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REMOVAL OF FUNGAL CONTAMINANTS AND THEIR DNA FROM THE SURFACE OF *DIAPHORINA CITRI* (HEMIPTERA: PSYLLIDAE) PRIOR TO A MOLECULAR SURVEY OF ENDOSYMBIONTS

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Endosymbionts play diverse roles in arthropod biology (Dale & Moran 2006), and molecular surveys to detect arthropod-associated microbes have bypassed the necessity of culturing them *in vitro* for identification (Darby & Welburn 2006). To date, no consistent method has been developed to eliminate microbial DNA from the external surfaces of arthropods prior to conducting molecular surveys of endosymbionts. This is problematic because resolving the relationships between a host and its endosymbionts can be complicated by polymerase chain reaction (PCR) amplification of DNA from contaminating external microbes (Thornhill et al. 2008). A variety of techniques that may kill microbes on arthropod surfaces have been reported, including washing with acetone (Fukatsu & Nikoh 1998), ethanol (Davidson et al. 1994; Van Borm et al. 2002), bleach (Davidson et al. 1994), formaldehyde (Duperchy & Zimmermann 2003), exposure to ultraviolet light (Van Borm et al. 2002), radiation (Duperchy & Zimmermann 2003) and antibiotic treatment (Duperchy & Zimmermann 2003). However, the effectiveness of these methods for eliminating DNA from external microbes so that it is not amplified in subsequent PCRs has not been evaluated.

DNA from contaminating external fungi should be eliminated, if possible, prior to determining if there are endosymbiotic fungi associated with a laboratory colony of the Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae). Examples of fungal endosymbionts have been reported in association with other hemipteran insects, including aphids and planthoppers (Noda et al. 1994; Fukatsu & Ishikawa 1995; Hongoh & Ishikawa 2000). A laboratory colony of *D. citri* (Skelley & Hoy 2004) was used to evaluate a variety of solutions (Table 1) for their ability to kill surface-inhabiting fungi and to eliminate their DNA so that they could not be detected using a high-fidelity PCR protocol. Adult *D. citri* in this colony support an abundance of external fungal growth, primarily on the ventral portion of their abdomens. This is due to being reared in high densities at a high relative humidity, which facilitates an accumulation of excreted honeydew that supports fungal growth (Fig. 1A).

Surface decontamination was conducted in a Plexiglass® hood that was first washed with 70% EtOH, then with bleach (6% sodium hypochlorite), and DNA Away (Molecular BioProducts, San Diego, CA). The protocol involved: (i) placing individual live insects into sterile 15-mL centrifuge tubes containing 5 mL of the decontamination solution and vortexing vigorously for 1 min; (ii) the solution was discarded, leaving the insects in the tube; (iii) insects were rinsed 3 X with 10 mL of sterile water, which was discarded after each rinse; (iv) insects were transferred to a sterile 1.5-mL centrifuge tube with a sterile pipette tip wetted with DNA extraction buffer prior to DNA extraction (Jeyaprakash & Hoy 2000). The efficacy of the surface decontamination treatments was determined with both culturing and molecular methods. Four replicates of 3 adult *D. citri* were treated with each decontamination solution and 2 replicates each were used in the following culturing and molecular experiments, respectively.

In the culturing experiments, adult *D. citri* treated with each decontamination solution were gently rolled on nutrient agar (NA) plates in a "Z" pattern with a sterile pipette tip and held in a growth chamber at 25°C with a 16L:8D photoperiod to determine if fungal growth occurred. No fungal growth was observed on the NA plates following treatment of *D. citri* with bleach alone or bleach + Tween 20; all other treatments failed to consistently kill the external fungi. Treatment with Tween 20 alone did not inhibit fungal growth on NA plates, indicating that bleach alone was sufficient to kill the external fungi. Following bleach treatment, scanning electron microscopy (SEM) revealed a reduction of fungi on the ovipositor of adult females of *D. citri* compared to untreated females (Fig. 1A-B).

