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Detection of Low-Level *Cardinium* and *Wolbachia* Infections in *Culicoides*

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Bacterial endosymbionts have been identified as potentially useful biological control agents for a range of invertebrate vectors of disease. Previous studies of *Culicoides* (Diptera: Ceratopogonidae) species using conventional PCR assays have provided evidence of *Wolbachia* (1/33) and *Cardinium* (8/33) infections. Here, we screened 20 species of *Culicoides* for *Wolbachia* and *Cardinium*, utilizing a combination of conventional PCR and more sensitive quantitative PCR (qPCR) assays. Low levels of *Cardinium* DNA were detected in females of all but one of the *Culicoides* species screened, and low levels of *Wolbachia* were detected in females of 9 of the 20 *Culicoides* species. Sequence analysis based on partial 16S rRNA gene and *gyrB* sequences identified "*Candidatus* Cardinium hertigii" from group C, which has previously been identified in *Culicoides* from Japan, Israel, and the United Kingdom. *Wolbachia* strains detected in this study showed 98 to 99% sequence identity to *Wolbachia* previously detected from *Culicoides* based on the 16S rRNA gene, whereas a strain with a novel *wsp* sequence was identified in *Culicoides narrabeenensis. Cardinium* isolates grouped to geographical regions independent of the host *Culicoides* species, suggesting possible geographical barriers to *Cardinium* movement. Screening also identified *Asaia* bacteria in *Culicoides*. These findings point to a diversity of low-level endosymbiont infections in *Culicoides*, providing candidates for further characterization and highlighting the widespread occurrence of these endosymbionts in this insect group.

Culicoides species are small hematophagous insects (Diptera: Ceratopogonidae), some of which are important vectors of viral and parasitic diseases of veterinary and medical importance (1). More than 50 different viruses have been isolated from *Culicoides* species, including bluetongue virus (BTV), Schmallenberg virus (SBV), and African horse sickness virus (AHSV), which cause significant impacts on livestock production through stock losses and trade restrictions (1). Capable of wind-borne displacement for several hundred kilometers, *Culicoides* spp. have a capacity for rapid long-distance transmission of disease and have recently been responsible for the establishment of enzootic BTV and SBV infections over vast new geographic areas (1, 2). Current control methods for *Culicoides* include breeding site removal and baiting of livestock and midge resting sites; however, these techniques are costly and labor-intensive and have various levels of success and permanence of control (3). Although vaccines are available for some *Culicoides*-transmitted viruses, such as BTV, the practicality of vaccination is limited by the large number of BTV serotypes and the potential for genome segment reassortment between live vaccines and naturally circulating virus strains (4). Inactivated vaccines are expensive and less potent but have been used effectively in Europe for control of BTV (5). Inactivated vaccines are not currently available for AHSV.

Insect vector control by use of endosymbiotic organisms has gained increasing attention in recent years (6). Bacterial endosymbionts present in arthropod species are capable of influencing host characteristics such as longevity and vector competence, as well as being involved in nutrient provisioning (7–9). The endosymbiont *Wolbachia pipientis* (*Alphaproteobacteria*) has attracted notable attention for its applicability to endosymbiont-based control of the dengue virus vector, *Aedes aegypti. Wolbachia* has been shown to successfully invade and be maintained in natural *A. aegypti* populations and to block virus transmission (8, 10–12).

Several previous studies have reported evidence of bacterial endosymbiont infection in *Culicoides* species. Screening studies conducted using conventional PCR assays detected *Wolbachia* DNA in a single *Culicoides paraflavescens* individual in Japan (13). "*Candidatus* Cardinium hertigii" (*Bacteroidetes*), another bacterial endosymbiont which also has a range of influences on its host insect, has been detected in four *Culicoides* species in Japan, two in Israel, and two in the United Kingdom (13–15). Endosymbiotic diversity in Australian *Culicoides* species has not been investigated previously, nor has a comparative analysis of *Cardinium* divergence in different *Culicoides* species from diverse geographical locations been reported.

Although conventional PCR has previously been used successfully to screen arthropods for endosymbionts (16–18), recent studies have demonstrated that this method can fail to detect low-level infections. More sensitive screening techniques, such as long PCR (19), nested PCR (20), or quantitative PCR (qPCR) (21), are therefore required. Low-level endosymbiont infections have been identified in a range of insects, including tsetse flies (22), *Drosophila* (23), cherry fruit flies (24) and planthoppers (25). Previous

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TABLE 1 PCR screening primers

Target group	Primer	Sequence (5′→3′)	Melting temp (°C)	Size (bp)	Reference
Alphaproteobacteria	Alf28F	ARCGAACGCTGGCGGCA	56	700	35
	Alf684R	TACGAATTTYACCTCTACA			
Cardinium	CAR-SP-F	CGGCTTATTAAGTCAGTTGTGAAATCCTAG	52	544	13
	CAR-SP-R	TCCTTCCTCCCGCTTACACG			
COI	BC1culicFm	GTAAAACGACGGCCAGTTCWACWAAYCAYAAARWTATTGG	50	692	32
	JerR2 m	CAGGAAACAGCTATGACCCAAARAATCARAAYARRTGTTG			
Housekeeper third loop of 28S rRNA gene	D3a	GACCCGTCTTGAAACACGGA	57	400	69
	D3ba	TCGGAAGGAACCAGCTACTA			
Wolbachia	Wol-F	TTGTAGCCTGCTATGGTATAACT	52	900	36
	Wol-R	GAATAGGTATGATTTTCATGT			
Wolbachia wsp (nested)	wsp-L-F2	TGGTCCAATAAGTGATGAAGAAACTAGCTACTACGTTCG	68	609	39
	wsp-L-R2	AAAAATTAAACGCTACTCCAGCTTCTGCACCAAC			
	wsp151F	TGGTTACAAAATGGACGACA	50	422	
	wsp599R	CACCAACAGTGCTGTAAAGAAC			

studies have suggested that at low levels of infection, endosymbionts are capable of influencing the host. For example, low levels of *Wolbachia* in *Drosophila paulistorum* semispecies have been shown to influence fecundity, sex ratios, and mate discrimination (23). However, other endosymbiont effects, including viral blockage and fitness effects, may depend on bacterial density (26).

In this study, a range of *Culicoides* species, collected predominately from southeastern Australia, were screened for evidence of *Cardinium* and *Wolbachia* infection. Global movement of *Cardinium* in *Culicoides* species was also investigated based on sequence divergence in multiple loci. Novel *Cardinium* and *Wolbachia* infections were identified in a range of *Culicoides* species, a high proportion of which were low-level infections. Nucleotide sequence analysis revealed that *Cardinium* detected in these samples was genetically similar to those previously discovered in Japan, Israel, and the United Kingdom, suggesting a global presence of a single *Cardinium* strain throughout a wide geographical range and in a range of *Culicoides* species.

