

## NEW *WOLBACHIA* ENDOSYMBIONTS FROM NEARCTIC AND NEOTROPICAL FLEAS (SIPHONAPTERA)

Katharina Dittmar and Michael F. Whiting

Department of Integrative Biology, Brigham Young University, 401 WIDB, Provo, Utah 84606. e-mail: kd98@email.byu.edu

**ABSTRACT:** Several species of fleas (Siphonaptera), ectoparasites of mammals and birds, have recently been shown to harbor species of *Wolbachia*. Here, we extend this data set to 20 more species of Siphonaptera (Rhopalopsyllidae, Stephanocircidae, Pulicidae, Ceratophyllidae, Ctenophthalmidae, Ischnopsyllidae, Leptopsyllidae, and Malacopsyllidae) from sylvatic populations throughout the Nearctic and Neotropical regions. Using polymerase chain reaction, we targeted the *Wolbachia* 16S ribosomal DNA (rDNA) gene. Applying maximum parsimony- and maximum likelihood-based algorithms, as well as statistical parsimony, we conducted a phylogenetic analysis of *Wolbachia* 16S rDNA to evaluate its position within the known *Wolbachia* spp. The analysis recovered the siphonapteran *Wolbachia* 16S rDNA sequences as a monophyletic group and shows multiple haplotype connections between the Neotropical and Nearctic *Wolbachia* strains of fleas.

*Wolbachia* spp. are a group of obligate intracellular bacteria (alpha 2 Proteobacteria, Rickettsiales) infecting a wide range of invertebrates, including insects, crustaceans, mites, and nematodes. They are assumed to account for a variety of effects in their hosts, such as cytoplasmic incompatibility (O'Neill et al., 1992), male killing (Hurst et al., 1999), thelytoky (Stouthammer et al., 1993), feminization (Rousset et al., 1992), speciation (Rokas et al., 2002), and local adaptation (Sinkins et al., 2000). Arthropod *Wolbachia* are predominantly vertically (maternally) transmitted; however, an intermediate rate horizontal transmission is thought to occur (Jiggins, 2000) because bacterial and host phylogenies are largely incongruent (Jiggins et al., 2002).

Recent studies proved the presence of *Wolbachia* in siphonapteran hosts (Jeyaprakash and Hoy, 2000; Gorham et al., 2003). 16S ribosomal DNA (rDNA) and *wsp* sequences were detected. Phylogenetic analysis of a part of 16S rDNA showed the paraphyly of these strains (Gorham et al., 2003), thus suggesting events of horizontal transmissions. On the basis of the known occurrence of *Wolbachia* spp. in Siphonaptera, we set out to extend their known geographical and organismal distribution. We analyzed 79 species of field-collected fleas from the Nearctic and Neotropical regions. Because the previous study only included a small piece of 16S rDNA in the analysis, it was our intention to increase the character set for the phylogenetic analysis by obtaining a longer piece of 16S rDNA.

It was with the supposed paraphyly of siphonapteran *Wolbachia* in mind that a phylogenetic analysis was conducted to assess the theory of horizontal transmission within siphonapteran *Wolbachia* strains in particular and among arthropod hosts in general.

### MATERIALS AND METHODS

#### Collection and DNA isolation

Siphonaptera were obtained from various hosts in localities throughout the Neotropical and Nearctic regions. This was done by trapping the animals with Sherman (H. B. Sherman Traps, Tallahassee, Florida) or Tomahawk live traps (Tomahawk Live Trap Co., Tomahawk, Wisconsin). The animals were combed, and fleas were collected with entomological featherweight tweezers. The animals were then released

back into their natural habitat. The fleas were stored in 96.7% ethanol in a -80 C freezer until extracted.

For extraction, total genomic DNA was isolated using the Qiagen DNeasy Kit® (Qiagen, Valencia, California). One specimen per species of flea was cut in half and placed in extraction buffer.

Voucher specimens are slide mounted and stored at the Monte L. Bean Life Science Museum in the collection of Dr. Michael Hastriter. Identification of the specimens was carried out according to currently available taxonomical keys. In 2 cases (*Jellisonia* sp. 1 and 2, females), a final species identification could not be made. In this genus, female specimens do not possess known morphological characters to allow species identification.

