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COMPARISON OF SHORT-TERM PRESERVATION AND ASSAY METHODS FOR THE MOLECULAR DETECTION OF *WOLBACHIA* IN THE MEDITERRANEAN FLOUR MOTH *EPHESTIA KUEHNIELLA*

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Methods proposed for the preservation of insect tissue for DNA analysis have included various concentrations of ethanol, Carnoy's solution, liquid nitrogen, and acetone (Post 1993; Dessauer 1996; Fukatsu 1999; Mtambo 2006). However, little attention has been paid to appropriate storage methods for future detection of endosymbiont DNA within an insect host (Fukatsu 1999). Some studies report successful amplification of bacterial DNA in a host after thousands of years (Salo et al. 1994; Fricker et al. 1997; Willerslev et al. 2004), but others have reported inconsistent amplification of bacterial DNA due to low titers of the bacteria in the host, difficulties with the DNA extraction process, PCR-inhibiting substances present in the insect gut, or storage method (Fukatsu 1999; Barnes et al. 2000; Bextine et al. 2004; Hoy & Jeyaprakash, unpublished data). Fukatsu (1999) suggested acetone storage was superior to ethanol as a preservation method for both the amplification of insect host DNA and the DNA of their endosymbionts.

Historically, standard PCR has been used to detect *Wolbachia* and other endosymbionts of arthropods; however, it has been demonstrated that amplification of *Wolbachia* DNA can be improved with High-Fidelity PCR (Jeyaprakash & Hoy 2000). Thus, both the specimen preservation technique and choice of assay method could be important in determining success when attempting to amplify endosymbiotic DNA in an insect host. The goal of this study was to compare molecular methods for the detection of *Wolbachia* in the Mediterranean flour moth *Ephestia kuehniella* (Keller) (Lepidoptera: Pyralidae), and potentially other endosymbiotic bacteria in their insect host, in preserved specimens over time. Standard, High-Fidelity (HF), and Real-Time Quantitative (RTQ) PCR methods were used to detect and quantify *Wolbachia* DNA from *E. kuehniella* specimens stored under 4 treatment conditions (2 in 95% ethyl alcohol (EtOH) and 2 in acetone) over a 2-yr storage period. Spectrophotometry readings were taken at each assay (n = 9 over a 2-yr period) to ensure consistency of concentration and quality of template DNA for each treatment. Stored samples were compared to fresh specimens at the end of the experiment.

A wild-type strain of *E. kuehniella* was reared on '*Plodia*' diet (Silhacek & Miller 1972) in a 16L: 8D photoperiod at 26°C and 70% RH at the USDA Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, FL. Live *E. kuehniella* (120 specimens) were anesthetized for a period of 5 min in a freezer at -20°C. Once the specimens were immobile, they were placed in 20 mL screw-capped vials (3 per vial). Half of the vials were filled with 95% EtOH, and the remaining vials were filled with 100% acetone (Fisher: Atlanta, GA), with a specimen to preservative ratio of 1:20 (v:v). Half of the vials filled with EtOH were placed in the freezer at -80°C and the remaining vials were placed in a rack within a plastic bag to prevent evaporation, and stored at room temperature (25-27°C). This procedure was repeated for the vials filled with acetone. After weeks 13, 22, 27, 32, 39, 51, 76, 92, and 101, respectively, 6 specimens from each treatment category were removed and placed into clean, disposable Petri dishes and the abdomens were separated from the thoraces with sterile razor blades. Each abdomen was placed on a Kimwipe (Fisher: Atlanta, GA) for about 30 s, and then transferred into a labeled 1.5-mL centrifuge tubes. Abdomens were macerated for 3 min with a grinding pestle made from a 1-mL pipette tip in a labeled 1.5-mL centrifuge tube. Genomic DNA was extracted from abdomens with a PureGene kit (Gentra Systems: Minneapolis, MN) according to the manufacturer's protocol. The extracted DNA was re-suspended in 250 mL of sterile double deionized water. Extractions were stored at -20°C overnight. The quality of the genomic DNA for every sample in each of the 4 treatments was assessed with a spectrophotometer and a dilution factor of 40 (2 µL of extracted DNA per 78 µL water). Total DNA concentration and absorbance (260:280 ratio) readings recorded for each set of extractions are listed in Table 1. At the start of the experiment, 20 live *E. kuehniella* specimens (10 males and 10 females) were tested for the presence of *Wolbachia* by HF PCR, and all specimens tested positive (100% infection). The HF PCR products were purified on a QIAquick PCR purification column (QIAGEN, Inc., Valencia, CA) and were cloned into the plasmid pCR2.1-TOPO according to the manufacturer's protocol (Invitrogen Corp., Carlsbad, CA). DNA sequencing was performed at the University of Florida ICBR Core Facility with a PERKIN-ELMER Applied Biosystems ABI PRISM Automated DNA sequencer. MacDNASIS software was used to evaluate the sequences (Hitachi Software Engineering America Ltd., San