MATERIALS AND METHODS

Insect collection. *Culicoides* insects were collected using either Centre for Disease Control (CDC) mini-light traps (BioQuip Products, Rancho Dominguez, CA) or yellow sticky traps (YST). Mini-light traps contained either green or UV light-emitting diodes (LEDs) and were powered by a 6-V motorbike battery (27, 28). Traps were positioned approximately 2 m above the ground at dusk and collected after dawn, consistent with previous trapping methodologies (29). A downdraught fan in the traps directed insects into a beaker containing approximately 200 ml of 80% ethanol. Contents of the beakers were collected daily. Before storage at −20°C, insects were sorted, identified, and stored individually in fresh 80% ethanol. By washing insects in ethanol, the risk of contamination was reduced. YST (Trappit) were elevated approximately 10 cm above the ground by a plastic stick and illuminated by a solar garden light. Insects were individually cut from the YST and placed in a tube containing enough solvent (De-Solv-it [orange oil]) (Orange-Sol, Chandler, AZ) to cover the sample. Tubes were incubated at 60°C for 5 min with intermittent inversion until the insect floated off the YST. Insects were removed and washed twice with 80% ethanol before being stored in fresh 80% ethanol at −20°C (I. Valenzuela personal communication). Collections were made from 305 traps (81 CDC traps and 224 YST) over a period of 33 trapping nights throughout southeastern Australia from January to April in 2013 and 2014. Samples of *Culicoides imicola* collected using CDC light traps were also obtained from Kenya and Madagascar through the International Livestock Research Institute, Kenya. *C. imicola* were identified morphologically based on wing patterning and through sequence analysis of the cytochrome oxidase subunit 1 (COI) region.

***Culicoides* identification.** Morphological identification was performed with the aid of the pictorial atlas of Australasian *Culicoides* wings (Diptera: Ceratopogonidae) (30). The *C. victoriae* species group was separated into a series of species denoted by wing identifier number in square brackets, such as *C. victoriae* [241], based on the wing pictures from reference 30. One *C. victoriae* species not present in the pictorial atlas was collected in Geelong, Victoria, Australia, and is denoted *C. victoriae* [172] (GenBank accession number KT338816). Genetic confirmation of *Culicoides* species was based on amplification and sequencing of the COI gene, which is commonly used for species determination of *Culicoides* and is capable of differentiating most *Culicoides* species (31, 32).

DNA extractions. *Culicoides* insects were removed from ethanol and air dried before extraction (33). The mouse tail protocol of the MagMax-96 DNA multisample kit (Applied Biosystems, Austin, TX) on a KingFisher Flex instrument (Thermo Scientific, Waltham, MA) was used with the following amendments. Individual *Culicoides* insects were incubated in 100 μl of proteinase K (PK) buffer and 10 mg/ml of proteinase K. Samples were homogenized with a FastPrep-24 instrument (MP Biomedicals, Santa Ana, CA) at 6.0 m/s for 20 s with approximately 10- by 1.0-mm zirconia-silica beads (BioSpec Products, Bartlesville, OK) before overnight incubation at 56°C. Elution was altered to 40 μl of both elution buffers 1 and 2. To assess possible contamination during extraction, every 11th well in the MagMax 96-well plate was left without a *Culicoides* sample but was still included in all downstream screening steps.

Conventional PCR screening. The primers (GeneWorks, SA, Australia) utilized included those for an initial housekeeper targeting the third loop of the 28S rRNA gene (D3a and D3ba), followed by the *Cardinium* 16S rRNA gene (CAR-SP-F and CAR-SP-R) (13), the *Alphaproteobacteria* 16S rRNA gene (Alf28F and Alf684R) (34, 35), the *Wolbachia* 16S rRNA gene (Wol-F and Wol-R) (36), and the cytochrome oxidase subunit 1 (COI) gene (BC1culicFm and JerR2m) (32) for species confirmation. Primer sequences can be found in Table 1. PCRs with 25-μl reaction mixtures were performed using 1 unit Platinum *Taq* DNA polymerase

TABLE 2 Primers for 16S rRNA gene and *gyrB* multiallele typing

Cardinium target	Primer	Sequence (5′→3′)	Amplicon size (bp)	Reference
16S rRNA gene amplicon 1	16SA1 F	AGCGGGACACTTCGGTGTTG	745	This study
	16SA1 R	TCATCGTTTACGGCGTGGAC		
16S rRNA gene amplicon 2	16SA2 F	CGTAGGCGGCTTATTAAGTC	900	This study
	16SA2 R	GTCCCAGTCGCTGGTCTAAC		
gyrB amplicon 1	*gyrB*A1 F	CATGGCGTGGGTATTTCTT	724	This study
	*gyrB*A1 R	CAGGTTTCTACCGCTCCTTG		
gyrB amplicon 2	*gyrB*A2 F	TGCGGATAAATCTGGTCTGC	841	This study
	*gyrB*A2 R	GCTGTACATACACGGCATCAAC		

(Invitrogen, Carlsbad, CA), following the manufacturer's protocol. PCR conditions were as follows: 1 cycle at 95°C for 2 min; 35 cycles of 95°C for 30 s, the annealing temperature indicated in Table 1 for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. Two negative controls with ultrapure water were included for all PCR screens to ensure that no contamination occurred. A single positive control was included for each PCR; DNA from *C. victoriae* [240] was used for 28S rRNA and COI PCRs, and DNA from *Drosophila simulans* was used for *Wolbachia* 16S rRNA and *Alphaproteobacteria* 16S rRNA PCRs. At the commencement of screening, no positive control was available for *Cardinium*; in later steps, *C. victoriae* [240] samples shown to be *Cardinium* positive were used as a control. Amplified products were separated on a 1% agarose gel containing 0.1% SYBR Safe DNA gel stain (Invitrogen, USA) and visualized with a G:BOX Syngene blue light visualization instrument.

Multiallele typing. Samples which tested positive for *Cardinium* by conventional PCR screening were typed for multiple alleles using the 16S rRNA and DNA gyrase subunit B (*gyrB*) genes. Four primers (16SA1F, 16SA1R, 16SA2F, and 16SA2R) (Table 2) were designed to amplify two overlapping regions of the *Cardinium* 16S rRNA gene, based on the Clustal W alignment of sequences from *Culicoides* (accession numbers AB506776 to AB506779, JN166961, and JN166962) available in the GenBank database. Primers were designed using Geneious v7.0.5 and covered 1,404 bp of the 16S rRNA gene, with a 243-bp overlap between the amplicons (37). Four primers (*gyrB*A1F, *gyrB*A1R, *gyrB*A2F, and *gyrB*A2R) targeting a 1,399-bp region of the *gyrB* gene with a 168-bp overlap between amplicons (Table 2) were designed using the same process as described above with sequences available in GenBank (accession numbers AB506791, AB506792, JN166963, and JN166964). PCRs were performed using the conditions previously stated; an annealing temperature of 50°C was used for the *Cardinium* 16S rRNA gene and *gyrB* primers.

For low-level *Wolbachia* detection, amplification was with a combination of heminested (16S rRNA gene) and nested (*wsp*) PCRs. Heminested primers for the 16S rRNA gene used two sets of previously published primers from Wol-F and Wol-R (36) and Rao-F and Rao-R (38). First-round amplification was performed using the Wol-F and Wol-R primers as previously outlined, generating an 897-bp amplicon. Second-round amplification yielded two amplicons, amplicon 1 (464 bp) (Wol-F and Rao-R) and amplicon 2 (495 bp) (Rao-F and Wol-R); both rounds of amplification had an annealing temperature of 52°C. A nested PCR developed by Hughes et al. (39) (Table 1) was used to amplify a 423-bp region of *wsp*. The nested protocol was altered to a two-step nested PCR instead of the previously outlined single-tube nested PCR (39). Two negative controls, both involving ultrapure water as the template, were run for first-round amplification, with the product used as the template for second-round amplification.

qPCR screening. Quantitative PCR (qPCR) was used to increase the level of sensitivity of detection of *Wolbachia* and *Cardinium*. Samples screened with the conventional assays were rescreened with the qPCR assays. A *Wolbachia* qPCR was obtained based on the primer and probe design of Rao et al. (38), who used this assay to detect *Wolbachia* in field-collected mosquitoes. The probe reporter dye at the 5′ end was changed to VIC (4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein) as opposed to FAM (6-carboxyfluorescein), but the same TAMRA (6-carboxytetramethylrhodamine) quencher dye was retained at the 3′ end. The *Wolbachia* primers and the probe reaction setup was modified to a 20-μl reaction mixture using 10 μl of TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA), 1 μl of both primers (0.3 μM forward primer and 0.9 μM reverse primer), 1 μl of probe (0.05 μM), 2 μl of template DNA, and 5 μl of ultrapure water.

