Wolbachia Infection in the Coffee Berry Borer (Coleoptera: Scolytidae)

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ABSTRACT A nested polymerase chain reaction protocol yielded positive detection of the maternally inherited cytoplasmic proteobacterium *Wolbachia* in total genomic DNA from coffee berry borers collected in Benin, Brazil, Colombia, Ecuador, El Salvador, Honduras, India, Kenya, Mexico, Nicaragua, and Uganda. *Wolbachia* was not detected in specimens from Cameroon, the Dominican Republic, Indonesia, Jamaica, and Peru. Amplified bands from India and Brazil were cloned and sequenced. The 438-bp sequence clearly conformed to *Wolbachia* group B and was nearly identical to that of *Ephestia kuehniella.* The possible implications of *Wolbachia* infection in the coffee berry borer are discussed.

KEY WORDS *Wolbachia*, *Hypothenemus*, coffee berry borer, endosymbionts

THE COFFEE BERRY BORER, *Hypothenemus hampei* (Ferrari), is the most important insect pest of coffee throughout the world. Endemic to Central Africa, it has now spread to most coffee growing regions (Baker 1999). Female adults bore a hole in the coffee berry and deposit their eggs; larvae feed on the endosperm, lowering its quality and possibly causing abscission of the berry (Le Pelley 1968). The entire life cycle occurs inside the berry, except when the newly molted adult females emerge in search of another berry to colonize. This cryptic life cycle makes control of this insect extremely difficult.

As is common in some Scolytidae tribes (e.g., Corthylini, Cryphalini, Dryocoetini, Hyorrhynchini, and Xyleborini; Kirkendall 1993), *H. hampei* (Cryphalini) exhibits a female-biased sex ratio, incestuous intraberry inbreeding (sib-mating), and dwarfed males (Bergamin 1943, Baker 1999). The female-biased sex ratio in Scolytidae is usually ascribed to haplodiploidy, whereby unfertilized eggs result in males, although this has been shown only for the Xyleborini and some members of the Dryocoetini (Entwistle 1964, Kirkendall 1993, Normark et al. 1999). Brun et al. (1995) have reported that male coffee berry borers appear to develop from fertilized eggs and have the same number of chromosomes as females, although only the maternal set of chromosomes is expressed. Another mechanism for skewed sex ratios favoring females has been ascribed to the effects of the maternally inherited intracellular proteobacterium *Wolbachia.* The mechanism, known as feminization or sex conversion—whereby genetic males become functional females—was originally reported in isopods (Rousset et al. 1992) and has recently been reported in *Ostrinia furnacalis* (Guenée) (Kageyama et al. 1998). *Wolbachia* also causes unidirectional or bidirectional cytoplasmic incompatibility, whereby fertility will depend on the *Wolbachia* strain present in the insect and on whether the male or female (or both) are infected (O'Neill et al. 1997; Bourtzis and O'Neill 1998). *Wolbachia* has also been shown to induce parthenogenesis in parasitic Hymenoptera (Stouthamer et al. 1990, Zchori-Fein et al. 1992); conflicting reports on parthenogenetic development have been reported for the coffee berry borer (Bergamin 1943, Browne 1961, Entwistle 1964, Muñoz 1989, Barrera et al. 1995).

Lastly, *Wolbachia* has been implicated in male killing, both during early and late developmental stages, in various insects (Hurst et al. 1997, Fialho and Stevens 2000, Hurst et al. 2000, Jiggins et al. 2000).

