

## Chapter 4

### Biochemical Separation of Plum Curculio Based on *Wolbachia* and *wsp* Gene Analysis

#### Introduction

Plum curculio, *Conotrachelus nenuphar* (Herbst), is an endemic pest of apples, apricots, blueberries, cherries, nectarines, peaches, plums, and quince in eastern North America. Two morphologically identical strains have been identified, a univoltine strain, occurring primarily in the northern U.S. and Canada, and a multivoltine strain, occurring primarily in the southern U.S. (Chapman 1938, Bobb 1952, Racette et al. 1992). The line dividing these populations, estimated by Chapman (1938), runs through western Virginia.

Stevenson and Smith (1961) report that crossing a univoltine (collected from New York State) female with a multivoltine (lab colony, originally collected in North Carolina) male resulted in decreased fecundity. Padula and Smith (1971) reported reproductive incompatibility when testing crosses of a multivoltine female and a univoltine male. Similar patterns of reproductive incompatibility have been described from several insect species infected with strains of *Wolbachia* (Werren 1998, Stouthamer et al. 1999, Bandi et al. 2001).

Originally described in 1924 by Hertig and Wolbach (Werren 1997) from the ovaries of *Culex pipiens* Linnaeus mosquitoes, this rickettsia-like (? -Proteobacteria) intracellular symbiont has been found in a large number of insects (up to 76% of 63 species studied by Jeyaprakash and Hoy 2000), mites, and nematodes. *Wolbachia* infections of reproductive tissue are common and have been associated with cytoplasmic incompatibility (leading to reduced numbers of viable offspring or a male-biased sex ratio), feminization of genotypic male offspring, female-biased sex ratios, parthenogenesis (Rousset et al. 1992, Bandi et al. 2001), thelytoky (Stouthamer et al. 1990, Vavre et al. 1999), and in some cases increased fecundity (Vavre et al. 1999).

Cytoplasmic incompatibility (CI) results when an infected male mates with an uninfected female or a female that is infected with an incompatible strain of *Wolbachia*. During embryonic meiosis the paternal genetic material is lost, and the resulting embryo is therefore haploid. In diploid species the embryo dies, reducing the number of viable offspring produced from a mating.

*Wolbachia* is currently classified by its *wsp* (*Wolbachia* surface protein) gene sequence (Zhou et al. 1998). The *wsp* gene mutates at a faster rate than the 16S rDNA and the *fts Z* (cell-cycle) genes previously used to classify *Wolbachia*. This allows for more definitive degree of strain separation within the clades. Zhou et al. (1998) used the sequences from the *wsp* gene to divide the genus *Wolbachia* into two supergroups (A and B), which correlate to the supergroups A and B defined by Werren et al. (1995) by the *fts Z* gene and to the supergroups I and II as defined by Stouthamer et al. (1993) by the 16S sequences. Within these clades, Zhou et al. (1998) created reference groups of *Wolbachia* based the similarity of the *wsp* gene sequence. All new *Wolbachia* isolates must be at least 97.5% similar in their *wsp* gene fragment sequence to belong to a reference group. The name of the reference group is generally derived from the first three letters of the first species from which that strain of *Wolbachia* was isolated. Novel strains of *Wolbachia* are named using “w” and the first three letters of the species from which it was isolated.

## **Methods and Materials**

### *Insects*

Univoltine plum curculio adults were obtained from Massachusetts (Dr. R. J. Prokopy, U. MA, Amherst), and multivoltine adults were received from Georgia (Dr. D. Horton, U. GA, Athens), and Florida (Dr. R. Mizell, U. FL, Monticello).

### *DNA extraction*

Weevils were placed in 300  $\mu$ l of TE (Tris-HCl, EDTA) buffer and frozen at  $-80^{\circ}\text{C}$  until processed. The insects were thawed and the TE buffer was removed before starting extraction. The DNA extraction protocol was modified from a protocol developed for *Drosophila melanogaster* by Ashburner (1989). Plum curculio adults were placed in individual 1.5 ml microcentrifuge tubes and ground with Kontés pestles in 300  $\mu$ l of homogenization buffer (10 mM Tris-HCl (pH 7.5), 60 mM NaCl, 10 mM EDTA with 5% sucrose) and homogenized. A volume of 300  $\mu$ l of lysis buffer (300 mM Tris-HCl, pH 7.5), 100 mM EDTA, 0.625% sucrose, 1% DEPC) was added and the samples were incubated for 15 min at  $70^{\circ}\text{C}$ . When cooled to room temperature, 90  $\mu$ l of