To develop a *Cardinium* qPCR, published *Cardinium* sequences isolated from *Culicoides* (accession numbers AB506776 to AB506779, JN166961, and JN166962) were aligned using Geneious v7.0.5 and a consensus sequence was obtained. Primer Express software v3.0.1. (Applied Biosystems) was used to design a TaqMan probe and primer pair to amplify a 111-bp region (Table 3) to specifically target *Cardinium* group C. The *Cardinium* primers and probes were calibrated to a final reaction volume of 20 μl consisting of 10 μl of TaqMan universal PCR master mix, 2 μl of forward and reverse primers (0.3 μM), 2 μl of probe (0.05 μM), 2 μl of template DNA, and ultrapure water. Optimal primer and probe concentrations for both assays were determined as in the protocol for the TaqMan universal PCR master mix. Triplicate reactions were performed on a QuantStudio 6 real-time PCR machine (Life Technologies, Carlsbad,

TABLE 3 Primers and probes for quantitative TaqMan assays

Primer or probe	Sequence (5′→3′)	Dye[a] Reporter	Dye[a] Quencher	Reference
Car F	ACGCCGTAAACGATGATTACTAGA	FAM	TAMRA	This study
Car R	TTCCTTTGAGTTTCACCCTTGC			
Car probe	ATGTACAACGTAGTTGTACGTGTCCAAGC			
Wol F	CCAGCAGCCGCGGTAAT	VIC	TAMRA	38
Wol R	CGCCCTTACGCCCAA T			
Wol probe	CGGAGAGGGCTAGCGTTATTCGGAATT			

[a] FAM, 6-carboxyfluorescein; VIC, 4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

CA). Cycling conditions included an initial incubation at 50°C for 2 min to activate uracil-*N*-glycosylase, followed by denaturation for 10 min at 95°C and then 45 cycles of 95°C for 15 s and a combined annealing and primer extension phase at 60°C for 1 min. Screening was conducted in a 96-well plate format, with 6 wells each plate being left for use as negative controls.

Cloned standards of *Cardinium* and *Wolbachia* were generated and used as positive controls, as follows. Conventional PCR was conducted using qPCR primers to amplify known *Wolbachia*-positive material from *Drosophila simulans* and *Cardinium*-positive material from *Culicoides victoriae* [240]. Amplicons were visualized on a 1% agarose gel and purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA). Purified DNA was ligated into pGEM-T Easy vector (Promega, Madison, WI) according to the manufacturer's protocol and then electroporated into competent *Escherichia coli* DH5α cells (New England BioLabs, Beverly, MA). Plasmid DNA was purified from positive clones using the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA) and sequenced using SP6 and T7 sequencing primers. Plasmid DNA clones were serially diluted before being used as positive controls. *Wolbachia* and *Cardinium* plasmids were also used to generate a standard regression curve based on 6 serial dilutions of the plasmid in each qPCR run, allowing relative quantification of infection. Plasmid copy numbers were calculated as per established protocols (Applied Biosystems).

Sensitivity comparison of conventional PCR and qPCR. Detection limits of conventional and quantitative PCRs were assessed by serially diluting *Wolbachia*-positive template DNA from *Drosophila simulans* and *Cardinium*-positive template DNA from *Culicoides victoriae* [240]. Ultrapure water was used to make 10-fold serial dilutions of each DNA template from neat to 10^{-9}. Duplicate 2-μl samples of each dilution were tested using the *Cardinium* conventional (primers CAR-SP-F and CAR-SP-R) (13) and quantitative assays (Table 3), as well as the *Wolbachia* conventional (primers Wol-F and Wol-R) (36) and quantitative assays (Table 3).

Sequencing. Nucleotide sequences of *Cardinium*-, *Wolbachia*-, and *Alphaproteobacteria*-positive amplicons were determined for species confirmation. DNA was purified from positive amplicons with a QIAquick gel extraction kit (Qiagen, Santa Clara, CA) according to the manufacturer's protocol. Fluorometric quantification of purified DNA was performed using a double-stranded DNA (dsDNA) HS assay on a Qubit (Invitrogen, Carlsbad, CA). Sequencing was performed using the BigDye Terminator v3.1 kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol, and sequence was generated via capillary Sanger sequencing on a 3500 Genetic Analyzer (Applied Biosystems).

A series of measures was implemented as an added precaution to reduce the risk of contamination and to ensure no false-positive detections. Clean-room working conditions were utilized, with insect identification, nucleotide extraction, master mix setup, amplification and postamplification handling all being performed in separate labs and with a nonamplified to amplified workflow being followed.

To compare the incidence of infections across sexes, contingency analyses were carried out for species where the infection was detected in at least one sex. Probabilities for detecting associations between infections and sex were based on exact tests.

Phylogenetic analysis. Phylogenetic relationships of *Cardinium* were analyzed using nucleotide sequences obtained from 16S rRNA and *gyrB* genes. Additional group A (isolated from *Ixodes scapularis* [AB001518 and AB506790], *Euides speciosa* [AB506775 and AB506788], *Tetranychus pueraricola* [AB241135 and AB506784], and *Oligonychus ilicis* [AB241130 and AB506783]) and group B (isolated from *Paenicardinium endonii* [DQ314214 and DQ314215]) *Cardinium* sequences obtained from GenBank were included to provide phylogenetic resolution. *Cardinium* 16S rRNA gene and *gyrB* sequences were concatenated head to tail, forming a supergene alignment. Concatenating two genes together increases the number of evolutionary changes on individual branches, which in turn can generate a phylogeny more precise than when the two genes are analyzed separately (40). The concatenated alignment was screened for the presence of recombination using the RDP, GENECONV, Bootscan, MaxChi, Chimaera, and SiScan methods implemented in Recombination Detection Program v4.39 (RDP4), with default parameters (41). A *Wolbachia* phylogeny was determined using the 16S rRNA gene and the *Wolbachia* surface protein gene (*wsp*). The *wsp* gene was included in phylogenetic analysis because it has been shown to have a much higher divergence rate than the 16S rRNA gene (18, 36). *Wolbachia* sequences from supergroup A (*Drosophila sechellia*), B (*Culex pipiens*), and C (*Dirofilaria immitis*) were included to see where *Culicoides Wolbachia* would be placed within the wider *Wolbachia* phylogeny, with supergroup C being used to root the trees. Host *Culicoides* phylogenetic analysis was based on mitochondrial cytochrome oxidase subunit I (COI).

All sequences were aligned by using the Clustal W algorithm in Geneious v7.0.5 (Biomatters Ltd.) (37). Aligned nucleotide sequences were analyzed using jModelTest2 v2.1.3, with the topology search taking the best of Nearest Neighbor Interchange (NNI) and Subtree Pruning and Regrafting (SPR) (42). Evolutionary models implemented to construct phylogenetic trees were selected based on the Akaike information criterion output from JModelTest2 (42) (number of substitution schemes, 11; base tree for likelihood calculations maximum-likelihood [ML] optimized; base tree search best). The Hasegawa-Kishino-Yano (HKY) model was selected for the *Cardinium* 16S rRNA gene, *Wolbachia wsp*, and the concatenation of the *Cardinium* 16S rRNA gene and *gyrB*. The general time-reversible (GTR) model was selected for the *Cardinium gyrB*, *Wolbachia* 16S rRNA gene, and *Culicoides* COI phylogeny. Phylogenetic ML trees were constructed using the PhyML plugin in Geneious v7.0.5 with 1,000 bootstrap replicates; the proportion of invariable sites and gamma distribution were both estimated (43).