TABLE 1. SUMMARY OF RTQ PCR AND SPECTROPHOTOMETRY DATA FOR *E. KUEHNIELLA* SPECIMENS STORED FOR A 2-YR PERIOD COMPARED TO FRESH SPECIMENS.

Storage method	*wsp*A copy number in abdomen (Mean ± SD)	Total DNA concentration (µg/mL) (Mean ± SD)[a]	260:280 (Mean ± SD)[a]
Acetone room temperature	$4.422 \times 10^5 \pm 1.276 \times 10^4$	17 ± 4.0	1.2 ± 1.0
Acetone -80°C	$4.361 \times 10^5 \pm 3.973 \times 10^4$	20 ± 1.0	1.2 ± 0.7
95% EtOH room temperature	$3.441 \times 10^5 \pm 2.295 \times 10^5$	18 ± 5.5	1.2 ± 0.4
95% EtOH -80°C	$4.245 \times 10^5 \pm 6.913 \times 10^4$	23 ± 7.3	0.8 ± 0.2
Fresh specimens[b]	$4.202 \times 10^5 \pm 2.218 \times 10^4$	25 ± 9.0	1.7 ± 0.0
Acetone combined[c]	$4.391 \times 10^5 \pm 2.751 \times 10^4$	19 ± 3.2	1.2 ± 0.8
95% EtOH combined	$3.843 \times 10^5 \pm 1.627 \times 10^5$	21 ± 6.5	1.0 ± 0.4
Room temperature combined	$3.931 \times 10^5 \pm 1.592 \times 10^5$	23 ± 8.3	1.5 ± 0.2
-80°C combined	$4.303 \times 10^5 \pm 5.256 \times 10^4$	22 ± 5.1	1.0 ± 0.5

[a]Total DNA concentration and 260:280 values are averages of individuals sampled (n = 6) and assayed over the 2-year period (n = 9 data points).

[b]Fresh specimens (n = 5) were analyzed at week 101.

[c]Combined values indicate averages of treatments combined (e.g., stored in acetone at room temperature + stored in acetone in -80°C freezer).

