File S1

Supporting Materials And Methods

Testing for the presence of *Wolbachia* by PCR

Following the previously published procedure (O'NEILL *et al*. 1992), insect ovary tissue was dissected, homogenized and incubated with 2 µl of proteinase K (10 mg/ml) for 30min at 37°C, followed by 5 min at 95°C. Samples were briefly centrifuged, and 1 µl of the supernatant was used as the template in subsequent PCR, with *Wolbachia* 16S rRNA-specific primers or universal bacterial 16S rRNA primers (see Table S2), with 30 cycles of 95 °C, 1 min, 52 °C 1 min, 72 °C 1 min.

SDS-PAGE and Western blotting

Gels were run in a Mini-Protean® II electrophoresis cell (Bio-Rad) then transferred to nitrocellulose membranes (Invitrogen) membranes using the iBlot™ dry blotting system (Invitrogen). After blotting, membranes were blocked in 5% non-fat milk in TBST buffer (150 mM M NaCl, 50 mM Tris-HCl, 0.05% Tween 20, pH 7.6) for 1 h at room temperature, then incubated with the primary antibody in 1% non-fat milk in TBST buffer overnight at 4 ° C. After washing 4 x 15 min in TBST buffer and blocking again in the blocking buffer for 1 h at room temperature, membranes were incubated in the presence of the secondary antibody for 1 h at room temperature, followed by further washes (4 x 15 min) in TBST buffer. Detection was performed with the Amersham ECL™ Western blotting analysis system (GE Healthcare).

Preparation of mitochondria for mtDNA isolation

Batches of 50-200 flies were crushed with one stroke of a Teflon pestle in a glass homogenizer in 4 ml HB medium (225 mM mannitol, 75 mM sucrose, 1 mM EDTA, 0.1% BSA, 10 mM Tris-HCl pH7.6) then homogenized with 8 more strokes. The homogenate was transferred to a 15 ml plastic centrifuge tube, filled up with HB, then centrifuged for 5 min at 1,000 g_{max} at 4 °C to pellet cell debris and nuclei. The supernatant was decanted to a new 15 ml tube and crude mitochondria were pelleted for 10 min at 12,000 g_{max} at 4 °C. The pellet was washed once, then resuspended in 300 µl HB and overlaid onto a 1.5 M/1 M sucrose (250 + 250 µl) step gradient and centrifuged for 1 h at 45,000 g_{max} at 4 °C. The mitochondrial layer was transferred to a 2 ml tube and one volume of HB was added. Mitochondria were pelleted for 5 min at 12,000 g_{max} at 4 °C, then processed immediately for DNA extraction.

Repeat analysis of the NCR by end-labeling and partial restriction digestion

Primers Dm14428F and Dm225R (see Table S2), designed to amplify the entire NCR, were individually end-labeled using T4 polynucleotide kinase (T4 PNK, Fermentas), and [γ-^{32}P]ATP (Perkin Elmer, 6000 Ci/mmol) under manufacturer's recommended conditions. Each 20 µl reaction contained, in manufacturer's Reaction Buffer A, 1 nmol of primer, 10 u of

enzyme and 3 µl (30 µCi) of labeled ATP. After incubation at 37 °C for 30 min, the reaction was stopped by the addition of 1 µl of 0.5 M EDTA (pH 8.0) and further incubation at 75 °C for 10 min. The unincorporated label was removed by gel filtration on Sephadex G-50 (GE healthcare). The mtDNA noncoding region was amplified from partially purified mtDNA with end-labeled primer pairs (for 'left' end: ^{32}P-Dm14428F and Dm225R, for 'right' end: Dm14428F and ^{32}P-Dm225R) using LongRange PCR kit (Qiagen), under the following conditions: 93 °C, 3 min (initial denaturation), followed by 35 cycles of 93 °C, 15 s (denaturation); 50 °C, 30 s (annealing); 60 °C, 6 min (extension), with final extension at 60 °C for 10 min. Restriction site mapping by partial digestion and gel electrophoresis was performed essentially as described previously (LEWIS *et al*. 1994). Briefly, after gel purification (GeneJet gel extraction kit, Fermentas), the radiolabeled fragments (20 ng in 20 µl) were partially digested with *Pac*I (1 unit, 1 x NEBuffer 1, Biolabs), *Hpy*188I (0.6 unit, 1 x NEBuffer 4, Biolabs) or *Swa*I (1 unit, 1 x buffer O, Fermentas). Reactions were incubated for 6 min (*Pac*I and *Hpy*188I at 37 °C, *Swa*I reaction at 30 °C), then terminated by the addition of EDTA to 20 mM. The reactions and 1 kb labeled DNA marker (GeneRuler^{TM} 1 kb ladder, 250-10,000 bp, Fermentas, labeled by [γ-^{32}P]ATP as above described above) were fractionated by 1.2% agarose gel electrophoresis in TAE buffer, after which the gel was dried (model 583, BIO-RAD) and exposed overnight at -80 °C (FUJI medical X-RAY film), with intensifying screen.