Mapping of *Cardinium* and *Wolbachia* in *Culicoides*. *Culicoides* organisms which tested positive for *Wolbachia* or *Cardinium* based on screening with the quantitative assays were plotted against capture location. Shapefiles were obtained from the Australian Bureau of Statistics, Australian Standard Geographical Classification (ASGC) Digital Boundaries, Australia (July 2011). The distribution map was created in quantum GIS (QGIS) Wien v2.8.1 (44).

Nucleotide sequence accession numbers. Sequences generated were deposited in GenBank. *Cardinium* accession numbers for the 16S rRNA gene are KR026906 to KR026918 and KR026920 to KR026923, and those for the *gyrB* gene are KR026924 to KR026935. *Wolbachia* accession numbers for the 16S rRNA gene are KR026936 to KR026941, and those for *wsp* are KR026942 to KR026951. Culicoides COI sequences of the *C. victoriae* group and *C. williwilli* were deposited in GenBank under accession numbers KT338814 to KT338822.

RESULTS

Endosymbiont assays. Increased sensitivity for the *Wolbachia* and *Cardinium* quantitative PCRs in comparison to the conventional assays was obtained. Serial dilution of *Wolbachia*- and *Cardinium*-positive samples resulted in a 100-fold-lower detection limit with the qPCR assays. Detections were confirmed by sequencing; however, the *Wolbachia* (62 bp of the 16S rRNA gene) and *Cardinium* (111 bp of the 16S rRNA gene) qPCR amplicons were too small for species discrimination. Developed nested PCRs for *Wolbachia* (16S rRNA gene and *wsp*) and *Cardinium* (16S rRNA gene and *gyrB*) were used to amplify enough material of the low-level detections to allow confirmation by sequencing.

Twenty *Culicoides* species were identified by morphological and genetic typing of insects collected in Victoria, Tasmania, and Queensland from January to April 2013 and January to April 2014 (Fig. 1). These samples, together with samples of *C. imicola* from Kenya and Madagascar, were analyzed for evidence of *Cardinium* and *Wolbachia* infection through the conventional and quantitative PCR assays.

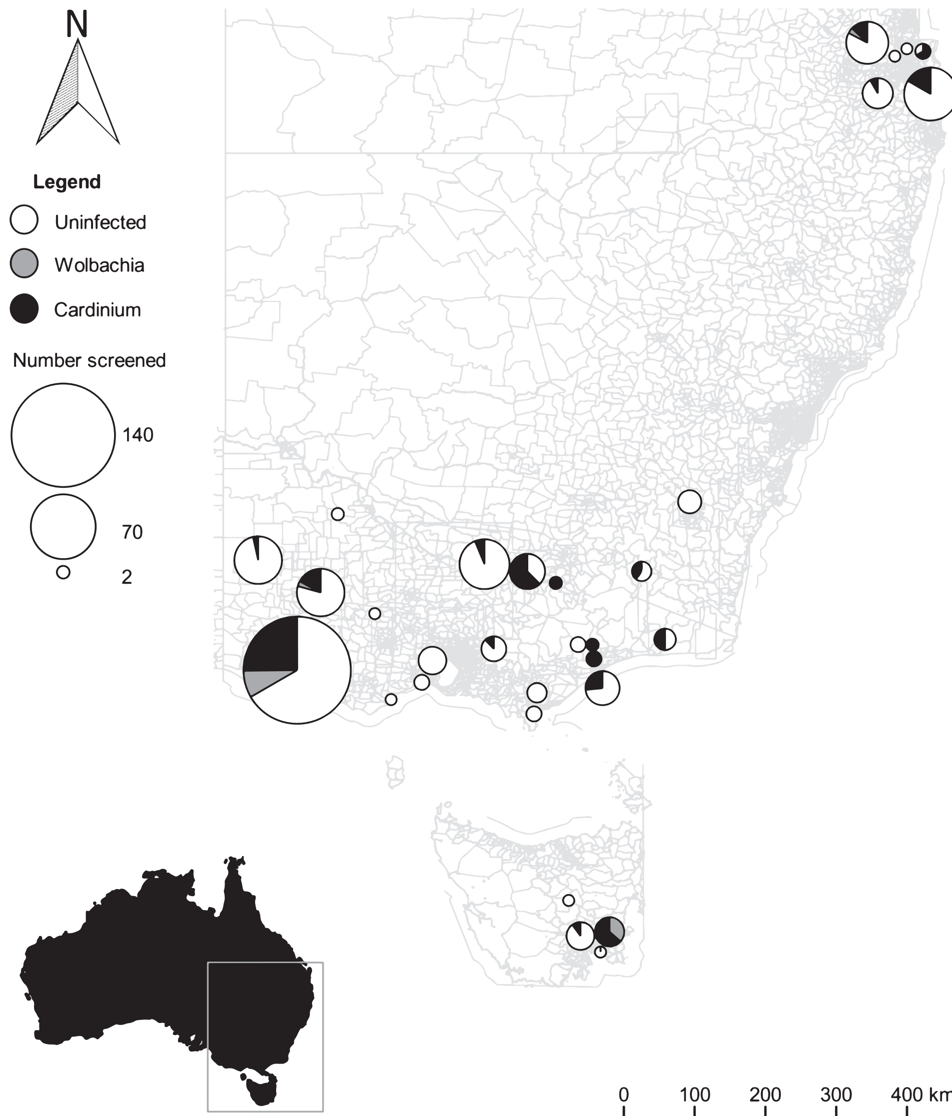

FIG 1 *Culicoides* trapping and endosymbiont distribution. A map depicting collection sites across Queensland, New South Wales, Victoria, and Tasmania is shown (shapefiles were obtained from the Australian Bureau of Statistics, ASGC Digital Boundaries, Australia, July 2011; the distribution map was created in QGIS Wien v2.8.1 [44]). At all sites an equal number of *Culicoides* insects were screened with both the *Cardinium* and *Wolbachia* quantitative assays. The size of the pie chart represents the number of individuals screened, with the proportion of the *Wolbachia* (gray) or *Cardinium* (black) wedge representing the number of *Culicoides* insects which tested positive for each endosymbiont. White circles indicate *Culicoides* insects which were screened but in which no *Wolbachia* or *Cardinium* was detected.

***Cardinium* screening.** The conventional PCR assay identified evidence of *Cardinium* infection in females of three species, *C. victoriae* [240] and *C. brevitarsis* from Australia and *C. imicola* from Kenya (Table 4). Rescreening the samples using the qPCR assay indicated a substantially higher prevalence of *Cardinium* infection, with positive samples detected in all *Culicoides* species except *C. victoriae* [172] (Table 4). The percentage of positive samples detected in females increased from 4% (14/360; confidence interval [CI], 2.23 to 6.28%) (conventional PCR) to 26% (92/360; CI, 21.25 to 30.25%) (qPCR), and detections in males increased from 0% (0/161; CI, 0 to 1.84%) (conventional PCR) to 14% (22/161; CI, 8.98 to 19.64%) (qPCR). A lower number of male *Culicoides* insects were screened due to their lower representation in collections. However, in those *Culicoides* species in which *Cardinium* was detected in individuals of both sexes, there was no significant difference between prevalence in males and females for *C. brevitarsis* (contingency analysis, $P = 0.63$ and df = 1), *C. bundyensis* (contingency analysis, $P = 0.33$ and df = 1), and *C. victoriae* [245] (contingency analysis, $P = 0.24$ and df = 1), although differences were marginally nonsignificant in the case of *C. parvimaculatus* (contingency analysis, $P = 0.065$ and df = 1) and *C. austropalpalis* (contingency analysis, $P = 0.067$ and df = 1) due to a higher prevalence in female *Culicoides*. For *C. marksi*, no infection was identified in males, but it was present in some females (contingency analysis, $P = 0.035$).