The external-inhabiting fungi of water-treated (control) *D. citri* were cultured on NA plates for 7 d and identified following high-fidelity PCR amplification (Barnes 1994; Jeyaprakash & Hoy

TABLE 1. TWO METHODS WERE USED TO EVALUATE IF SURFACE DECONTAMINATION SOLUTIONS KILLED EXTERNAL FUNGI AND ELIMINATED THEIR DNA FROM A LABORATORY COLONY OF ADULT *D. CITRI*. FOLLOWING TREATMENT WITH EACH DECONTAMINATION SOLUTION, ADULT *D. CITRI* WERE USED TO INOCULATE NUTRIENT AGAR (NA) PLATES THAT WERE MONITORED FOR FUNGAL GROWTH. A HIGH-FIDELITY PCR PROTOCOL WAS USED TO AMPLIFY THE FUNGAL 28S RRNA GENE (LARGE RIBOSOMAL SUBUNIT: LSU) WITH TEMPLATE DNA EXTRACTED FROM TREATED ADULT *D. CITRI*.

	Fungi[1]	
Decontamination Solution	Growth on NA	PCR: LSU
Acetone (>99%)	+	+
Acetone + Tween 20 (0.1%)	+	+
Bleach (6% sodium hypochlorite)	—	—
Bleach + Tween 20	—	—
DNA Away (Molecular BioProducts, San Diego, CA)	+	+
DNA Away + Tween 20	+	+
DNA lysis buffer (Gentra Systems, Minneapolis, MN)	+	+
DNA lysis buffer + Tween 20	+	+
Ethanol (100%)	+	+
Ethanol + Tween 20	+	+
Tween 20	+	+
Water (control)	+	+

[1]Fungi: Two different fungi were identified on the surface of adult *D. citri* based on their 28S rRNA gene sequences (*Penicillium* sp. and *Cladosporium* sp.).

2000) of the fungal 28S rRNA gene (large ribosomal subunit: LSU) with primers LS1 and LR5 (Hausner at al. 1993; Rehner & Samuels 1995). PCR products were cloned, analyzed with a restriction fragment length polymorphism (RFLP) technique, and sequenced according to Jeyaprakash et al. (2003). After *Rsa*I digest, 2 unique banding patterns were detected among the 10 clones selected, and clones representing each unique RFLP were sequenced. The first sequence was 856 bp (Genbank Accession EU500238) and produced significant BLAST alignments to the LSU gene of multiple Ascomycete species in the genus *Penicillium***.** Fungi in this genus are associated with citrus and can cause citrus green mold, a damaging post-harvest disease (Smilanick et al. 2006). The second sequence was 857 bp (Genbank accession EU500239) and was 100% identical (857/857 bp) to a homologous region of the LSU gene of the Ascomycete *Cladosporium cladosporioides* (GenBank accession DQ008145), the causal organism of *Cercospora* leaf spot in olives. Previously, a *Cladosporium* species was reported as a pathogen of *D. citri* during periods of high relative humidity (Aubert 1987). These fungi are likely only external contaminants and are not fungal endosymbionts of *D. citri*.

In the molecular experiments, DNA was extracted from treated insects and used in the PCR to determine if surface decontamination eliminated the external fungal DNA. No PCR amplification products were obtained for the LSU with DNA isolated from *D. citri* treated with bleach or bleach + Tween 20, indicating that bleach treatment eliminated or damaged the fungal DNA sufficiently so it was not amplified. This also showed that there are no obligate fungal endosymbionts associated with a laboratory colony of *D. citri* or, if so, the titer of the fungal DNA was below the sensitivity of the high-fidelity PCR protocol used here. PCR products were obtained for the fungal LSU using template DNA isolated from *D. citri* treated with all of the other decontamination solutions tested, demonstrating that these treatments were insufficient to eliminate the DNA from these external fungi.