#### Polymerase chain reaction and cloning

Three microliters of extraction product was electrophoresed on a 1% agarose gel to estimate the quality and amount of genomic DNA, and sample dilutions were performed accordingly. Bacterial 16S rDNA was amplified using the universal primers 27F and 1492 (Lane, 1991) in conjunction with the Ultrapure dNTP Mix (Amersham Bioscience, San Francisco, California). The polymerase used in the reaction was AmpliTaq Gold® (PE Applied Biosystems, Foster City, California). The amplification protocol was as follows: 12 min at 95 C for the initial denaturation, 40 cycles of 1 min at 95 C, 1 min at 50 C, and 1 min at 72 C, followed by a final extension of 5 min at 72 C. The total reaction volume was 50 µl. A negative control (without DNA) was run with each sample group to ensure lack of contamination. The reactions were performed on a DYAD® Peltier Thermal Cycler (MJ Research, San Francisco, California).

Double-stranded polymerase chain reaction (PCR)-amplified products were screened on a 2% agarose gel, and distinctive bands were excised, purified with a GeneClean III Kit (BIO101, Vista, California), and cloned into a PCR2.1-TOPO® vector (TOPO TA Cloning® Kit, Invitrogen, Carlsbad, California). Fifteen individual inserts per sample (flea) were then reamplified with M13 primers and directly sequenced using the Perkin-Elmer ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Excess dye terminator was removed with Sephadex plates, and sequences were fractionated by polyacrylamide gel electrophoresis on an ABI PRISM 377 automated DNA sequencer (PE Applied Biosystems) at the DNA Sequencing Center at Brigham Young University.

#### Data analysis

Sequence analysis and editing were performed with the Sequencher 4.1.1 software (Gene Codes Corp., Ann Arbor, Michigan). Contigs were assembled from forward and reverse sequences and aligned using the default settings for gap and mismatch penalties, with subsequent manual adjustments. The acquired consensus sequences were submitted to BLAST searches on GenBank (Altschul et al., 1997) to determine matches with other *Wolbachia* spp. sequences.

The complete data set includes 20 *Wolbachia* 16S rDNA sequences from our analysis as well as 24 available *Wolbachia* and *Rickettsia*-*Ehrlichia* sequences from GenBank (AB085179, AJ306315, AY227741,

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TABLE I. Flea samples from the Nearctic and Neotropical region, including taxonomic information and host association. The numbers correspond to the numbers given in Figure 2.

No.	Family	Genus	Species	Sex	Location	Host
1	Ceratoophyllidae	<i>Orchopeas</i>	<i>leucopus</i>	M	U.S.A. (Utah)	<i>Peromyscus</i> sp.
2		<i>Jellisonia</i>	sp. 1	F	Honduras	Mouse
3		<i>Jellisonia</i>	sp. 2	F	Mexico	<i>Oryzomys</i> sp.
4	Ctenophthalmidae	<i>Oropsylla</i>	<i>hirsuta</i>	M	U.S.A. (Colorado)	<i>Cynomys</i> sp.
5		<i>Adoratopsylla</i>	<i>i. intermedia</i>	F	Brazil	<i>Metachirus</i> sp.
6	Ischnopsyllidae	<i>Myodopsylla</i>	<i>gentilis</i>	F	U.S.A. (Utah)	<i>Myotis</i> sp.
7	Leptopsyllidae	<i>Geusibia</i>	<i>ashcrafti</i>	F	U.S.A. (California)	<i>Ochotona</i> sp.
8	Malacopsyllidae	<i>Peromyscopsylla</i>	<i>selensis</i>	M	U.S.A. (Idaho)	<i>Peromyscus</i> sp.
9		<i>Phtiropsylla</i>	<i>agenoris</i>	M	Argentina	<i>Zaedyus pichyi</i>
10	Pulicidae	<i>Ctenocephalides</i>	<i>felis</i>	F	Peru	<i>Canis familiaris</i>
11		<i>Pulex</i>	<i>irritans</i>	M	U.S.A. (Utah)	<i>Cynomys</i> sp.
12		<i>Pulex</i>	<i>simulans</i>	F	Peru	<i>Cavia aperea</i>
13		<i>Xenopsylla</i>	<i>cheopis</i> 1	F	Brazil	<i>Rattus</i> sp.
14	Rhopalosyllidae	<i>Xenopsylla</i>	<i>cheopis</i> 2	F	Brazil	<i>Rattus</i> sp.
15		<i>Echidnophaga</i>	<i>gallinacea</i>	M	Mexico	<i>Urocyon</i> sp.
16		<i>Polygenis</i>	<i>pradoi</i>	M	Brazil	<i>Akodon</i> sp.
17	Stephanocircidae	<i>Ectinorus</i>	<i>alejoi</i>	M	Peru	<i>Lagidium</i> sp.
18		<i>Craneopsylla</i>	<i>minerva wolffheuglia</i>	F	Venezuela	<i>Rhipidomys</i> sp.
19		<i>Sphinctopsylla</i>	<i>ares</i>	F	Chile	<i>Oryzomys</i> sp.
20		<i>Plocopsylla</i>	<i>achillea</i>	F	Peru	<i>Akodon</i> sp.