8 M potassium acetate was added and the samples were incubated for 30 min in ice. The samples were then centrifuged at 20800 g at 4°C for 10 min. The supernatant was removed to a fresh microcentrifuge tube without disturbing the surface lipid or pellet. The supernatant was extracted twice with an equal volume of 1:1 phenol/chloroform and spun at 20800 g at 4°C for 5 min. One extraction was done with chloroform only to remove residual phenol in the supernatant. DNA was precipitated by adding 2 volumes of absolute ethanol and incubated at room temperature for 5 min. The samples were centrifuged at 20800 g at room temperature for 5 min. The supernatant was discarded. The pellet was washed with 400  $\mu$ l of 70% ethanol and allowed to dry. The dried pellet was resuspended in 50  $\mu$ l of TE (Tris-HCl EDTA) buffer.

RNA was removed by adding 1.5  $\mu$ l of RNase Cocktail A (Ambion, Austin) to each sample and incubated for 1 hour at 37°C to degrade the RNA. The samples were column-purified (Microcon® Centrifugal Filter Devices, Millipore Corporation, Bedford) to remove RNA fragments. Aliquots of 10  $\mu$ l of each sample were electrophoresed on an 0.8% agarose gel containing ethidium bromide to visually assess the quality of the DNA. Samples were also tested using a GeneQuant DNA Calculator (Pharmacia Biotech, Piscataway) spectrophotometer to estimate the concentration of DNA calculated from absorbance at 260 nm. Quality of DNA was inferred from the appearance of a single, discrete, high molecular weight band following electrophoresis and from the ratio of absorbance at 260 nm (DNA and RNA) to the absorbance at 280 nm (protein). The ideal range of this ratio is 1.4- 1.8, and lowest ratio of quality accepted in these studies was 1.4. Samples with a ratio lower than 1.4 were not used in this study.

#### *Polymerase Chain Reaction (PCR)*

DNA fragments were amplified using targeted PCR techniques from the 16S rDNA using universal eubacterial primers (Table 4, Heddi et al. 1999) and primers designed to amplify the 16S rDNA region in *Wolbachia* and the *wsp* gene (Table 5, Hsiao 1996, Heddi et al. 1999, Zhou et al. 1998)

Table 4. Universal eubacteria primers

Primer <sup>1</sup>	Primer Sequence	GenBank Accession Number
BacF	5'-AGA GTT TGA TCA TGG CTC AG-3'	Nucleotides numbering 8-27, <i>Escherichia coli</i> (GenBank accession no J01859)
BacR	5'-TAC CTT GTT ACG ACT TCA CC-3'	Nucleotides 1487-1506

1. Heddi et al. 1999

#### *Universal Eubacterial*

PCR was carried out in a total volume of 50  $\mu$ l containing 300 nM BacF and BacR primers (Heddi et al. 1999; Table 4), 10 ng of plum curculio DNA, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTP mix (PE Applied Biosystems, Foster City), 5  $\mu$ l 10x PCR buffer (PE Applied Biosystems), 1.5 units of AmpliTaq<sup>TM</sup> Gold (PE Applied Biosystems) and distilled water to a volume of 50  $\mu$ l. Amplification was completed with the following thermal profile on a GeneAmp<sup>®</sup> PCR System 9700 (PE Applied Biosystems): 94°C for 10 min, then 35 cycles of 94°C 30 sec, 53°C 45 sec, 72°C 45 sec, final extension 72°C for 7 min and held at 4°C. A total of 10  $\mu$ l of PCR product was electrophoresed through 1.5% agarose containing ethidium bromide to verify the presence and determine the size of the amplicon.

*Wolbachia* 16S rDNA.

Two rounds of PCR, one round with primers 99F and 994R and the next round with primers Wolf and WolR, were used to amplify a fragment of *Wolbachia* 16S rDNA. PCR was carried out in a total volume of 50  $\mu$ l for both rounds of amplification. For the first round, I used 300 nM 99F and 994R primers (Hsiao 1996; Table 5), 10 ng of plum curculio DNA, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTP mix (PE Applied Biosystems), 5  $\mu$ l 10x PCR buffer (PE Applied Biosystems), 1.5 units of AmpliTaq<sup>TM</sup> Gold (PE Applied Biosystems) and distilled water to a volume of 50  $\mu$ l. Amplification was completed with the following cycling profile on a GeneAmp<sup>®</sup> PCR System 9700 (PE Applied Biosystems): 94°C for 10 min, then 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 45 sec, final extension 72°C for 7 min and held at 4°C.