Bruno, CA). *Wolbachia* sequences were identical to *Wolbachia* sp. group A (GenBank Accession #AB024570.1) from *E. kuehniella* by BLAST. For each specimen in all 4 treatments, we amplified 605 bp of the *wsp*A gene using wsp-F, 5'-TGGTCCAATAAGTGATGAAGAAACTAGCTA-3' and wsp-R, 5'-AAAAATTAAACGCTACTCCAGCTTCTGCAC-3' primers. All PCRs were performed in a total reaction volume of 50 mL containing 50 mM Tris (pH 9.2), 16 mM ammonium sulfate, 1.75 mM MgCl2, 350 mM each of dATP, dGTP, dTTP, dCTP, 400 picomoles of primers (wsp-F and wsp-R), 1 unit of *Pwo* and 5 units of *Taq* DNA polymerases (Jeyaprakash & Hoy 2000). Negative controls were included in all PCRs to test for contamination. The HF PCR cycling profile was 1 denaturing cycle at 95°C for 2 min, 10 cycles each of denaturation at 94°C for 10 s, annealing at 65°C for 30 s, and extension at 68°C for 1 min and 25 cycles each of denaturation at 94°C for 10 s, annealing at 65°C for 30 s and extension at 68°C for 1 min, plus an additional 20 s added for every consecutive cycle, using a PERKIN-ELMER DNA Cycler 480. A standard PCR analysis also was conducted at each sample date and results were compared to those of the HF PCR analyses (Jeyaprakash & Hoy 2000). In order to estimate *Wolbachia* density in *E. kuehniella*, the *wsp*A gene copy number was measured from abdomens of 4 individuals from each treatment after a 2-yr storage period, and compared to the copy number in abdomens from 4 fresh specimens by RTQ PCR using a MyiQ Single-Color Real-time PCR Detection System (Bio-Rad: Hercules, CA). Primers and probes for RTQ PCR analysis of the *Wolbachia* target DNA (surface protein gene *wsp*A) were designed with the Primer3 Output v. 0.4.0 software (Rozen & Skaletsky 2000). The forward primer WSP-RTF (5'-CTATCACTCCATATGTTGGTGTTGGTGTTG-3') corresponded to the region from base 327-356 of the *wsp*A sequence and the reverse primer WSP-RTR (5'-CTCCTTTGTCTTTCTCACCAACGCTTTTAT-3') to the region from 519-548 of the same sequence. The length of the amplification product was 222 bp. The Taqman real-time PCR protocol was performed in a final volume of 25 µL and contained: 1 µL of template DNA, 10 µL of Power SYBR Green PCR Master Mix (Applied Biosystems: Foster City, CA), 7.24 µL double deionized water, 0.16 µL Accuzyme DNA polymerase (Bioline: Randolph, MA), and 0.8 µL of each primer, and was carried out in 96-well plates (Applied Biosystems: Foster City, CA) that had been sealed with film, and centrifuged for approximately 30 s (100 rpm) prior to RTQ PCR. The RTQ PCR cycling profile consisted of 10 min at 95°C followed by 50 cycles of 10 s at 95°C, 30 s at 60°C, 30 s at 68°C, followed by a melt cycle for which conditions were 15 s at 95°C, 1 min at 65°C, and 15 s at 95°C. A standard curve was calculated by using a standard plasmid sample that contained the *wsp*A gene at concentrations of [10^2 to 10^9] copies/ mL. The number of molecules in the samples was determined from the threshold cycles (C_T) in the PCR based on the standard curve. Negative controls were included in all PCRs to test for contamination.

Wolbachia (*wsp*A) was detected in all *E. kuehniella* individuals (n = 6) in each treatment (n = 4) over the 2-yr period (9 total sample times) with both standard and HF PCR (data not shown). The average *wsp*A copy number (Table 1) found in *E. kuehniella* abdomens (n = 20) for each of the 4 treatments and from 4 abdomens from fresh specimens with RTQ PCR was lower than previously reported in the testis of different strains of *E. kuehniella* (*groEL* copy number in testis: $5.2\text{-}7.5 \times 10^6$ ± 2.4; Ikeda et al. 2003). This is not surprising given the potential variation in abundance of *Wol-*

bachia within an insect host depending on strain, sex, life cycle, and tissues or organs sampled (Ijichi et al. 2002). The *wsp*A copy number in each of the 20 individuals sampled was not significantly different among preservation treatments based on C_T values from the RTQ PCR (ANOVA, F = 0.53, df = 4, P = 0.72).

The HF PCR amplicon band intensity for the material stored in 95% EtOH at room temperature for 2 yr appeared lower for 3 of the 6 specimens sampled (compare Fig. 1A and 1B). When compared to HF PCR, a reduction in band intensity for standard PCR after the 2-yr storage period for all treatments also was observed (data not shown).

The average DNA concentration/sample taken at the last sample date (week 101) ranged from 17 to 23 µg/mL and the 260:280 ratio of the stored samples ranged from 0.8 to 1.2; fresh specimens had a DNA concentration of 25 and a 260:280 ratio of 1.7 based on the same extraction protocol (Table 1). Average total DNA concentrations and 260:280 ratios taken at the last sample date (week 101) were not significantly different between treatments (ANOVA, F = 1.4, df = 4, P = 0.28 and ANOVA, F = 1.7, df = 4, P= 0.18, respectively).