AF093510, AJ012646, AB038370, AB025965, AF045190, L02887, U17060, AF035160, AY0075551.1, U80584, L02882, AF220604, Z49261, AF487892, AJ010276, AJ509026, AF178037, D84558, U03777, X61768, and AY158006).

GenBank accession numbers for *Wolbachia* strains submitted from this study are AY335917 (ex: *Jellisonia* sp. 1), AY335918 (ex: *Myodopsylla gentilis*), AY3359179 (ex: *Echidnophaga gallinacea*), AY335920 (ex: *Sphinctopsylla ares*), AY335921 (ex: *Geusibia ashcrafti*), AY335922 (ex: *Jellisonia* sp. 2), AY335923 (ex: *Ctenocephalides felis*), AY335924 (ex: *Orchopeas leucopus*), AY335925 (ex: *Oropsylla hirsuta*), AY335926 (ex: *Pulex irritans*), AY335927 (ex: *Craneopsylla minerva wolffheuglia*), AY335928 (ex: *Peromyscopsylla selensis*), AY335929 (ex: *Adoratopsylla intermedia intermedia*), AY335930 (ex: *Polygenis pradoi*), AY335931 (ex: *Ectinorus alejoi*), AY335932 (ex: *Phtiropsylla agenoris*), AY335933 (ex: *Plocopsylla achillea*), AY335934 (ex: *Pulex simulans*), AY335935 (ex: *Xenopsylla cheopis* 1), and AY335936 (ex: *Xenopsylla cheopis* 2).

Previous phylogenetic analysis indicates that *Wolbachia* spp. are closely related to *Rickettsia* spp. and *Ehrlichia* spp. (Anderson and Karr, 2001). Because both *Rickettsia* and *Wolbachia* belong to the Rickettsiaceae, we included some *Rickettsia* spp. 16S rDNA sequences in the analysis and chose a representative of the Anaplasmataceae (*Cowdria (Ehrlichia) ruminantium*) as the outgroup.

The phylogeny of the *Wolbachia* sequences was inferred using maximum likelihood (ML), maximum parsimony (MP), and statistical parsimony (SP) analyses (Templeton et al., 1992). The best-fit model of nucleotide substitution for the ML analysis was selected using the hierarchy of likelihood ratio tests implemented in Modeltest 3.0 (Posada and Crandall, 1998). MP and ML heuristic searches were conducted with 1,000 random sequence addition replicates. Nodal support was estimated using the bootstrap approach (Felsenstein, 1985) with 1,000 replicates. The MP and ML analyses were performed with PAUP 4.03b (Swofford, 2000). The SP nesting design was constructed with the program TCS 1.0 according to the rules given in Crandall (1996) and Templeton (1998). Thus, we were able to resolve the connection of haplotypes across the Neotropical and Nearctic regions. At this point, our sample size does not permit credible inferences regarding the phylogeographic or population history of the strains, rendering further analysis (GeoDis: Posada et al., 2000) unwarranted.

