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WOLBACHIA-ASSOCIATED THELYTOKY IN *DIAPHORENCYRTUS ALIGARHENSIS* (HYMENOPTERA: ENCYRTIDAE), A PARASITOID OF THE ASIAN CITRUS PSYLLID

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Wolbachia is an obligate intracellular α-proteobacterium associated with arthropods and nematodes (O'Neill et al. 1997; Bazzochi et al. 2000). *Wolbachia* is transovarially transmitted by females to their progeny, and infections often are associated with reproductive anomalies in their host (O'Neill et al. 1997). In parasitoids, *Wolbachia* can cause cytoplasmic incompatibility (Stouthamer et al. 1999), thelytoky (parthenogenesis) (Stouthamer et al. 1990), and alter aspects of fecundity (Grenier et al. 2002).

In Florida, colonies of the thelytokous endoparasitoid *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae) (Shafee, Alam and Agarwal) and the arrhenotokous ectoparasitoid *Tamarixia radiata* Waterston (Hymenoptera: Eulophidae) were imported from Taiwan and Vietnam, respectively, and released in a classical biological control program against the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) (Hoy & Nguyen 1998, 2000; Hoy et al. 1999; Skelley & Hoy 2004). Worldwide, these parasitoids have a significant impact on reducing populations of *D. citri*, which is the most economically important citrus pest in regions where it vectors citrus greening disease (Chien 1995; Halbert & Manjunath 2004).

Jeyaprakash and Hoy (2000) detected *Wolbachia* in the imported population of *D. aligarhensis*. We hypothesized that *Wolbachia* causes thelytoky in *D. aligarhensis*, and this was tested by attempting to eliminate *Wolbachia* with antibiotics following the previous work of Stouthamer et al. (1990). This research is important because *D. aligarhensis* populations are low in Florida, and this could be due to its low reproductive rate (Skelley & Hoy 2004) which may be influenced by *Wolbachia*.

A laboratory colony of *D. aligarhensis* was maintained as follows. Ten small citrus trees (20-50 cm tall) grown in 15.2-cm diameter pots were pruned each week, fertilized with Peter's 20-20-20 (N-P-K) water-soluble fertilizer (United Industries, St. Louis, MO), and placed in wooden-framed mesh cages (0.76 m × 0.91 m × 1.11 m) in a greenhouse at 20-32°C with a 16L:8D photoperiod. Adult female psyllids oviposited on the new growth (flush) produced by the trees. Adult *D. aligarhensis* were aspirated and released into the cages when immature *D. citri* reached the first or second instar. After emergence, adult *D. aligarhensis* were fed pure clover honey smeared on small strips of Kimwipes (Kimberly-Clark, Roswell, GA) and used to initiate the next generation. During a 4-year rearing period, all *D. aligarhensis* observed in this colony were females (J. Meyer, personal observation).

A preliminary toxicity test indicated that 10 mg/mL tetracycline + honey did not negatively influence longevity in adult female *D. aligarhensis*, so this dosage was adopted for this experiment. For 3 consecutive generations, 50 newly-emerged female *D. aligarhensis* were administered pure clover honey + 10 mg/mL tetracycline hydrochloride (Sigma Chemical Co., St. Louis, MO) (Stouthamer et al. 1990) for 24 h at 70-75% RH, 24-25°C with a 16L:8D photoperiod. Treated parasitoids were released into a separate cage and maintained as described above. After the third generation, approximately 60 adult male *D. aligarhensis* were observed and collected.

Female and male *D. aligarhensis* were placed on a glass slide and submerged in 95% EtOH or Euparal mounting medium (BioQuip, Rancho Domingez, CA) for photography with the Auto-Montage Pro system with software ver. 5.02 (Synoptics, Frederick, MD). Morphological differences were observed between female and male *D. aligarhensis* (Fig. 1). The male abdomen was small and all black, but the female abdomen was larger and was yellowish and black (Fig. 1A, D). Both the geniculate antennae (Fig. 1B, E) and genitalia (Fig. 1C, F) of female and male *D. aligarhensis* were structurally distinguishable. The antennae of male *D. aligarhensis* in an arrhenotokous population from Asia (Shafee et al. 1975) were similar to those observed in male *D. aligarhensis* produced here.