Detections of *Cardinium* by conventional PCR were seen at the lowest quantification cycle (C_q) value of 26.58 in *C. williwilli* (equating to 23,000,000 copies) and the highest C_q value of 34.06

TABLE 4 Prevalence of *Cardinium* and *Wolbachia* detected in samples from *Culicoides* species based on conventional and quantitative PCR screening

Species	No. positive/total (% positive)[a] by:							
	Conventional PCR				Quantitative PCR			
	Cardinium		*Wolbachia*		*Cardinium*		*Wolbachia*	
	Female	Male	Female	Male	Female	Male	Female	Male
C. austropalpalis	0/63	0/32	0/63	0/32	**7/63 (11)**	**3/32 (9)**	**1/63 (2)**	0/32
C. narrabeenensis	0/14		0/14		**1/14 (7)**		**3/14 (21)**	
C. marksi	0/50	0/24	0/50	0/24	**8/50 (16)**	0/24	**1/50 (2)**	0/24
C. parvimaculatus	0/20	0/20	0/20	0/20	**8/20 (40)**	**2/20 (10)**	**4/20 (20)**	0/20
C. dycei	0/5		0/5		**2/5 (40)**		0/5	
C. williwilli	**1/4 (25)**	0/1	0/4	0/1	**4/4 (100)**	**1/1 (100)**	0/4	0/1
C. henryi	0/11		0/11		**7/11 (64)**		**1/11 (9)**	
C. brevitarsis	**1/22 (5)**	0/9	0/22	0/9	**6/22 (27)**	**1/9 (11)**	**5/22 (23)**	0/9
C. imicola (Madagascar)	0/9	0/2	0/9	0/2	**3/9 (33)**	0/2	**2/9 (22)**	0/2
C. imicola (Kenya)	**1/1 (100)**				**1/1 (100)**			
C. wadai	0/2	0/1	0/2	0/1	**1/2 (50)**	0/1	0/2	0/1
C. bundyensis	0/39	0/23	0/39	0/23	**6/39 (15)**	**6/23 (26)**	**1/39 (3)**	**2/23 (9)**
C. victoriae [172]	0/15	0/13	0/15	0/13	0/15	0/13	0/15	0/13
C. victoriae [245]	**1/34 (3)**	0/11	0/34	0/11	**10/34 (29)**	**1/11 (9)**	**3/34 (9)**	0/11
C. victoriae [241]	0/18	0/9	0/18	0/9	**1/18 (5)**	0/9	**1/18 (6)**	0/9
C. victoriae [240]	**11/18 (61)**	0/1	0/18	0/1	**13/18 (72)**	0/1	0/18	0/1
C. victoriae [true]	0/8	0/2	0/8	0/2	**1/8 (12)**	0/2	0/8	0/2
C. antennalis		0/12		0/12		**8/12 (67)**		**6/12 (50)**
Culicoides (*Molestus* group) species no. 2	0/2		0/2		**1/2 (50)**		0/2	
C. marmoratus	0/6		0/6		**6/6 (100)**		**1/6 (17)**	
C. multimaculatus	0/19		0/19		**6/19 (31)**		0/19	
Total	14/360 (4)	0/160	0/359	0/160	92/360 (26)	22/160 (14)	23/359 (7)	8/160 (5)

[a] Boldface indicates endosymbiont-positive results.

(equating to 24,000 copies) in *C. victoriae* [240] (see Table S1 in the supplemental material). However, the *Cardinium* qPCR assay provided detections at the lowest C_q value of 42.68 in *C. parvimaculatus*, which equates to approximately 290 copies of the *Cardinium* target region. In *Culicoides* species which had detections of *Cardinium* in both sexes, there was no significant difference (based on *t* tests) in C_q values and hence *Cardinium* copy number between sexes in *C. bundyensis* ($P = 0.22$, df $= 10$) and *C. austropalpalis* ($P = 0.29$, df $= 8$), although there was a difference for *C. parvimaculatus* ($P = 0.018$, df $= 8$).

***Wolbachia* screening.** No evidence of *Wolbachia* infection was detected in any of the *Culicoides* samples tested using the conventional PCR. In contrast, evidence of low-level *Wolbachia* infections was detected in 10 species of *Culicoides* when individual samples were screened using the qPCR assay (Table 4). Across all species tested, the percentages of detected positive females (7%, 23/353; CI = 4.27 to 9.46) and males (5%, 8/160; CI = 2.34 to 9.27) were similar (contingency test, $P = 0.55$ and df $= 1$). *Culicoides bundyensis* was the only species in which evidence of *Wolbachia* infection was detected in both sexes, with similar proportions of positive males and females (contingency test, $P = 0.54$ and df $= 1$).

Detections of *Wolbachia* by qPCR were seen to range from the lowest C_q value of 30.53 in *C. antennalis* (equating to 11,600 copies) to the highest C_q value of 41.30 (equating to 22.4 copies) in *C. brevitarsis* (see Table S1 in the supplemental material). Detections of *Wolbachia* were rarely seen in both sexes of the same *Culicoides* species; hence, no comparison of C_q value and sex was performed.

There was evidence of low-level dual infections with *Wolbachia* and *Cardinium* in a number of *Culicoides* species. These included five individuals of *C. antennalis*, four individuals of *C. brevitarsis* and *C. parvimaculatus*, three individuals of *C. bundyensis* and *C. victoriae* [245], and single individuals of *C. imicola* (Madagascar), *C. marksi*, *C. austropalpalis*, and *C. marmoratus* (Table 5). The dual infections in single individuals occurred at the expected frequency on the basis of a random association between the infections.

***Alphaproteobacteria* screening.** Screening by conventional PCR was also performed using the broad-range *Alphaproteobacteria* primer set. The assay provided evidence of infection in individuals representing four *Culicoides* species: *C. austropalpalis*, in which 30% (13/43; CI = 17.96 to 45.09%) of females and 11% (3/28; CI = 2.79 to 26.45%) of males were positive; *C. marksi*, in which 7% (2/29; CI = 1.17 to 20.97%) of females and 5% (1/19; CI = 0.26 to 23.33%) of males were positive; *C. brevitarsis*, in which 22% (2/9; CI = 3.90 to 56.21%) of females and 50% (1/2; CI = 2.5 to 97.5%) of males were positive; and *C. bundyensis*, in which 18% (5/27; CI = 7.11 to 36.38%) of females were positive. The amplicons generated in the assay were sequenced. BLAST analysis indicated that amplicons obtained from *C. marksi* and *C. brevitarsis* shared a high percentage of nucleotide sequence identity (99%) with the 16S rRNA gene of an *Asaia* sp. bacterium isolated from *Aedes albopictus* mosquitoes (GenBank accession number JX445140.1).