For the second round, I used 300 nM of the internal Wolf and WolR primers (Heddi et al. 1999; Table 5), 2  $\mu$ l of product from the first round of PCR, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTP mix (PE Applied Biosystems), 5  $\mu$ l 10x PCR buffer (PE Applied Biosystems), 1.5 units of AmpliTaq<sup>TM</sup> Gold (PE Applied Biosystems) and distilled water to a volume of 50  $\mu$ l. The cycling parameters were the same as above. A total of 10  $\mu$ l of PCR product was electrophoresed on a 1.5% agarose gel containing ethidium bromide to estimate size of the amplified DNA fragment.

*wsp* gene

PCR was carried out in a total volume of 20 $\mu$ l containing 300 nM *wsp* primers, 81F and 691R (Zhou et al. 1998; Table 5), 5-10 ng of weevil DNA, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTP mix (PE Applied Biosystems), 2  $\mu$ l 10x PCR buffer, 0.5 unit AmpliTaq<sup>TM</sup> Gold (PE Applied Biosystems), and 13.5  $\mu$ l distilled water. Amplification was completed with the following cycling profile on a GeneAmp<sup>®</sup> PCR System 9700 (PE Applied Biosystems): 94°C for 10 min, then 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, final extension 72°C for 7 min and held at 4°C. A total of 10  $\mu$ l was run on a 1.5% agarose gel containing ethidium bromide to estimate the size of the amplified DNA fragment.

Table 5. *Wolbachia* 16S rDNA and *wsp* gene primers

Primer	Primer Sequence	Reference
WolF	5'-CGG GGG AAA AAT TAT TGC T-3'	Heddi et al, 1999
WolR	5'-GAC TGT AAT ACA GAA AGT AAA-3'	Heddi et al., 1999
99 F	5'-TTG TAG CCT GCT ATG GTA TAA CT-3'	Hsiao, 1996
994 R	5'-GAA TAG GTA TGA TTT TCA TGT-3'	Hsiao, 1996
81F	5'-TGG TCC AAT AAG TGA TGA AGA AAC-3'	Zhou et al, 1998
691R	5'-AAA AAT TAA ACG CTA CTC CA-3'	Zhou et al, 1998

### *Cloning and DNA Sequencing*

Fragments amplified with the universal eubacterial primers, *Wolbachia* 16S rDNA specific primers, and the *wsp* gene primers were prepared for cloning as follows: 1-2  $\mu$ l of the PCR reaction was directly ligated into TOPO-TA<sup>®</sup> vector (Invitrogen, Carlsbad) without purification. The vector was introduced into bacterial cells. Cells, which tested positive for transformation, were cultured overnight in LB medium containing ampicillin. Plasmids containing the PCR product were extracted from bacterial transformants as follows: At 20800 g, a 1.5 ml aliquot of the overnight culture was centrifuged for 10 sec to pellet the cells. The supernatant was decanted, leaving 50-100  $\mu$ l of supernatant with the pellet. The aliquot was vortexed to re-suspend the cells, 300  $\mu$ l of TENS (TE buffer pH 8, 0.1 N NaOH, 0.5% SDS) were added and vortexed for 15-20 sec until the mixture became sticky. To the aliquot, 150  $\mu$ l of 3M sodium acetate pH 5.2 was added and the aliquot vortexed 15-20 sec to mix. The tubes were placed on ice for 5-10 min before centrifuging for 5 min at 20800 g to pellet cell debris and chromosomal DNA. The supernatant was transferred to a fresh tube and mixed well with 900  $\mu$ l of 100% ethanol pre-cooled to  $-20^{\circ}\text{C}$ . The aliquot was centrifuged 5 min at 20800 g to pellet plasmid DNA and RNA (white pellet). The supernatant was discarded and the pellet rinsed twice with room temperature 70% ethanol. The pellet was allowed to dry for 10 min and then resuspended in 20-40  $\mu$ l TE buffer. RNase cocktail was added to the aliquot and then the aliquot was placed in  $37^{\circ}\text{C}$  water bath for 1 hour.

The purified plasmids were sent to Davis Sequencing (UC Davis). For each population, three individuals were used. From the first individual, three clones were sequenced. From the second and third individuals, one clone was sequenced. This gave five partial sequences for the *wsp* gene from the *Wolbachia* associated with individuals

from each population. These sequences were aligned and compared to generate a consensus sequence for that population. The consensus sequences were used in the phylogenetic analyses.

### *Phylogenetic Analysis*

The nucleotide sequence of the *wsp* gene was used to analyze the phylogenetic relationships of the *Wolbachia* from the three populations of plum curculio using CLUSTAL W (Thompson et al. 1994) and PAUP\*4 (Sinauer Associates, Sunderland, MA).