## RESULTS

During the screening for siphonapteran obligate bacterial symbionts and pathogens, 20 of 79 screened species or specimens were positive for *Wolbachia*. We identified 20 different *Wolbachia* 16S rDNA sequences, which were present in 8 families distributed in North and South America (Table I).

All *Wolbachia* 16S rDNA sequences obtained from individual cloning colonies were identical for each flea species, and the amplified sequences from the 20 species ranged between 1,310 and 1,314 bp.

The best-fit model of nucleotide substitution chosen by Modeltest 3.0 (Posada and Crandall, 1998) was the HKY85 model (Hasegawa et al., 1985), including rate variation among sites as modeled by the gamma distribution (Yang, 1996). Three separate ML analyses resulted in 3 trees with similar likelihood scores. The MP analysis recovered 216 equally parsimonious trees. All ML trees showed identical topologies, with slight differences in branch length, and poor resolution of the ingroup. In all trees, the siphonapteran *Wolbachia* 16S rDNA samples were recognized as a monophyletic, yet internally highly unresolved, group nested within the arthropod *Wolbachia* spp. (Fig. 1). All siphonapteran strains form a single separate network in the SP analysis (Fig. 2); thus, their sequences are more closely related to one another than to the rest of the sequences used in this analysis. The network supports multiple connections between Neotropical and Nearctic siphonapteran *Wolbachia* haplotypes (Fig. 2). The bootstrap support values for the *Rickettsia* group, the *Wolbachia* ingroup, and the siphonapteran *Wolbachia* group are high; the remaining nodes have moderate to weak support (Fig. 1).

## DISCUSSION

Our investigation expands the currently available sequence data and distributional records for *Wolbachia* infections

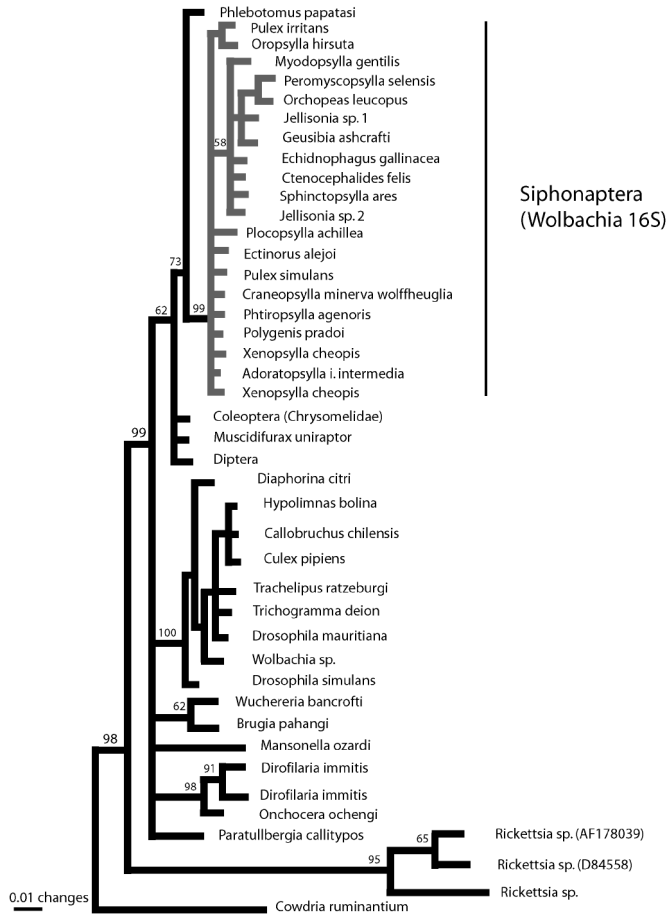


FIGURE 1. ML phylogram of the *Wolbachia* data set. Numbers above the branches correspond to bootstrap support values.

throughout the Siphonaptera. Previous records included only Pulicidae, Rhopalopsyllidae, and Ceratophyllidae from the United States (Jeyaprakash and Hoy, 2000; Gorham et al., 2003).

The discovery of these bacterial symbionts across Nearctic and Neotropical Siphonaptera in sylvatic populations indicates a potentially widespread association with fleas in general (Fig. 2). It was not our intent to provide rates of prevalence because only single specimens were studied per population and species.

