margaret River 2-WA   GATGAATACATCCCACTAGtgggttggggtgatccatctccccctaatccaAGTGTATTCCATCAgcaaag-
Augusta 1-WA          GRTGAATACATCCCACTAGtgggttgggg-gatc-ayytyccccctaatccaAGTGTATTCCATCAgcaaag-
Augusta 2-WA          GATGAATACATCCCACTAGtgggttggggtgatmcayctyccccctaatccaAGTGTATTCCATCAgcaaag-

                      22222222222333333333333333333333333333
                      89999999999000000000011111111112222222
                      90123456789012345678901234567890123456

Aedes aegypti         -----ctaactaactccctataGTAGGCCTCAAATAAT
Sydney 1-NSW          tacctcgaacgcaaata--ca-GTAGGCCTCAAATAAT
Sydney 2-NSW          tacctcgaacgcatata--ca-GTAGGCCTCAAATAAT
Sydney 3-NSW          twcctcgaacgcaaata--ca-GTAGGCCTCAAATAAT
Sydney 4-NSW          tacctcgaacgcatata--ca-GTAGGCCTCAAATAAT
Purakanui Bay 1-NZ    ----tcgaacgcata----ca-GTAGGCCTCAAATAAT
Purakanui Bay 2-NZ    ----tcgaacgcatata--ca-GTAGGCCTCAAATAAT
Purakanui Bay 4-NZ    ----tcgaacgcatata--ca-GTAGGCCTCAAATAAT
Purakanui Bay 5-NZ    ----tcgwacggatata--ca-GTAGGCCTCAAATAAT
Devonport Tas.        ----tcgaacgcatata--ca-GTAG-CCTCAAATAAT
Kingston Tas.         --cctcgaacgcata----ca-GTAGGCCTCAAATAAT
Low Head Tas.         tacctcgaacgcatata--ca-GTAGGCCTCAAATAAT
Marrawah Tas.         --cctcgaacgcata----ca-GTAGGCCTCAAATAAT
Walkerville 1-Vic.    tacctcgaacgcatata--ca-GTAGGCCTCAAATAAT
Walkerville 2-Vic.    tacctcaaacgcatata--ca-GTAGGCCTCAAATAAT
Walkerville 3-Vic.    tacctcraacgcatata--ca-GKTGGGCTCAAATAAT
Wardang Island 1-SA   tacctcgaacgcatatataca-GTAGGCCTCAAATAAT
Wardang Island 2-SA   tacctcgaacgcatatataca-GTAGGCCTCAAATAAT
Wardang Island 3-SA   ----tcgaacgcatatataca-GTAGGCCTCAAATAAT
Wardang Island 4-SA   tacctcgaacgcatatataca-GTAGGCCTCAAATAAT
Rottnest Island 1-WA  --cctcgaacgcata----caaGTAGGCCYCAAATAAT
Rottnest Island 1-WA  --cctcgaacgcata----caaGTAGGCCCCAAATAAT
Rottnest Island 1-WA  --cctcgaacgcata----caaGTAGGCCCCAAATAAT
Rottnest Island 1-WA  --cctcgaacgcata----caaGTAGGCCTCAAATAAT
Margaret River 1-WA   --cctcgaacgcata----caaGTAGGCCTCAAATAAT
Margaret River 2-WA   --cctcgaacgcata----caaGTAGGCCTCAAATAAT
Augusta 1-WA          --cctcgaacgcata----caaGTAGGCCTCAAATAAT
Augusta 2-WA          --cctcgaacgcata----caaGTAGGCCTCAAATAAT
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