### **Results**

The universal eubacterial primers yielded robust amplification with the univoltine and multivoltine individuals tested (n=20). A Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1997) analysis of the sequence showed homology with *Pseudomonas* spp. but not *Wolbachia*. No further studies have been done with the *Pseudomonas* spp.

The *Wolbachia* 16S rDNA-specific primers were tested with DNA from univoltine (MA) and both multivoltine (GA, FL) strains. Fragments (650-750 bp; Figure 13) of the 16S rDNA gene were amplified from DNA samples of each multivoltine individual tested (n=20). When a BLAST analysis was performed on the sequence of these fragments, they showed strong homology with *Wolbachia* 16S rDNA. The univoltine strain gave inconsistent results when tested with the 16S rDNA primers: some DNA samples tested yielded no detectable amplicon. Further, sequence of one amplicon from a reaction with univoltine strain DNA showed homology with a human 60S ribosomal protein. In contrast to results with the 16S rDNA primers, both univoltine and multivoltine weevil DNA samples (n=30) yielded amplicons (550-650 bp; Figure 14) when tested with the *wsp* gene primers.

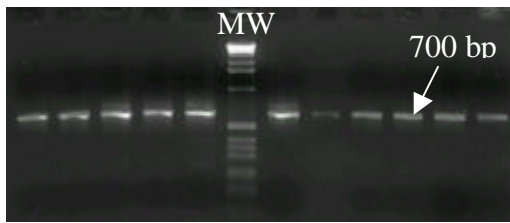


Figure 13. Amplified fragments of the 16S rDNA gene after the second round of PCR

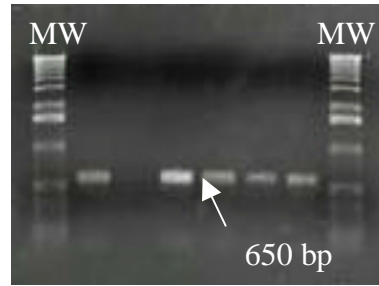


Figure 14. Amplified fragments of the *wsp* gene.

The three consensus sequences (Table 6) were aligned with 26 other previously described partial *wsp* sequences using the CLUSTAL W program (Thompson et al. 1994) followed by manual adjustment. Regions with ambiguous alignments were excluded from the data set, resulting in a 507-nucleotide site data set. The phylogenetic tree (Figure 15) was constructed using PAUP\* 4 (Sinauer Associates, Sunderland, MA). The data set was analyzed by maximum parsimony with branch and bound searches for the nucleotide sequences. Bootstrap analysis was performed with 100 replications.

Excluding *Bemisia tabaci* 2, *B. tabaci* 1, *Dryinid* wasp, *Aleurotrachelus* sp. because they were repetitious, the data set of the remaining 24 nucleotide sequences was transformed into predicted protein sequence data set of 169 characters. A phylogenetic tree (Figure 16) of predicted protein sequence was constructed using PAUP\* 4 (Sinauer Associates, Sunderland, MA). The data set was analyzed by the neighbor-joining method using a heuristic search. Bootstrap analysis was performed with 100 replications.

Although the arrangement of some taxa varies from the tree generated using the nucleotide sequences and parsimony method, the predicted protein sequence tree supports the groupings of the taxa in the tree based on nucleotide sequences. The likelihood of the relationships is strengthened by using two methods, parsimonious (nucleotide sequences) and neighbor joining (predicted protein sequences), which are based on different assumptions (Hoy et al. 1994, Hillis et al. 1996).

Phylogenetic analysis suggests that the univoltine plum curculios carry a different strain of *Wolbachia* than do the multivoltine weevils (Figures 15 & 16). The strain of *Wolbachia* amplified from the Massachusetts weevils is in supergroup B and most

closely related to the strain wTen-B1 amplified from *Perithemis tenera* (Say) (Odonata). Table 7 shows the percentage similarity among all strains included in the analysis (numbers above the line describe percent nucleotide similarity, below the line describe percent predicted protein similarity). At the nucleotide level, the strain of *Wolbachia* carried by the Massachusetts curculios is 91.48% similar to wTen-B1 (Table 7). According to Zhou et al. (1998) the strains must be 97.5% similar to be in the same reference group. Therefore the strain carried by the Massachusetts weevils is in a different reference group from that carried by *P. tenera*. Following the conventional naming of *Wolbachia* strains and reference groups (Stouthamer et al. 1999, Jeyaprakash and Hoy 2000) I have named this strain associated with the Massachusetts plum curculios “wNen” and the reference group “Nen”.