The monophyly of the flea *Wolbachia* strains and thus the apparent association of a particular group of *Wolbachia* strains with fleas stand in contrast to previously published results. Typically, arthropod *Wolbachia* spp. show little association or phylogenetic congruence with particular families of hosts. Closely related strains have been observed in phylogenetically disparate hosts, and related hosts are reportedly infected by distantly related *Wolbachia* spp. (West et al., 1998; Zhou et al., 1998). The analysis of Gorham et al. (2003) supports this hypothesis and suggests an apparent paraphyly of the siphonapteran *Wolbachia* strains, resulting in the lack of phylogenetic congruence with the hosts at an ordinal level. They used a relatively short (491 bp) and conserved part of the 16S rDNA sequence. Sequence analysis shows that of these characters, only 19 are parsimony informative versus 314 characters in our data set. Also, Gorham

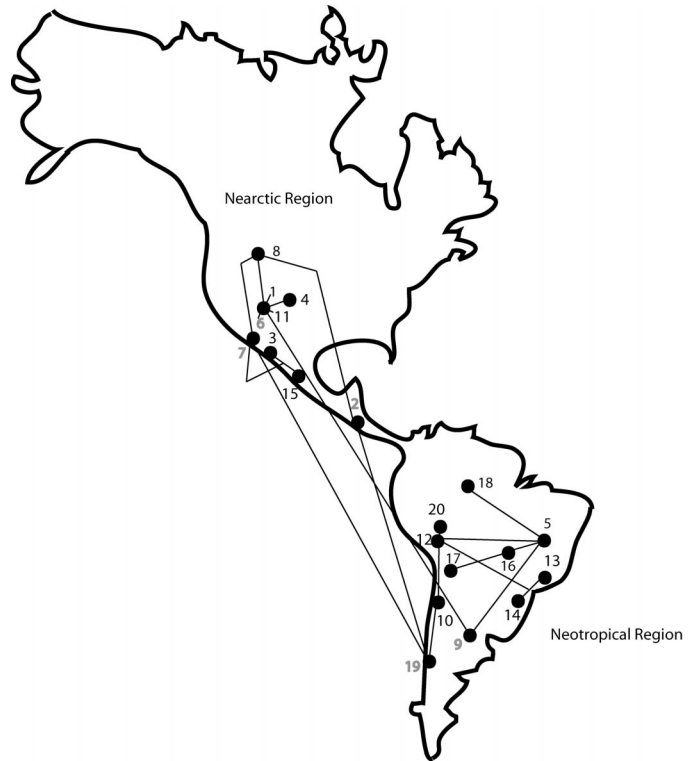


FIGURE 2. Geographical locations of the siphonapteran *Wolbachia* sp. sampling sites, and SP haplotype network. The numbers correspond to Table I. The network clearly indicates several independent haplotype connections between the Neotropical and Nearctic *Wolbachia* populations (2, 6, 7, 9, and 19).

et al. (2003) based their tree reconstruction on neighbor joining (Saitou and Nei, 1987) as implemented in PAUP (Swofford, 2000). This purely algorithmic approach has repeatedly been shown to be problematic because it converts discrete data into distance matrices, thus comparing overall similarity (amount of difference) rather than special similarity (sharing of unique characters) (Farris et al., 1996). A similarity test (PAUP: Swofford, 2000) of the data of Gorham et al. (2003) shows that all the sequences fall into a 96.7–100% similarity interval with our data. These sequences also group within the siphonapteran *Wolbachia* when added to our data set. This suggests that Gorham et al. (2003) probably sequenced similar strains of the same group or species of *Wolbachia*.

Several conclusions can be drawn from these results. Our data clearly suggest the association of a particular group of *Wolbachia* strains with fleas. The group seems to infect a wide range of flea species, distributed over great geographic distances. This is further supported by the recovery of a single network of all sequences in the SP analysis.