Wolbachia has been detected using the polymerase chain reaction (PCR) in 15–20% of insect species sampled (Werren et al. 1995, O'Neill et al. 1997, Werren 1997, Zhou et al. 1998, Werren and Windsor 2000), although a method known as long-PCR resulted in *Wolbachia* detection in 76% of species sampled (Jeyaprakash and Hoy 2000). *Wolbachia* has been detected in several Coleopterans (Wade and Chang 1995, Giordano et al. 1997, Fialho and Stevens 2000, Jeyaprakash and Hoy 2000, Malloch et al. 2000), but only one report of detection in Scolytidae (*Ips typographus* L.) has been published (Stauffer et al. 1997). We were inter-

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ested in using molecular techniques to document the presence of *Wolbachia* in the coffee berry borer to determine whether *Wolbachia* might be involved in the skewed-sex ratio exhibited by the insect, which would expand knowledge on the basic biology of the insect.

Materials and Methods

Insects. Coffee berry borers were obtained from infected coffee berries collected in the field in Benin (Niaouli), Brazil (Piracicaba), Cameroon (Atok), Colombia (Santa Rosa de Cabal, Risaralda), Dominican Republic (La Cumbre, Santiago), Ecuador (Via Chone, Santo Domingo, Pichincha), El Salvador (Finca El Espino, Antiguo Cuscatlán, Libertad), Honduras (Yojoa Flora, Peña Blanca, Cortés), India (Elkhill Estate, Siddapur, Kodagu District, Karnataka), Indonesia (Sumber-Asin, Malang), Jamaica (Saint Andrews Parish), Kenya (Coffee Research Foundation in Ruiru, Thika District), Mexico (Finca La Alianza, Chiapas), Nicaragua (km 14, Matagalpa), and Uganda (Muwoko, Mubende District). The extensive coffee berry borer collection from Peru allowed us to analyze males and females separately, but due to the female-biased sex ratio, most of the insects from all other countries were females, and no attempt was made to separate any males, if present. Insects were placed in absolute ethanol while still alive and stored at room temperature until DNA was extracted.

PCR Assay. Total genomic DNA was extracted from insects using the DNeasy Tissue Kit (Qiagen, Valencia, CA). Total genomic DNA from *Drosophila simulans* Sturtevant (Riverside) naturally infected with *Wolbachia* was used as a positive control. Negative controls consisted of samples lacking DNA template. All experiments were repeated at least twice.

A nested PCR protocol was used, with primers designed to amplify the *Wolbachia wsp* gene fragment (Braig et al. 1998, Zhou et al. 1998): 81F (5′-TGGTCCAATAAGTGATGAAGAAAC), 691R (5′-AAAAATTAAACGCTACTCCA), and 522R (5′-ACCAGCTTTTGCTTGATA). PCRs were done in 20 μl reactions volumes consisting of 1 μl DNA template, 17 μl Platinum PCR Supermix (Gibco BRL, Gaithersburg, MD), and 1.0 μl each of primers 81F and 691R (20 pmol/μl). The thermal profile used in the GeneAmp PCR System 9700 thermocycler (PE Applied Biosystems, Norwalk, CT) was as follows: 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR product was diluted 1:100 and used for the second PCR with primers 81F and 522R in 20 μl reaction volumes as described above. Bands were visualized under UV illumination in a 1% agarose gel stained with ethidium bromide. A 50-bp DNA ladder (Gibco BRL, Gaithersburg, MD, catalog No. 10416–014) was used to determine the size of the amplified DNA band. Before running nested PCR, primers 12SA1 and 12SB1 (specific for insect mitochondrial 12S rRNA) were used to assess the quality of the DNA (O'Neill et al. 1992; results not shown).

Cloning and Sequencing. DNA bands (≈450 bp) from PCR products, corresponding to coffee berry borer specimens from India and Brazil were used for cloning and sequencing. The QIAEX-II Gel Extraction Kit (Qiagen) was used to isolate the DNA fragments from the 1% agarose gel. The TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA) was used for cloning and transformation of the DNA fragments. The solutions containing the DNA fragments and the vector were incubated overnight in prewarmed LB plates with 50 μg/ml of kanamycin. Hundreds of colonies were obtained after this procedure. Four colonies from each sample (four Brazilian and four Indian) were selected and cultured overnight in LB medium containing 50 μg/ml of kanamycin. To isolate plasmid DNA, the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI) was used. The DNA fragments were sequenced using an ABI-3700 Automatic Sequencer (PE Applied Biosystems). BLASTX and BLASTN sequence analyses (Altschul et al. 1997) were performed to compare the CBB sequence with other sequences in the GenBank database.