The strains of *Wolbachia* associated with Florida and Georgia plum curculios are in supergroup A (Figures 15 & 16). These *Wolbachia* strains are most closely related to the strains carried by *Dacus destillatoria* (wDes), *Callosbruchus chinensis* (Linn.) (wAus), and *Bactrocera* sp. (wAscD). Analysis of the nucleotide sequences shows the *Wolbachia* strain associated with the Georgia curculios to be unique (at most a 98.81% similarity, Table 7). The strain associated with the Florida curculios is almost identical (99.6%, a difference in two nucleotides, Table 7) to the strain associated with *D. destillatoria*, and cannot be considered unique. Neither of the strains of *Wolbachia* associated with the Florida and Georgia plum curculios differ by more than 2.5% from the *Wolbachia* strains carried by *D. destillatoria*, *C. chinensis*, and *Bactrocera* sp.; therefore, these strains all belong in the reference group “Des”. I have named the strain of *Wolbachia* associated with the Georgia curculios “wCnen” (first letter of genus name and first three letters of species name).

Table 6. Consensus sequences of *Wolbachia wsp* gene fragment from associated plum curculio hosts.

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Massachusetts Weevils

5'TGGTCCAATAAGTGATGAAGAACTAGCTACTACGTTTCGTTTACAATACAACGGTGAAATTT  
TACCCCTCTTTACACAAAAGTTGATGGTATTACAAATGCAACAGGTAAAGAAAAGGATAGTCCC  
TTAACAAGATCTTTTATAGCTGGTGGTGGTGCATTTGCTATAAAAATGGACGACATTAGAGTTG  
ATGTTGAAGGGCTTTACTCACAATTGGCTAAAGATGCAACTGTAGTATCTGATAACAAGGCTG  
CAGATAGTGTAACAGCATTTCGGGATTGGTTAACGTTTATTACGATATAGCGATTGAAGATA  
TGCCTATCACTCCATACATTGGTGTGGTGTGGTGCAGCATATATCAGCAATCCTTCAAAGC  
TGATGCAGTTAAAGAGCAAAAAGGATTTGGTTTTGCTTATCAAGCAAAAGCTGGTGTAGTTA  
TGATGTAACCTCCAGAAATCAAACCTTTGCTGGTGCCTCGCTATTTTGGTTCTTATGGCGCTAGT  
TTTAATAAAGAAACAGTATCAGCTACTAAAG-3'

Georgia Weevils

5'TGGTCCAATTAAGTGATGAAAGAACTAGCTACTACGTTTCGTTTGCAAATACAACGGGTGA  
AATTTTAACCTCTTTTCACAAAAGTTGATGGGTATTGCACCATAAATCAGGCAAAGACAATA  
ATAGTCCCTTAAAAGCATCTTTTATAGCTGNCGGTGGTGCCTTTGGTTATAAAAATGGACGACA  
TCAGGGTTGACGTTGAAGGACTTTACTCATGGTTGAATAAAGATGCAGATGTAGTAGGTGATA  
CAGTTGCAGAAAGCTTAACAGCAATTTACAGGATTAGTTAACGTTTATTACCGATGTAGCGATT  
GAAGACATGCCTATCACTCCATATGTTGGTGTGGTGTGGTGCAGCGTATATTAGCACACCTT  
TGGCAACTGCTGTGAGTAGTCAAAAATGGTAAATTTGCTTTTGCTGGTCAAGCAAGAGAGCTG  
GTGTTAGTTACGATGTAACCTCCAGAAAGTCAAACCTTTACGCTGGTAGCTCGCTATTTTCCGG  
KTCTTATGGTGCTAACTTTGATAAAACTGACAAAGACGGCAAAGGGGAACTCAAAGTTCTTT  
ACAGCACTGTTGGTGCAGGAAGCTGGAGTAGCG-3'

Florida Weevils

5'TGGTCCAATAAGTGATGAAGAACTAGCTACCTACGTTTCGTTTGCAATACAACGGTGAAATT  
TTACCTCTTTTACAAAGTTGATGGTATTGCACATAAATCAGGCAAAGACAATAATAGTCCCTT  
AAAAGCATCTTTTATAGCTGGCGGTGGTGCCTTTGGTTATAAAAATGGACGACATCAGGGTTGA  
CGTTGAAGGACTTTACTCATGGTTGAATAAAGATGCAGATGTAGTAGGTGATACAGTTGCAGA  
AAGCTTAACAGCAATTTACAGGATTAGTTAACGTTTATTACGATGTAGCGATTGAAGACATGCC  
TATCACTCCATATGTTGGTGTGGTGTGGTGCAGCGTATATTAGCACACCTTTGGCAACTGCT  
GTGAGTAGTCAAAAATGGTAAATTTGCTTTTGCTGGTCAAGCAAGAGCTGGTGTAGTTACGAT  
GTAACCTCCAGAAGTCAAACCTTTACGCTGGAGCTCGCTATTTCCGGTTCTTATGGTGCTAACTTTG  
ATAAACTGACAAAGACGGCAAAGGGGAACTCAAAGTTCTTTACAGCACTGTTGGTGCAGAA  
GCTGGAGTAGCGTTTAATTTTT