Moreover, certain ecological parameters of Siphonaptera make an infestation with distantly related strains from other arthropods unlikely. Fleas are parasites and as adults feed exclusively on vertebrate blood. Therefore, chances to become infected horizontally with distantly related strains of *Wolbachia* at this stage of development, e.g., from Hymenoptera, are negligible. Because flea larvae are not known to be predatory, they also would rarely be subjected to infections with distantly re-

lated strains from other insects. Predation as a mode of horizontal transmission has been reported (Johanovicz and Hoy, 1996) but makes it more likely that predatory insect larvae become infected with siphonapteran *Wolbachia* strains than vice versa. However, transient infection due to accidental ingestion of infected organisms, such as nematode eggs or soil mites, cannot be entirely ruled out and certainly deserve further examination. Other monophyletic clades of *Wolbachia* are known from *Trichogramma* wasp species or isopods (Schilthuisen and Stouthammer, 1997; Bouchon et al., 1998; Cordaux et al., 2001; Jiggins et al., 2002). In both these host groups, *Wolbachia* causes distortions of sex ratio or sexuality. This may make the transmission to closely related taxa more likely (Jiggins et al., 2002). Unfortunately, currently, there are only limited studies about such effects in fleas. Gorham et al. (2003) concluded that there was no significant difference in infection prevalence between the flea sexes and there is no distortion of sex ratios.

Generally, it might be useful to investigate other arthropods sharing the same habitat, e.g., spiders, pseudoscorpions, and beetles, because they are known to prey on soil-dwelling organisms, e.g., flea larvae, of all kinds. Potential predators may have the same *Wolbachia* strains as fleas. Because fleas are closely related to Boreidae–Mecoptera, it may be of interest to determine if this group is also naturally infected with *Wolbachia*. This might give indications concerning the time line of *Wolbachia* infection among Siphonaptera and clarify whether it is derived from a single ancestral infection event.

The entire clade of siphonapteran *Wolbachia* is highly unresolved, and the lack of data derived from several individuals of a population make a prediction regarding phylogenetic host–parasite relations and potential horizontal transmissions within the Siphonaptera not feasible at this point.

The essential problems of interpreting currently available arthropod *Wolbachia* phylogenies is not only the fact that the available sequence data constitutes only a fraction of all potential insect hosts (sample size) but also the difficulty of relating phylogenetic events of *Wolbachia* speciation to their host association, which is often obscured and altered because of recurrent horizontal transmissions. Only the extension of the data set, involving not only more species and localities but also more informative genes, will provide answers to yet unresolved questions.