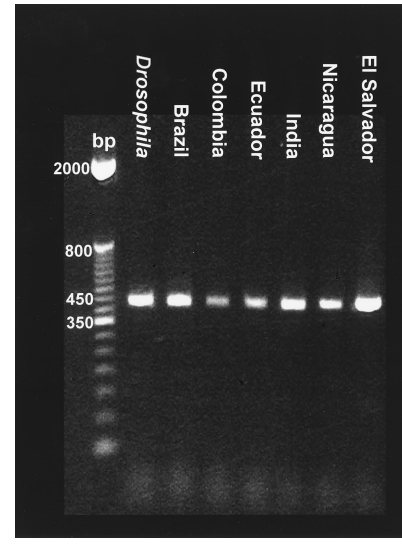

Fig. 1. *Wolbachia* detection in total genomic coffee berry borer DNA extracted from specimens collected in Brazil (lane 3), Colombia (lane 4), Ecuador (lane 5), India (lane 5), Nicaragua (lane 7), and El Salvador (lane 8). Lane 1 is the DNA ladder (50 bp; Gibco BRL), and lane 2 is a positive *Wolbachia* control from *Drosophila.*

Results and Discussion

An ≈450-bp band was obtained from the nested PCR using total genomic DNA from coffee berry borers originating in Benin, Brazil, Colombia, Ecuador, El Salvador, Honduras, India, Mexico, Nicaragua, and Uganda (Fig. 1). The positive *Drosophila* control, yielded a band of similar size. No amplification was obtained from any of the negative controls (not shown). Cloning and sequencing of four clones from each sample (India and Brazil) revealed a 438-bp sequence (Fig. 2, GenBank Accession AF389084). There were sequence differences between clones, but these were interpreted to be PCR artifacts: one Brazilian clone had a G instead of an A at position 74, and a T instead of an A at position 72. One Indian clone had

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TGGTCCAATA AGTGATGAAG AAACTAGCTA CTATGTTCGT TTGCAATATA
 G  P  I   S  D  E  E   T  S  Y   Y  V  R   L  Q  Y  N

ATGGTGAAGT TTTACCTTTT AAAACAAAGA TTGATGGTGT TACATATAAA
  G  E  V   L  P  F   K  T  K  I   D  G  V   T  Y  K

TCAGGTAAGG ACAACAATAG TCCCTTAAAA CCATCTTTTC TAGCTGGAGG
S  G  K  D   N  N  S   P  L  K   P  S  F  L   A  G  G

TGGTGCATTT GGTTATAAAA TGGATGATAT CAGGGTTGAT GTTGAAGGAC
 G  A  F   G  Y  K  M   D  D  I   R  V  D   V  E  G  L

TTTACTCACA ATTGAGTAAA GATGCAGATG TAGTAGATAC TTCTCCAGCA
  Y  S  Q   L  S  K   D  A  D  V   V  D  T   S  P  A

GTTGTAGAAA GTTTAACAGC ATTTTCAGGA CTAGTTAATG TTTATTACGA
V  V  E  S   L  T  A   F  S  G   L  V  N  V   Y  Y  D

                          *
TATAGCAATT GAAGATATGC CTATCACCCC ATATGTTGGT GTTGGTGTTG
 I  A  I   E  D  M  P   I  T  P   Y  V  G   V  G  V  G

GTGCAGCGTA TGTAAGCAAT CCTTTAGTAA CAGAGATTAC TGGTGATAAA
  A  A  Y   V  S  N   P  L  V  T   E  I  T   G  D  K

AAATCTGGAT TTGGTTTTGC TTATCAAGCA AAAGCTGG
K  S  G  F   G  F  A   Y  Q  A   K  A
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Fig. 2. Consensus sequence (438 bp) for *Wolbachia* isolated from Indian and Brazilian coffee berry borers.