DNA was isolated from adults and soft-bodied nymphs of *D. citri*, which each host a suite of previously described eubacterial endosymbionts (Subandiyah et al. 2000; Meyer et al. 2007), to determine if external exposure to bleach interfered with PCR amplification of host genomic or endosymbiont DNA. Bleach treatment was conducted on adults of the thelytokous *D. citri* parasitoid *Diaphorencyrtus aligarhensis* (Shafee, Alam and Agarwal) (Hymenoptera: Encyrtidae), which hosts the endosymbiotic bacterium *Wolbachia* (Jeyaprakash & Hoy 2000; Meyer & Hoy 2007), and tested similarly. For the host genomic DNA analyses, 2 replicates of 3 of these insects each were treated with bleach or water (control) and used for DNA extraction and PCR detection of the single-copy nuclear *actin* gene (Hoy et al. 2000). For the bacterial endosymbiont DNA analyses, the 16S rRNA gene of the primary endosymbiont (Subandiyah et al. 2000) and the *Wolbachia wspA* gene (Braig et al. 1998) were amplified from

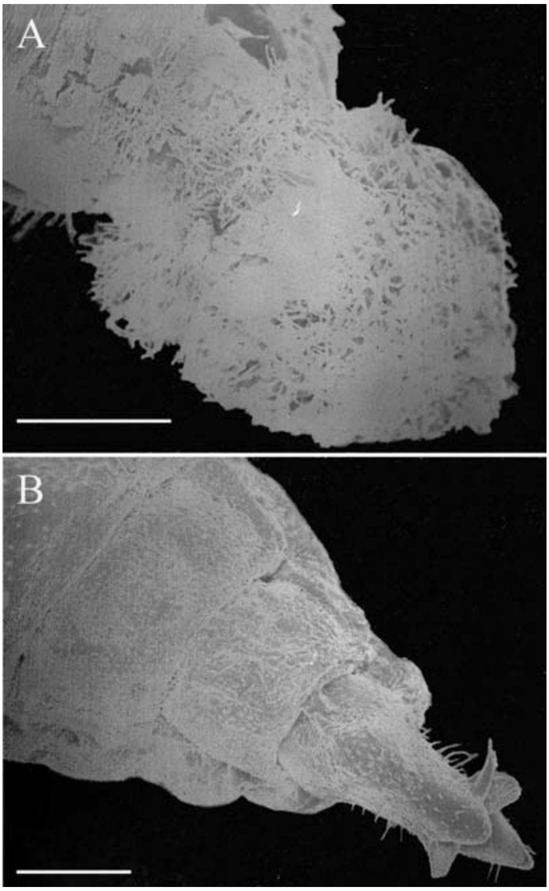

Fig. 1. SEM of the ovipositor of an adult female *D. citri* from the laboratory colony. (A) untreated female, showing abundant fungal growth; (B) bleach-treated female, showing removal of most of the fungal growth. Scale: (A) 0.1 µm; (B) 0.2 µm.

adults and nymphs of *D. citri* and adult *D. aligarhensis*, respectively. In summary, for each replicate, bleach treatment did not interfere with PCR amplification of either host genomic or endosymbiont DNA from adults or nymphs of *D. citri* or adult *D. aligarhensis*. Both the host genomic and endosymbiont DNA was PCR amplified in the control experiments (water treatment) for these insects, as expected.

Together, these experiments demonstrated that the surface decontamination protocol with bleach treatment reduced the amount of fungal cells from the external surfaces of *D. citri* and reduced the titer and/or quality of external fungal DNA so that it was not amplified in a high-fidelity PCR, despite the fact that this PCR protocol typically can detect as few as 100 copies of target DNA 100% of the time (Jeyaprakash & Hoy 2000). Following bleach treatment, no fungal endosymbionts were identified in the molecular survey of our laboratory colony of *D. citri*. Also, bleach treatment did not prohibit PCR amplification of host genomic or eubacterial endosymbiont DNA from *D. citri* or its parasitoid. This was consistent with the findings of Linville and Wells (2002), where bleach treatment of maggots reduced the amount of external contaminating DNA and did not interfere with subsequent mitochondrial DNA analysis of food consumed by the insect. Surface decontamination with bleach may be useful to reduce false positives caused by contaminating external-inhabiting microbes in future molecular surveys of endosymbionts in arthropods or other organisms. Further research is needed to determine if bleach treatment is effective in removing DNA from other microbial taxa that inhabit the external surfaces of arthropods, such as Eubacteria.

Summary

Bleach treatment reduced the quantity of intact DNA from external fungi sufficiently so that it was not amplifiable with a high-fidelity PCR protocol, while maintaining the integrity of insect genomic and eubacterial endosymbiont DNA for downstream PCR applications.

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