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#### LITERATURE CITED

- ALTSCHUL, S. F., T. L. MADDEN, A. A. SCHÄFER, J. ZHANG, Z. ZHANG, W. MILLER, AND D. J. LIPMAN. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acid Research* **25**: 3389–3402.
- ANDERSON, B. E., AND T. L. KARR. 2001. *Wolbachia*: Evolutionary novelty in a rickettsial bacteria. *BioMed Central Evolutionary Biology* **1**: 775–780.
- BOUCHON, D., T. RIGAUD, AND P. JUCHAULT. 1998. Evidence for widespread *Wolbachia* infection in isopod crustaceans: Molecular identification and host feminization. *Proceedings of the Royal Society of London Series B* **265**: 1081–1090.
- CORDAUX, R., A. MICHEL-SALAZAT, AND D. BOUCHON. 2001. *Wolbachia* infection in crustaceans: Novel hosts and potential routes for horizontal transmission. *Journal of Evolutionary Biology* **14**: 237–243.
- CRANDALL, K. A. 1996. Multiple interspecies transmission of human and simian T-cell leukaemia/lymphoma virus type I sequences. *Molecular Biology and Evolution* **13**: 115–131.
- FARRIS, J. S., V. A. ALBERT, M. KALLERSJO, D. LIPSCOMB, AND A. G. KLUGE. 1996. Parsimony jackknifing outperforms neighbor-joining. *Cladistics* **5**: 417–419.
- FELSENSTEIN, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**: 783–791.
- GORHAM, C. H., Q. Q. FANG, AND L. A. DURDEN. 2003. *Wolbachia* endosymbionts in fleas (Siphonaptera). *Journal of Parasitology* **89**: 283–289.
- HASEGAWA, M., H. KISHINO, AND T. YANO. 1985. Dating the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* **22**: 160–174.
- HURST, G. D. D., F. M. JIGGINS, AND J. H. G. SCHULENBERG. 1999. Male killing *Wolbachia* in two species of insects. *Proceedings of the Royal Society of London Series B* **266**: 735–740.
- JEYAPRAKASH, A., AND M. A. HOY. 2000. Long PCR improves *Wolbachia* DNA amplification: Wsp sequences found in 76% of sixty-three arthropod species. *Insect Molecular Biology* **9**: 393–405.
- JIGGINS, F. M. 2000. The cause and evolutionary consequences of sex ratio distortion in African butterflies. Ph.D. Thesis. University of Cambridge, Cambridge, U.K., 215 p.
- , J. K. BENTLEY, M. E. N. MAJERUS, AND G. D. D. HURST. 2002. Recent changes in phenotype and patterns of host specialization in *Wolbachia* bacteria. *Molecular Ecology* **11**: 1275–1283.
- JOHANOVICZ, D. L., AND M. A. HOY. 1996. *Wolbachia* in a predator-prey system: 16S ribosomal DNA analysis of two phytoseiids (Acari: Phytoseiidae) and their prey (Acari: Tetranychidae). *Genetics* **89**: 435–441.
- LANE, D. J. 1991. 16S/23S rRNA sequencing. *In Nucleic acid techniques in bacterial systematics*, E. Stackebrandt and M. Goodfellow (eds.). John Wiley & Sons, Chichester, U.K., p. 115–175.
- O'NEILL, S. L., R. GIORDANO, A. M. E. COLBERT, T. L. KARR, AND H. M. ROBERTSON. 1992. 16S-ribosomal RNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proceedings of the National Academy of Sciences of the United States of America* **89**: 2699–2702.
- POSADA, D., AND K. A. CRANDALL. 1998. Modeltest: Testing the model of DNA substitution. *Bioinformatics* **14**: 817–818.
- , ———, AND A. R. TEMPLETON. 2000. GeoDis: A program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology* **9**: 487–488.
- ROKAS, A., R. J. ATKINSON, J. L. NIEVES-ALDREY, S. WEST, AND G. N. STONE. 2002. The incidence and diversity of *Wolbachia* in gall-wasps (Hymenoptera; Cynipidae) on oak. *Molecular Ecology* **11**: 1815–1829.
- ROUSSET, F., D. BOUCHON, B. PINTUREAU, P. JUCHAULT, AND M. SOLIGNAC. 1992. *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. *Proceedings of the Royal Society of London Series B* **250**: 91–98.
- SAITOU, N., AND M. NEI. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**: 406–425.
- SCHILTHUISEN, M., AND R. STOUTHAMMER. 1997. Horizontal transmission of parthenogenesis-inducing microbes in *Trichogramma* wasps. *Proceedings of the Royal Society of London Series B* **264**: 361–366.
- SINKINS, S. P., AND S. L. O'NEILL. 2000. *Wolbachia* as a vehicle to modify insect populations. *Insect Transgenic Methods and Applications* **10**: 271–287.
- STOUTHAMMER, R., J. A. J. BREEUWER, R. F. LUCK, AND J. H. WERREN. 1993. Molecular identification of microorganisms associated with parthenogenesis. *Nature* **361**: 66–68.
- SWOFFORD, D. L. 2000. PAUP\*: Phylogenetic analysis using parsimony (\*and other methods). Sinauer Associates, Sunderland, Massachusetts.
- TEMPLETON, A. R. 1998. Nested clade analysis of phylogeographic data: Testing hypothesis about gene flow and population history. *Molecular Ecology* **7**: 381–397.
- , K. A. CRANDALL, AND C. F. SING. 1992. A cladistic analysis of

- phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* **132**: 619–633.
- WEST, S. A., J. M. COOK, J. H. WERREN, AND H. C. J. GODFRAY. 1998. *Wolbachia* in two insect-parasitoid communities. *Molecular Ecology* **7**: 1457–1465.
- YANG, Z. 1996. Among-site rate variation and its impact on phylogenetic analysis. *Trends in Ecology and Evolution* **11**: 367–372.
- ZHOU, W. F., F. ROUSSET, AND S. O'NEILL. 1998. Phylogeny and PCR based classification of *Wolbachia* strains using *wsp* gene sequences. *Proceedings of the Royal Society of London Series B* **265**: 509–515.