an A instead of a G at position 149. Interestingly, one Brazilian clone and two Indian clones had a T instead of a C at position 327 (marked by the asterisk in Fig. 2). A T instead of a C at that position would make this sequence identical to that of the *Wolbachia wspB* gene isolated from *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) (Sasaki 1999). This sequence clearly indicates that the coffee berry borer *Wolbachia* belongs to *Wolbachia* sp. group B (Sasaki 1999).

To further investigate the negative amplification in specimens from Cameroon, the Dominican Republic, Indonesia, Jamaica, Kenya, and Peru new primers were designed (pbf2: 5′-TAGCTACTATGTTCGTTTGC; pbr1: 5′–CTCTGTTACTAAAGGATT GC) based on the cloned fragment sequence from Brazil and India. PCR products from nested reactions using primers 81F/691R followed by 81F/522R were used with primers pbf2 and pbr1 to increase sensitivity and yielded positive amplification in all samples that were previously positive as well as in coffee berry borer DNA from Kenya whose amplification had previously been negative. PCR experiments conducted using DNA from samples collected from Cameroon, Dominican Republic, Jamaica, Indonesia, and Peru with primers 81F/691R followed by pbf2/pbr1 were still negative. The lack of amplification in these specimens could be due to the actual absence of *Wolbachia* in insects from sites sampled in these countries or to a different nucleotide sequence in the *Wolbachia* DNA which prevents annealing with the primers used. It is also possible that the quality of *Wolbachia* DNA from these samples might have been compromised, or its titer might have been low. As stated by Jeyaprakash and Hoy (2000), standard PCR techniques frequently produce false negatives, thus requiring more sensitive detection techniques. Our results provide evidence for the presence of *Wolbachia* in specimens from 11 countries and of infection with a B group *Wolbachia* in coffee berry borer specimens from India and Brazil.

As is the case with other insects, *Wolbachia* may be playing a significant role in the biology of the coffee berry borer. An interesting phenomenon, termed functional haplodiploidy, has been described for this insect (Brun et al. 1995) which has two related consequences for the paternally derived set of chromosomes in males. First, these chromosomes condense

during spermatogenesis and always fail to be incorporated in the sperm. Second, in males, these chromosomes condense and are never expressed in somatic tissues. Although additional investigations have also reported the elimination of paternal genes in the coffee berry borer (Borsa and Kjellberg 1995), there is no apparent karyotypic difference between male and female coffee berry borers. Thus, it seems reasonable that the cause of this phenomena, as well as the sex determination mechanism itself, may be cytoplasmatic, and may involve *Wolbachia.* This is, in fact, consistent with recent hypotheses on the mode of action for *Wolbachia*-induced cytoplasmic incompatibility. These suggest an effect on chromosomes by selectively removing or modifying host proteins involved in chromosomal condensation and de-condensation before and after zygote formation (Bourtzis and O'Neill 1998, Feder et al. 1999). Furthermore, the work by Peleg and Norris (1972a, 1972b) and Norris and Chu (1980) and Normark et al. (1999) suggests that a maternally inherited bacterium might be involved in sex determination in the scolytid *Xyleborus ferrugineus* (F.).

Past research on coffee berry borer genetics, coupled with the detection of *Wolbachia* in coffee berry borers from different countries and evidence of production of male-only progenies when antibiotics are used in rearing diets, suggest that *Wolbachia* might be playing a role in sex determination in the coffee berry borer. Additional studies confirming the elimination of the female-biased sex ratio in the progeny of coffee berry borer females treated with antibiotics, as well as detection of *Wolbachia* in inbreeding but not in outbreeding members of Scolytidae, would provide additional support to the hypothesis stating that *Wolbachia* is involved in sex determination.

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