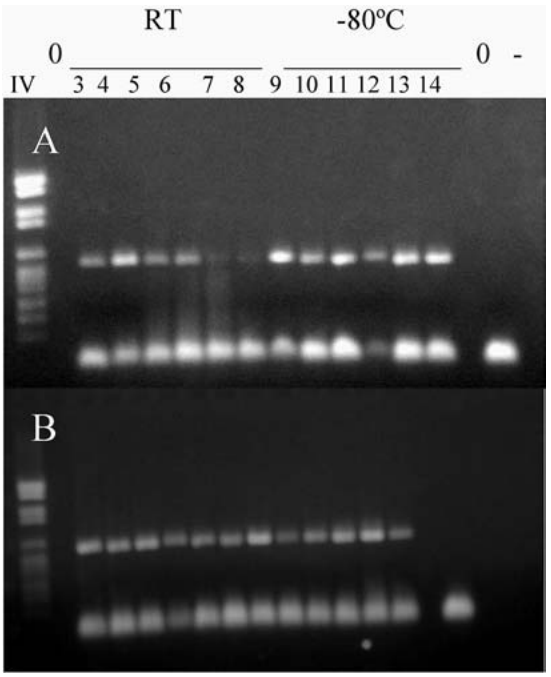

Fig. 1. Examination of DNA preservation and amplification by HF PCR of the *wsp*A gene fragment (605 bp) in *E. kuehniella*. (A) HF-PCR amplification of 12 individual *E. kuehniella* specimens following a 2-yr storage period in 95% EtOH at room temperature (lanes 3-8) and at -80°C (lanes 9-14). Blank lanes = 0, negative control lanes = -. (B) HF PCR amplification of 12 individual *E. kuehniella* specimens following a 2-yr storage period in acetone at room temperature (lanes 3-8) and at -80°C (lanes 9-14). IV = HyperLadder (Bioline MA), blank lanes = 0, negative control lanes = -.

Surprisingly, over the 2-yr storage period, a regression analysis showed an increase in total DNA concentration over the sample dates (data not shown) (slope = 0.340, y-intercept = 8.360, and R^2 = 0.05 for specimens stored in acetone at RT; slope = 1.365, y-intercept = 7.203, and R^2 = 0.5 for acetone at -80°C; slope = 1.469, y-intercept = 5.750, and R^2 = 0.3 for 95% EtOH at RT; slope = 1.132, y-intercept = 8.226, and R^2 = 0.3 for 95% EtOH at -80°C), perhaps due to dehydration of the specimens over time allowing for easier maceration of specimens. A decrease in the quality of the total DNA was observed (data not shown) for all 4 treatments (slope = -0.005, y-intercept = 1.28, and R^2 = 0.005 for specimens stored in acetone at RT; slope = -0.071, y-intercept = 1.62, and R^2 = 0.5 for acetone at -80°C; slope = -0.021, y-intercept = 1.55, and R^2 = 0.1 for 95% EtOH at RT; slope = -0.070, y-intercept = 1.55, and R^2 = 0.3 for 95% EtOH at -80°C) suggesting the quality of the DNA was compromised over time due to storage in each of the 4 treatments.

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Summary

Our results are consistent with previous studies (Fukatsu 1999) suggesting acetone and 95% EtOH are adequate short-term specimen preservation methods for detecting endosymbiotic bacterial DNA within an insect host. Although not statistically significant, an apparent reduction in *Wolbachia wsp*A copy number and HF PCR band intensity of the symbiont DNA isolated from specimens stored in 95% EtOH at room temperature was observed (Fig. 1A, Table 1). Averages in the total DNA concentration and purity of the stored specimens at week 101 were not significantly different among replicates from the same treatment nor were they significantly different between treatments. Regression analyses revealed an increase in total DNA concentration over time for each treatment, and a decrease in absorbance over time. Additionally, a reduction in band intensity was observed for specimens stored in 95% EtOH at room temperature at the last sample date (week 101), suggesting long-term storage in 95% EtOH at room temperature may not be an optimal storage method because it may damage the DNA or inhibit the PCR. The results reported here are specific to *E. kuehniella*, but

they indicate that when *Wolbachia* infection density in the host is high and the DNA extraction method is consistent, both standard and HF PCR are adequate for detecting *Wolbachia* after a 2-yr storage period in both acetone and 95% EtOH.

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