Long PCR and mtDNA sequencing

The coding region of the mitochondrial genome was amplified in three overlapping fragments (each about 5.5 kb in size) by long-PCR (LongRange PCR kit, Qiagen), using partially purified mtDNA as template. The cycling conditions were: 93 °C, 3 min (initial denaturation); then 35 cycles of 93 °C, 15 s (denaturation); 50 °C, 30 s (annealing); 60 °C, 6 min (extension), with final extension at 60 °C for 10 min. Each PCR product was verified by 0.8% (w/v) agarose gel electrophoresis, then purified over mini-spin columns and used as templates for cycle sequencing using primers as indicated (Table S2). The mtDNA fragments were sequenced in both directions using Big Dye v3.1 chemistry and a 3130xl genetic analyzer (Applied Biosystems), with primer sequences as listed in Table S2. The sequences were aligned manually in win_serialcloner1-3 (Serial Basics), then analyzed using the phred/phrap/consed package (GORDON *et al*. 1998). Each deviation from the reference sequence was confirmed by a second PCR and sequencing reaction. The NCR and its flanking sequences were sequenced separately, including the portion of the SSU rRNA not already covered by sequencing of coding region fragment 3.

For sequencing of the NCR, repeat arrays I and II, plus their flanking regions, were amplified from mtDNA template in separate, overlapping PCR reactions, then analyzed by a combination of cloning of restriction fragments and sequencing with overlapping primers, as indicated below. In general, at least three independent PCR reactions and over 200 clones were analyzed to infer the sequence of each sub-region from each strain analyzed. Nucleotide differences found only in single clones were assumed to represent mutations arising during PCR/cloning, but might also represent low-level

heteroplasmy. PCR used an initial denaturation step at 93 °C for 3 min, followed by 35 cycles of 93 °C for 15 s, 42 °C for 30 sec, 60 °C for 1-3 min depending on the length of the desired product, with a final extension at 60 °C for 10 min. PCR products were purified by gel electrophoresis (GeneJet gel extraction kit, Fermentas). The 'left' end of the NCR, between SSU rRNA and repeat array I, plus the short region of the SSU rRNA gene not already covered by sequencing of coding region fragment 3, was inferred by direct sequencing of the product amplified with primer pair Dm14428F/Dm17556R, using overlapping primers Dm14428F, Dm14570F, Dm14721F and Dm14787R. The PCR for type I repeats, which also confirmed the reverse-strand portion of the flanking region and start of the SSU rRNA gene, was performed using primer pair (Dm14721F and Dm17556R). After gel purification, the product was digested with *Ssp*I (1 X buffer G, Ferments) or *Pac*I at 37 °C overnight, then ligated into plasmid vector pNEB193 (New England Biolabs), which had been digested, respectively, with *Sml*I (*Pme*I, Fermentas) at 30 °C or *Pac*I (New England Biolabs) at 37 °C, then dephosphorylated with CIAP (Fermentas) and purified by gel electrophoresis. The order of repeat I elements was inferred from overlaps of the restriction sites used for cloning (see Fig. S4F). It was further confirmed by PCR using primer pair Dm15578F/Dm17295R, and an elongation time of 90 s, which generated a mixture of 3 fragments of approximately 330, 700 and 1100 bp, which were independently cloned and sequenced. The region between repeat array I and repeat array II was amplified using PCR primer pairs Dm15578F/Dm17556R and Dm15578F/Dm17833R, and sequenced using unique sequencing primers covering this region on both strands (Table S2). Repeat array II was amplified by four different primer pairs which, under varying extension times, generate products of different sizes (see Fig. S4G). These were independently cloned and sequenced, to infer the final order of repeat II elements, which are very similar. The primer pairs used for repeat array II were: Dm15578F/Dm17833R, Dm17556F/Dm18026R, Dm17717F/Dm17833R, and Dm18933F/Dm225R. PCR products were cloned into pCR4 vector (TA Cloning Kit, Invitrogen) after gel purification, and sequenced using universal M13 forward and reverse primers. The shortest product from primer pair Dm15578F/Dm17833R allowed unambiguous assignment of the first portion of the ‘first’ copy of repeat II (the one closest to the repeat I array). The next shortest product from this primer pair extends into the second copy of repeat II, allowing full assignment of the first copy and the junction with the second. Similarly, the two shortest products from primer pair Dm18933F/Dm225R allow unambiguous assignment of the final copy of repeat II (the one closest to the $tRNA^{Ile}$ gene), as well as the ‘right’ end of the NCR between repeat array II and $tRNA^{Ile}$, extending into the coding region. The various products from the two other primer pairs allow unambiguous assignment of the sequence and order of the remaining repeat II elements. All sequences were assembled using win_serialcloner1-3.