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Table 7. Pairwise comparison of nucleotide and predicted protein sequence similarities based on *wsp* gene sequence analysis. Above the line is percent similarity of predicted protein sequence. Below the line of asteriks is percent similarity of nucleotide sequence.

	wDes	wAus	wAscD	Florida	Georgia	wTen	Massachusetts
<i>D. destillatoria</i>	*	98	94.7	100	80.7	72.7	74
<i>C. chinensis</i>	99	*	92.7	98	79.3	71.3	72.7
<i>Bactrocera</i> sp.	98.81	97.82	*	94.7	78.67	72.67	72
Florida	99.6	98.6	98.41	*	80.7	72.7	74
Curculio							
Georgia	98.8	97.8	97.6	98.4	*	62.7	64
Curculio							
<i>P. tenera</i>	81.7	81	81.2	81	80.2	*	97.3
Massachusetts	77.4	77	77.2	77	76.2	91.5	*
Curculio							

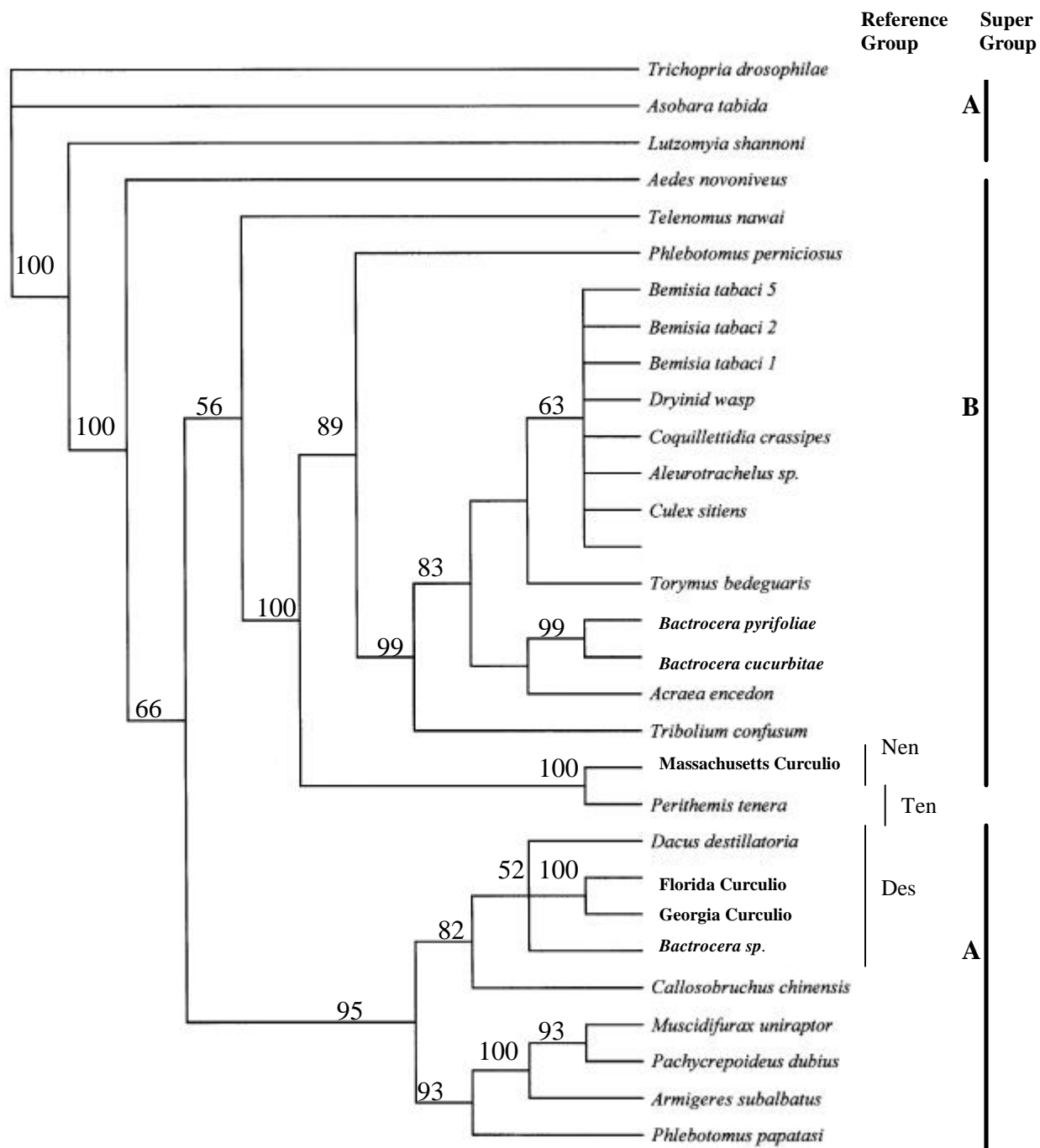


Figure 15. *Wolbachia* strains shown by host. Parsimonious midpoint rooted tree generated for 29 *Wolbachia* strains using a CLUSTAL W alignment of nucleotide sequences. All bootstrap values detected were above 50% for 100 replicates. Branches without bootstrap values were collapsed for bootstrap analysis. A bootstrap value of 94-100 shows good support for the relationship to the right of the number. A total of five clones from three individuals (3 clones from a single individual, 1 clone from each of two individuals) were used to generate a consensus sequence each for “Massachusetts curculio”, “Florida curculio”, and “Georgia curculio”. All other sequences were obtained by a BLAST search of GenBank. All strains within a group are less than 2.5 % divergent.