***Cardinium* phylogenetic analysis.** Partial 16S rRNA and DNA gyrase subunit B gene sequences were used to construct the *Car-*

TABLE 5 Morphological grouping of *Culicoides* species with *Cardinium* and *Wolbachia* detections by qPCR

			No. positive/total (% positive)[a]					
			Cardinium		*Wolbachia*		Dual infected[b]	
Subgenus	Complex or group	Species	Female	Male	Female	Male	Female	Male
Avaritia	Boophagus complex	*C. wadai*	**1/2 (50)**	0/1	0/2	0/1		
	Imicola complex	*C. brevitarsis*	**6/22 (27)**	**1/9 (11)**	**5/22 (23)**	0/9	**4/22 (18)**	
		C. imicola (Madagascar)	**3/9 (33)**	0/2	**2/9 (22)**	0/2	**1/9 (11)**	
		C. imicola (Kenya)	**1/1 (100)**					
Marksomyia	Marksi group	*C. dycei*	**2/5 (40)**		0/5			
		C. marksi	**8/50 (16)**	0/24	**1/50 (2)**	0/24	**1/50 (2)**	
		C. parvimaculatus	**8/20 (40)**	**2/20 (10)**	**4/20 (20)**	0/20	**4/20 (20)**	
Unplaced	Antennalis group	*C. antennalis*		**8/12 (67)**		**6/12 (50)**		**5/12 (42)**
	Ornatus group	*C. marmoratus*	**6/6 (100)**		**1/6 (17)**		**1/6 (16)**	
	Williwilli group	*C. austropalpalis*	**7/63 (11)**	**3/32 (9)**	**1/63 (2)**	0/32	**1/63 (2)**	
		C. narrabeenensis	**1/14 (7)**		**3/14 (21)**		0/14	
		C. williwilli	**4/4 (100)**	**1/1 (100)**	0/4	0/1		
	Victoriae group	*C. bundyensis*	**6/39 (15)**	**6/23 (26)**	**1/39 (3)**	**2/23 (9)**	**1/39 (3)**	**2/23 (9)**
		C. henryi	**7/11 (64)**		**1/11 (9)**		0/11	
		C. multimaculatus	**6/19 (31)**		0/19			
		C. victoriae [172]	0/15	0/13	0/15	0/13		
		C. victoriae [241]	**1/18 (5)**	0/9	**1/18 (6)**	0/9	0/18	
		C. victoriae [240]	**13/18 (72)**	0/1	0/18	0/1		
		C. victoriae [245]	**10/34 (29)**	**1/11 (9)**	**3/34 (9)**	0/11	**3/34 (9)**	
		C. victoriae [true]	**1/8 (12)**	0/3	0/8	0/3		

[a] Boldface indicates endosymbiont-positive results.
[b] *Culicoides* species which tested positive for both *Wolbachia* and *Cardinium* by qPCR.

dinium phylogeny. Based on the 505-bp 16S rRNA gene amplicon, all Australian *Cardinium* isolates grouped with the previously described sequences of *Culicoides* group C *Cardinium hertigii* from Israel (*C. oxystoma* and *C. imicola*), Japan (*C. arakawae*, *C. lungchiensis*, *C. peregrinus*, and *C. ohmorii*), and the United Kingdom (*C. punctatus* and *C. pulicaris*) (see Fig. S1a in the supplemental material). A low level of nucleotide sequence divergence was observed, ranging from 100% identity to 98.94% identity (5 nucleotide substitutions), between *C. brevitarsis* collected in Brisbane, Australia, and *C. oxystoma* (GenBank accession number JN166962) collected in Israel.

Phylogenetic analysis of the 1,088-bp region of the *gyrB* gene also indicated that the Australian *Cardinium* isolates cluster with group C *Cardinium hertigii* (see Fig. S1b in the supplemental material). There was generally a higher level of sequence divergence in *gyrB* than in the 16S rRNA gene, but there was 100% identity observed between some *Cardinium gyrB* sequences. The highest divergence (95.87% identity or 45 nucleotide substitutions) occurred between *Cardinium* sequences from *C. multimaculatus* from Australia and *C. imicola* from Israel (GenBank accession number JN166963) and Kenya. In this analysis, the *Cardinium* sequences grouped predominately to the geographical region from which the host *Culicoides* had been collected, with the exception of *C. williwilli* from Brookfield in Queensland, which was most similar to *Cardinium* detected in *C. arakawae* from Japan (Fig. 2; see Fig. S1b in the supplemental material). Translation of the *gyrB* gene sequences indicated that the majority (90%) of amino acid substitutions in the gyrase B protein were synonymous.

A 1,563-bp amplicon assembled by concatenation of the 16S rRNA and *gyrB Cardinium* genes (Fig. 2) generated a phylogeny

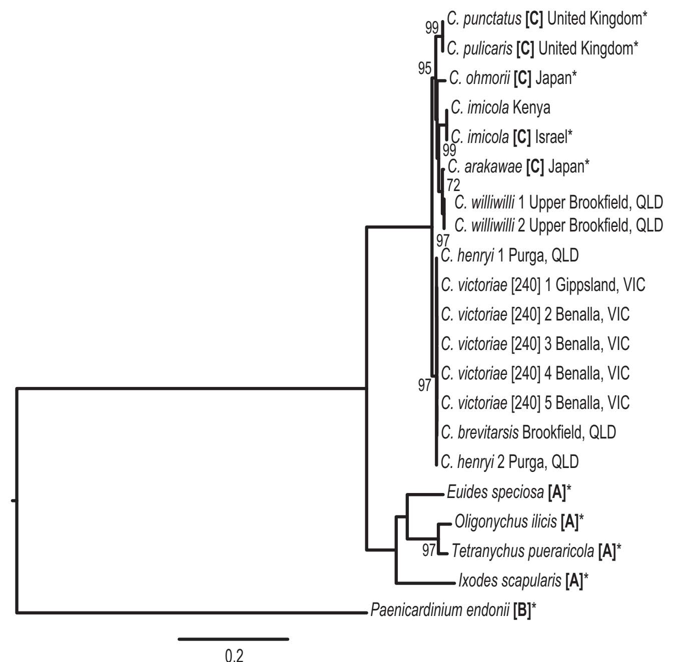

FIG 2 Maximum-likelihood concatenated *Cardinium* tree. The phylogenetic tree was constructed from the 1,563-bp concatenated 16S rRNA gene and *gyrB Cardinium* amplicon sequences. The sequences were aligned using the Clustal W algorithm employing the HKY substitution model based on JModelTest2 analysis with 1,000 bootstrap replicates. Bootstrap proportions of ≥70% are indicated beside nodes. Three *Cardinium* groups were included: group A, found in arthropods; group B, found in plant parasitic nematodes; and group C, found in *Culicoides*. The capture location is indicated beside the species name. Asterisks denote sequences from GenBank; all other sequences were generated in this study.

consistent with the phylogenetic trees constructed using the individual genes (see Fig. S1a and b in the supplemental material). The highest divergence of the concatenated sequences (97.05% identity or 47 nucleotide substitutions) was between *C. multimaculatus* from Australia and *C. ohmorii* from Japan, *C. imicola* from Israel, and *C. imicola* Kenya. The concatenated tree was assessed for the presence of recombination using the program RDP v.4.39, but no putative recombination events were detected.

***Wolbachia* phylogenetic analysis.** Phylogenetic analysis of *Wolbachia* (Fig. 3) based on partial sequences of the 16S rRNA gene (390-bp amplicon) and the *wsp* gene (351-bp amplicon) was done through nested and heminested PCRs. The success of amplifying sequences from low-level *Wolbachia* infections was lower than for *Cardinium*, and only 16S rRNA gene or *wsp* amplicons were obtained for many samples. The 16S rRNA gene sequences displayed a high level of homology to the only other *Wolbachia* sequence reported previously from *Culicoides*, which was from *C. paraflavescens* collected in Japan (accession number AB506794). The highest level of divergence from this sequence (98.72% identity) was with *Wolbachia* from *C. victoriae* [241] collected in Australia. The *wsp* gene sequences displayed generally lower levels of sequence identity, with greatest divergence (79.5% identity) observed in a group of *Culicoides narrabeenensis* isolates which were collected in Brisbane, Australia. BLAST analysis of *C. narrabeenensis* samples showed the closest sequence identity of 98% to *Wolbachia* isolated from *Phengaris teleius* (accession number JX470438), which is placed in *Wolbachia* supergroup B (45).