Molecular analyses were used to determine if *Wolbachia* was eliminated from male *D. aligarhensis*. DNA was isolated from each of 3 individual female and male *D. aligarhensis* with PUREGENE reagents (Gentra Systems, Minneapolis, MN) according to the manufacturer's protocol. A 25-µL high-fidelity polymerase chain reaction (PCR) was conducted according to Hoy et al. (2001) to detect the *wsp* gene of *Wolbachia* with the primers *wsp* 81F (5'-TGGTCCAATAAGTGATGAAGAAAC-3') and *wsp* 691R (5'-AAAAATTAAACGCTACTCCA-3') (Braig et al. 1998). For a DNA template control, the mitochondrial *cytochrome c oxidase I* gene (COI) was amplified with the primers CI-J1632

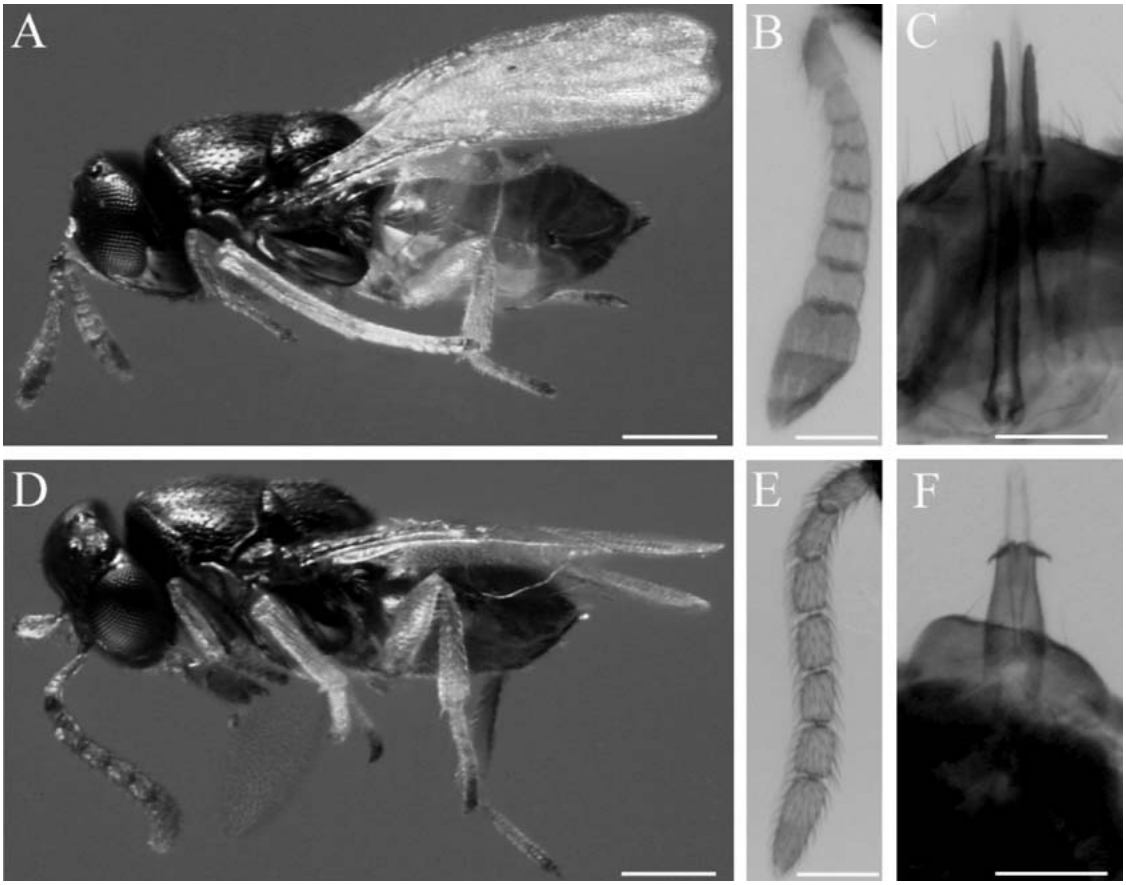

Fig. 1. Adult female compared to a male *D. aligarhensis* produced by tetracycline treatment. (A) Adult female; (B) Antenna: adult female; (C) Terminal abdominal segment: adult female (ventral view); (D) Adult male; (E) Antenna: adult male; (F) Terminal abdominal segment: adult male (dorsal view). Scale: (A, D) 0.25 mm; (B, C, E, F) 0.1 mm.

(5'-TGATCAAATTTATAAT-3') and CI-N-2191 (5'-GGTAAAATTAAAATATAAACTTC-3') (Kambhampati & Smith 1995). PCR amplification products were analyzed, purified, cloned, and sequenced according to Hoy & Jeyaprakash (2005).

The *wsp* gene was PCR-amplified from all female *D. aligarhensis* (n = 3) but not from any males (n = 3) (Fig. 2). The mitochondrial COI gene was amplified from all samples indicating that the DNA was adequate for the PCR (Fig. 2). No amplification products were detected in the negative control for both the *wsp* and COI genes.