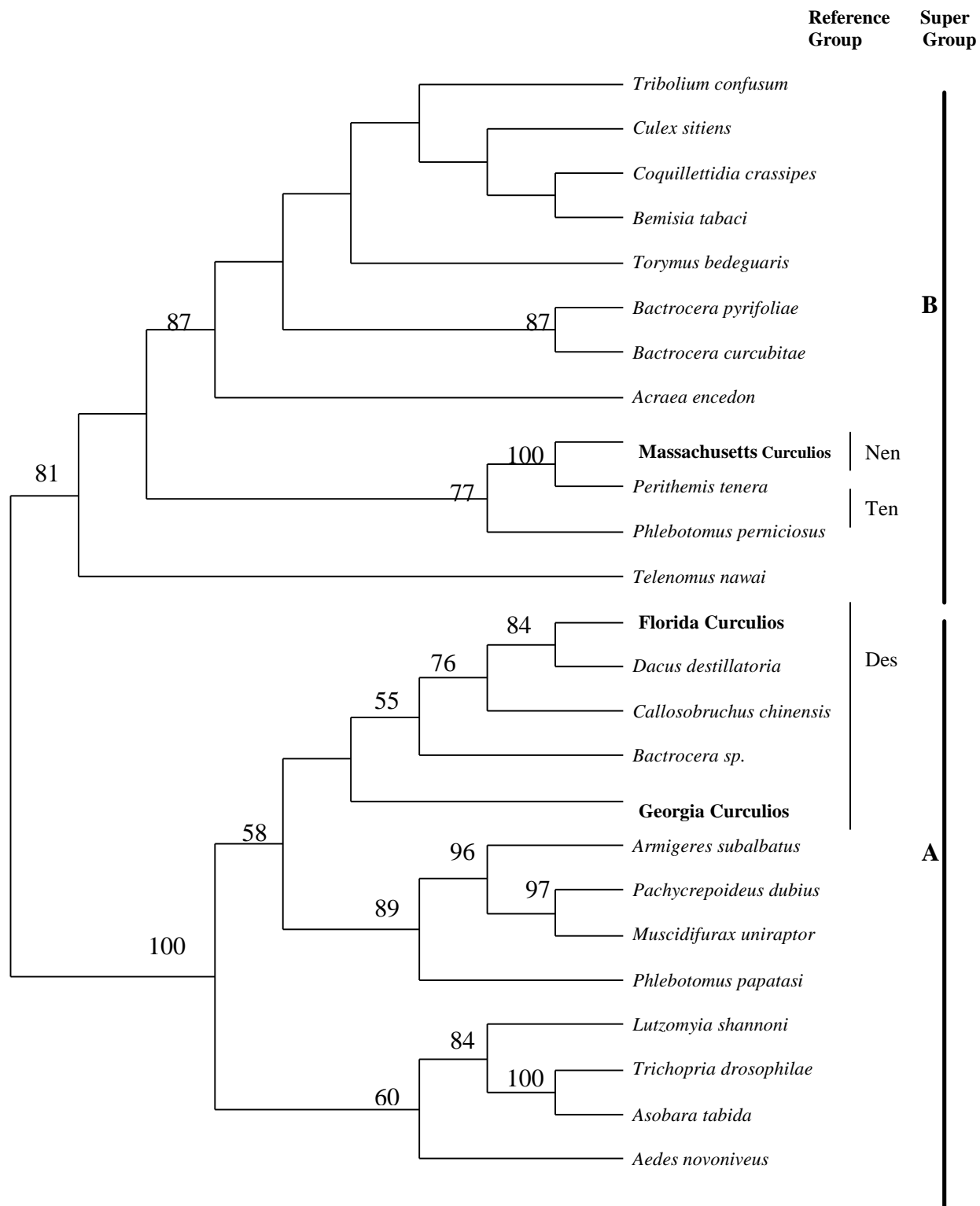


Figure 16. Neighbor joining mid-point rooted tree generated for 24 *Wolbachia* strains using a CLUSTAL W alignment of predicted protein sequences. All bootstrap values detected were above 50% for 100 replicates. Branches without bootstrap values were collapsed for bootstrap analysis.

## Discussion

The strains of *Wolbachia* associated with the populations of plum curculio can be used to develop an assay to differentiate between the univoltine and multivoltine strains of plum curculio. Once this assay has been developed it can be used to detect and monitor the populations of plum curculio in Virginia. Zhou et al. (1998) designed primers that can distinguish between *Wolbachia* infections in supergroup A and supergroup B. Use of these primers and restriction enzyme analysis will allow identification of different populations of plum curculio and possible novel strains of *Wolbachia* within these populations.

It is unknown how much of an effect the host has on *Wolbachia*. It is known that *Wolbachia* strains are closely linked to mitochondrial haplotypes in *Drosophila* spp. (O'Neill 1997). This research has shown that mitochondria exert some control over which strains of *Wolbachia* establish in a host. If the mitochondria in plum curculio have evolved to exert this type of control, then populations with the same mitochondrial haplotype will be associated with the same strain of *Wolbachia*. If the haplotypes are specific to the voltinism of the populations of plum curculio (i.e. univoltine populations are associated with mitochondrial haplotype A and multivoltine populations are associated with haplotype B) a mitochondrial haplotype assay could be another way to test field-collected individuals for voltinism. These data could also support the theory of a *Wolbachia*-driven speciation plum curculio

It is known that *Wolbachia* is associated with reproductive shifts of its hosts. However, little is known about the fitness costs to the female who carries *Wolbachia*. The costs can vary depending upon the species. This raises the question of the fitness cost of being infected and providing the bacteria with the resources with which to live and reproduce. The fitness cost is high for an adult curculio that mates with another carrying an incompatible strain of *Wolbachia*, as the fecundity is reduced. Overwintering in the univoltine