Occurrence of endosymbionts within the genus *Culicoides* and location. *Wolbachia* and *Cardinium* detections were investigated across the *Culicoides* genus to determine if there was a specific association with a particular subgenus. This was done based on the morphological classification of *Culicoides* species, largely based on wing classification (Table 5) (30). *Wolbachia* was detected sporadically across different subgenera, with no obvious pattern identified (Table 5). *Cardinium* was seen to infect all species except *C. victoriae* [172], with a low prevalence in other *C. victoriae* species. This was further investigated by constructing a phylogenetic tree based on COI of the *C. victoriae* group, with the absence of *Cardinium* not restricted to a particular lineage (see Fig. S2 in the supplemental material). As expected, *Cardinium* detections were more common across *Culicoides* species than across *Wolbachia*. Finally, to determine if there was an association with the presence of *Wolbachia*, *Cardinium*, and geographical location, infection prevalence was plotted against capture site (Fig. 1), with no obvious association apparent.

DISCUSSION

The genus *Culicoides* is one of the least studied of the major dipteran vector groups, with limited information known about their endosymbionts. Recent studies have identified *Wolbachia* and *Cardinium* in *Culicoides* species collected in Japan, Israel, and the United Kingdom. These studies used conventional PCR assays to reveal a relatively low prevalence of both *Wolbachia* (1/34) and *Cardinium* (8/34) (13–15). In the present study, 20 species of *Culicoides*, collected predominately in southeastern Australia, were screened for the presence of these endosymbionts. Previously established screening methodologies (13) were utilized to identify *Cardinium* in samples of *C. victoriae* [240], *C. victoriae* [245], *C. williwilli*, *C. imicola* (Kenya), and *C. brevitarsis*; however, these methods failed to detect *Wolbachia* in individuals of any of the species tested. Bacteria of the species *Asaia* were identified in *C. marksi* and *C. brevitarsis* samples by conventional PCR screening utilizing *Alphaproteobacteria* primers. *Asaia* species have previously not been identified in *Culicoides*. *Asaia* has been detected in *Anopheles* mosquitoes, colonizing the salivary glands and midgut of the insect (46). Unlike *Wolbachia* and *Cardinium*, *Asaia* species can be cultured on medium and easily colonize insects. Hence, these bacteria have been suggested as potential paratransgenesis control agents for insect vectors (46, 47).

Through the use of qPCR assays, previously undetected low-level *Cardinium* and *Wolbachia* infections were found in *Culicoides* species. Low-level detections were due to an improvement in detection sensitivity of approximately 100-fold gained through utilizing qPCR assays, compared to conventional PCR screening methodologies. The qPCR screening identified a significant number of low-level *Wolbachia* and *Cardinium* infections in *Culicoides*, with the improved sensitivity increasing detection prevalence. Using conventional PCR, Nakamura et al. (13) reported a *Cardinium* infection prevalence of 16% in *Culicoides*, but our analysis indicates that this is likely to be an underestimate of the true prevalence. Following a similar pattern, *Wolbachia* has previously been detected in only a single *C. paraflavescens* individual (13), whereas we have detected *Wolbachia* in 7% of female and 5% of male *Culicoides* insects that were screened.

This study also detected evidence of a range of low- and high-level infections in *Culicoides*, with no discernible pattern identified to explain this variability. High-density *Cardinium* infections detected by conventional PCR were found in *C. brevitarsis*, *C. victoriae* [240], *C. victoriae* [245], *C. williwilli*, and *C. imicola* Kenya, with target copy numbers ranging from ≈120,000 to 47,140,000 based on relative quantification. Low-level infections detected only by qPCR and confirmed by nested PCR were found in a range from ≈125 to 60,000 target copies per *Culicoides* insect. Changes in endosymbiont density can be a result of a range of factors, such as the bacterial strain (48, 49), host age (49, 50), sex (50), and temperature (51, 52). Endosymbiont density based on C_q values was investigated with *Culicoides* species that had *Cardinium* infections in both sexes. No difference was seen between sexes in *C. bundyensis*, *C. austropalpalis*, and *C. victoriae* [240], whereas *C. parvimaculatus* females had a higher density, but this result is dependent on low numbers (only 2 males and 8 females). Morag et al. (12) provided evidence that temperature could affect endosymbiont density, based on a lower prevalence of *Cardinium* in *Culicoides* in arid regions than in Mediterranean regions. Low-level endosymbiont infections can be highly localized within the insect host, such as in some *Drosophila* species (53), highlighting the importance of screening the whole insect instead of using only abdomens (54). Although these infections occur at a low level, they may still have a significant impact on their host insect, as has been shown for *Wolbachia* in *Drosophila paulistorum* semispecies, which can influence fecundity, sex ratio, and mate discrimination (23). Studies on the effect of *Cardinium* in *C. imicola* have found no infection impact on *Culicoides* survival under optimal, starvation, heat, and antibiotic treatments and no effect on wing size (55). However, detection in that study involved conventional PCR screening, and perhaps an effect on *Cardinium* was overlooked because low-level endosymbiont infections were not identified or other effects of *Cardinium* were not characterized.

Based on 16S rRNA gene and *gyrB* sequences, *Cardinium hertigii* was the only *Cardinium* strain identified in this study. Specific