PCR products from the COI gene of both female and male *D. aligarhensis* were cloned and sequenced, and the resulting 552-bp sequences were 100% identical (GenBank accession EF431956). This indicated that the males were the same species as the female *D. aligarhensis* treated with tetracycline and that another parasitoid had not unexpectedly invaded the laboratory colony.

The morphological and molecular data support our hypothesis that *Wolbachia* causes thelytoky in our laboratory colony of *D. aligarhensis*, the first report of this phenomenon in this genus. Male production also has been documented following elimination of *Wolbachia* from other thelytokous parasitoids in the families Encyrtidae (Pijls et al. 1996), Trichogrammitidae (Stouthamer et al. 1990), Scelionidae (Arakaki et al. 2000), Eulophidae (Argov et al. 2000), and Aphelinidae (De Barro & Hart 2001). It is possible that the titer of *Wolbachia* in male *D. aligarhensis* was below the sensitivity of the high-fidelity PCR assay, which detects as few as 100 copies of the target template 100% of the time and as few as 10 copies 50% of the time (Hoy et al. 2001), but, if so, this titer reduction could still result in male production. No bacterial symbionts other than *Wolbachia* were detected in a molecular survey of the imported population of *D. aligarhensis* (Meyer 2007). Although unlikely, it cannot be excluded that uni-

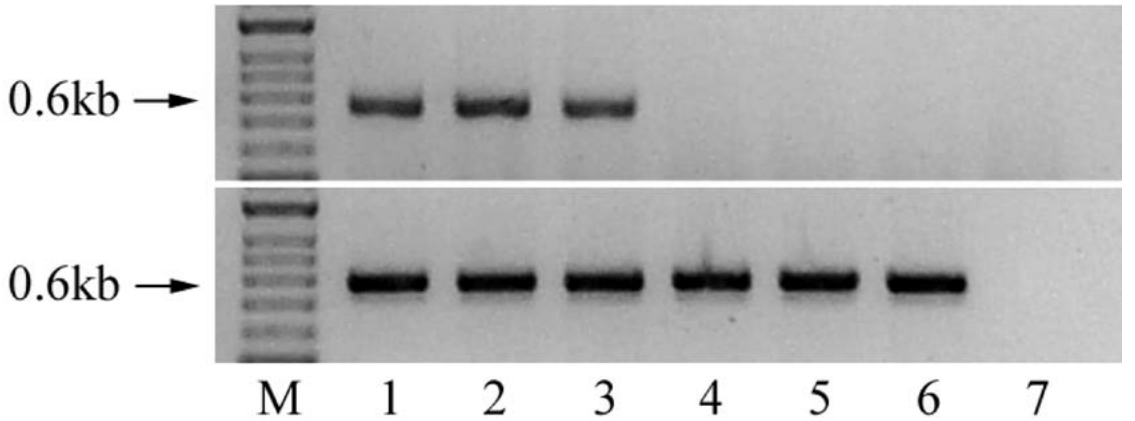

Fig. 2. PCR amplification of the *wsp* gene of *Wolbachia* and the mitochondrial *cytochrome c oxidase I* (COI) gene from DNA isolated from female and male *D. aligarhensis*. *Wolbachia* appears to be missing in males of *D. aligarhensis* produced by tetracycline treatment of their mothers, indicating that *Wolbachia* is associated with thelytoky in this population. (Top) *wsp* gene of *Wolbachia*; (Bottom) mitochondrial COI gene. Lane: (M) DNA size Marker (Hyperladder II: Bioline Randolph, MA); (1-3) individual females of *D. aligarhensis*; (4-6) individual males of *D. aligarhensis*; (7) no-DNA control.

dentified microbial species in *D. aligarhensis* also influence thelytokous reproduction.

Male and female *D. aligarhensis* exhibited mating behavior when they were held together in a 50-mL centrifuge tube (Meyer 2007). First the male faced the female, then moved behind and climbed on the female, and finally attempted to copulate by bending its abdomen to contact the female abdomen. Males attempted to mate with multiple females. Further studies are needed to determine if males produce viable sperm that are transferred to the female during mating, and if a *Wolbachia*-free bisexual line of *D. aligarhensis* can be produced.

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Summary

Wolbachia is associated with thelytokous reproduction in *D. aligarhensis*. Male *D. aligarhensis* were produced following antibiotic treatment of females in a thelytokous colony. The males lacked *Wolbachia*, were morphologically distinguishable from females, and exhibited mating behavior.

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