strain must also be figured into the fitness costs for both the curculios and the symbiont. Many adult plum curculios don't survive overwintering, which lowers both their fitness and the fitness of the *Wolbachia*. This raises the question of *Wolbachia* moderating or overriding in the multivoltine populations of plum curculio what is an obligatory diapause in univoltine populations of weevils.

*Wolbachia* strains have been implicated in driving speciation in *Nasonia* spp. wasps. Bordenstein et al. (2001) showed that the *Wolbachia*-induced incompatibility preceded any other pre-zygotic or post-zygotic barriers in *Nasonia* wasps. Shoemaker et al. (1999) suggested that *Wolbachia* causing unidirectional incompatibility coupled with a pre-zygotic barrier and a post-zygotic barrier could drive speciation in *Drosophila recens* Wheeler and *Drosophila subquinaria*. Stevenson and Smith (1961) reported that crosses of univoltine female and multivoltine male plum curculios produced 73% fewer offspring than same-strain crosses. This could be attributed to the effect of incompatible *Wolbachia* infections. However, Padula and Smith (1971) reported histological data in crosses of multivoltine female and univoltine male plum curculios that the sperm degenerated and the spermathecal gland collapsed to varying degrees. This is much more severe than the proposed “sperm recovery” mechanism and has much higher costs associated with it for the female weevil that mates with a male infected with a different *Wolbachia* strain. *Wolbachia* has not been implicated in causing such histological changes in any other species. More research needs to be done to determine if the degeneration of the sperm and the breakdown of the spermathecal gland are a separate pre-zygotic barrier from the *Wolbachia* infection. This mechanism of histological changes should be included in possible effects or impacts of *Wolbachia* that could drive speciation. This research would involve not only curing the infection by the use of antibiotics and/or heat treatment, but also infecting another insect species with the strain of *Wolbachia* of interest and recording any shifts in reproduction or phenotype (Werren 1997).

Currently the data are insufficient to support a hypothesis of *Wolbachia*-driven speciation of the two populations (univoltine and multivoltine).

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