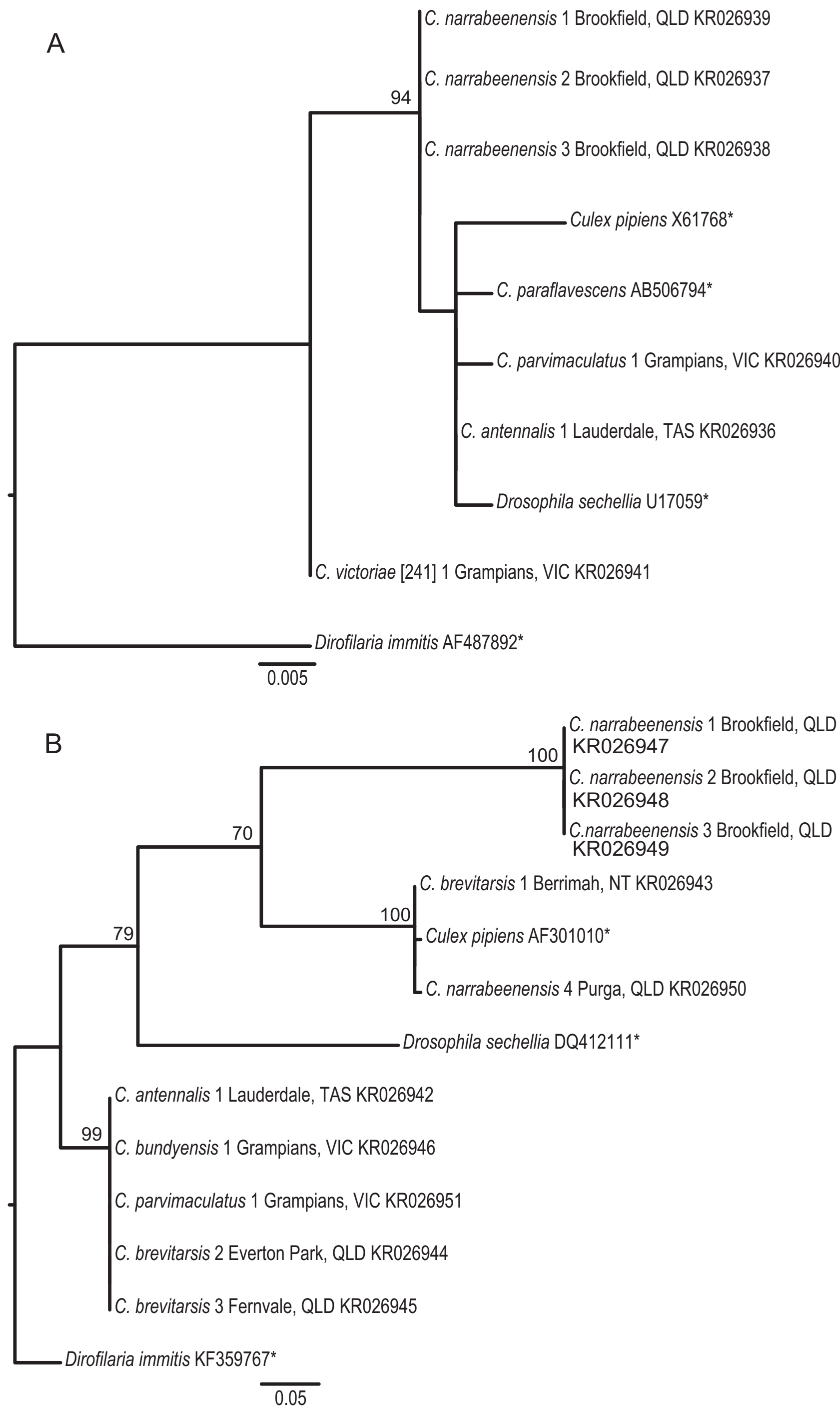

FIG 3 Maximum-likelihood phylogenetic trees of *Wolbachia*. Phylogenetic trees based on a 390-bp region of the 16S rRNA gene (A) and a 351-bp region of the *wsp* gene (B) are shown. Sequences were aligned using the Clustal W algorithm employing the GTR substitution model (16S rRNA gene) and the HKY substitution model (*wsp*) based on JModelTest2 analysis with 1,000 bootstrap replicates. Bootstrap proportions of ≥70% are indicated beside nodes. Three supergroups were also included for reference; *Drosophila sechellia* (*Wolbachia* supergroup A), *Culex pipiens* (*Wolbachia* supergroup B), and *Dirofilaria immitis* (*Wolbachia* supergroup C). The *Wolbachia* sequence from *Dirofilaria immitis* was used to root the trees. The capture location is indicated beside the species name. Asterisks denote sequences obtained from GenBank; all other sequences were generated in this study.

to *Culicoides*, group C *Cardinium* has previously been reported with <3.1% sequence divergence for 16S rRNA gene and <19% for *gyrB* (13). This pattern of similar *Cardinium* and *Wolbachia* endosymbiont strains in closely related hosts has been seen in other infected insects (56–59).

The phylogeny generated by analysis of *gyrB* sequences showed *Cardinium* strains clustering primarily geographically rather than by host *Culicoides* species. For example, strains from species in the *Avaritia* subgenus (*C. imicola* and *C. brevitarsis*) were not positioned together, but similar *Cardinium* strains were detected in *C.*

brevitarsis and several species in the *C. victoriae* species group (*C. henryi*, *C. multimaculatus*, and *C. victoriae*) from Australia (see Fig. S1b in the supplemental material). A similar pattern has been seen in *Wolbachia* infecting ants from a range of geographical locations, with closely related *Wolbachia* strains typically confined to the geographical location of the host insects (60), indicating that *Wolbachia* populations can be isolated by geographic barriers (60). There were only two exceptions to this general trend: *Cardinium* strains from Australian *C. williwilli* did not cluster with strains from other Australian *Culicoides* species, and strains from *C. arakawae* and *C. ohmorii* in Japan did not cocluster in the phylogeny (Fig. 2; see Fig. S1b in the supplemental material). Also, there was no evidence of *Wolbachia* or *Cardinium* infections being restricted to a particular geographical region (Fig. 1). A higher number of individuals infected with *Cardinium* from different geographical regions as well as analysis of other, more rapidly evolving genes will be required to examine this association more definitively.

To determine *Wolbachia*'s placement within the currently recognized 10 *Wolbachia* supergroups, 16S rRNA gene and *wsp* sequences were analyzed (61). *Wolbachia* sequences from *Culicoides* were seen to place in either *Wolbachia* supergroup A or B, which is consistent with the single *Wolbachia* isolate from *C. paraflavescens* (13). To provide further *Wolbachia* species discrimination, additional genes should be screened, such as that for cell division protein FtsZ (62). Nested primers for *ftsZ* were tested in this study with no success. A high sequence similarity was also seen between some *Wolbachia* isolates infecting *Culicoides* and those infecting *Culex pipiens* obtained from GenBank (Fig. 3A and B). This is not surprising, as highly related, and even genetically identical, *Wolbachia* strains are occasionally found in distantly related hosts (60). The cooccurrence of *Wolbachia* and *Cardinium* was detected in a number of individual *Culicoides* insects. *Wolbachia* and *Cardinium* are commonly seen to coinfect the same insect host species (57, 63, 64), but the nature of the interaction of these endosymbionts within the host insects can be unclear (65).

The *Culicoides* genus is divided into a number of subgenera based on morphology. *Wolbachia* was seen to occur sporadically throughout the *Culicoides* genus. On the other hand, *Cardinium* was seen to occur in almost every species. This sporadic occurrence of endosymbionts, such as *Wolbachia*, has been seen before in other insect species and could be a consequence of a weak cytoplasmic incompatibility (CI) phenotype (66). It was also noted that *Cardinium* was not detected in samples of *C. victoriae* [172] (see Fig. S2 in the supplemental material), but this could also be due to the relatively low numbers of individuals screened (Table 4). The reported higher prevalence of *Cardinium* than of *Wolbachia* across the *Culicoides* genus is consistent with previous observations (13). Taxonomical grouping based on wing morphology was used to construct a *Culicoides* phylogeny; a COI sequence-based phylogeny was also investigated but proved to be inadequate for separating species and clades, consistent with previous studies (31). However, COI was informative for resolving taxa at a finer level within the *C. victoriae* group (see Fig. S2 in the supplemental material).

Conventional PCR has previously been shown to be an inadequate method for accurately profiling endosymbiont distribution in insect populations (19). The detection of low-level endosymbiont infections requires more sensitive screening assays, such as nested, long, or quantitative PCR (19, 67). However, techniques such as nested and long PCR increase the risk of obtaining false positives through contamination (68). For this study, qPCR was the preferred method, as it has been shown previously that increased sensitivity can be achieved with a lower risk of contamination. Regardless of the technique used when working with low-level infections, additional precautions must be implemented; in this study we used a nonamplified to amplified workflow in separate labs and multiple negative controls within each procedure.

This study identified evidence of low-level endosymbiont infections of both *Cardinium* and *Wolbachia* in *Culicoides* species. Low-level endosymbionts were detected infrequently and sporadically within *Culicoides* populations, as has been observed in other insect species, such as *Drosophila equinoxialis*, *Drosophila paulistorum*, *Pityogenes chalcographus*, *Perkinsiella saccharicida*, and *Perkinsiella vitiensis* (20, 23, 25). This sporadic occurrence could be a consequence of a weak cytoplasmic incompatibility (CI) phenotype. Unfortunately, due to difficulties in maintaining *Culicoides* colonies, there is limited knowledge yet available on the effect that *Cardinium* or *Wolbachia* may have on the host.

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