

Incidence of *Wolbachia* and *Cardinium* Endosymbionts in the *Osmia* Community in Korea

Gilsang Jeong¹, Kyeongyong Lee², Jiyoung Choi¹, Seokjo Hwang¹, Byeongdo Park¹, Wontae Kim¹, Youngcheol Choi¹, Ingyun Park², and Jonggill Kim^{1*}

¹Laboratory of Environmental Entomology, ²Laboratory of Pollinating Insect, Department of Agricultural Biology, National Academy of Agricultural Science, Rural Development Administration, Suwon 441-853, Republic of Korea

(Received August 6, 2008 / Accepted November 20, 2008)

Sex ratio distorting endosymbionts induce reproductive anomalies in their arthropod hosts. They have recently been paid much attention as firstly texts of evolution of host-symbiont relationships and secondly potential biological control agents to control arthropod pests. Among such organisms, *Wolbachia* and *Cardinium* bacteria are well characterized. This study aims at probing such bacteria in the *Osmia* community to evaluate their potential utilization to control arthropod pests. Among 17 PCR tested species, *Osmia cornifrons* and a parasitic fly are infected with *Wolbachia* and a mite species is infected with *Cardinium*. Phylogenetic tree analyses suggest that horizontal transfer of the bacteria occurred between phylogenetically distant hosts.

Keywords: *Wolbachia*, *Cardinium*, sex ratio distortion, biological control, phylogenetic analyses

Vertically transmitted endosymbiotic microorganisms that distort sex of their hosts for increasing their frequency are widespread in arthropods (Werren *et al.*, 1995; Weeks *et al.*, 2003; Zchori-Fein and Perlman, 2004; Hilgenboecker *et al.*, 2008). Most notable are *Wolbachia* and *Cardinium* bacteria. The two bacteria have abilities to induce several distinct reproductive anomalies in their hosts (Stouthamer *et al.*, 1999; Zchori-Fein and Perlman, 2004; Jeong and Suh, 2008) (Table 1). Researchers have envisioned application potential of such endosymbionts to control pest species as an environmentally sound tool so called paratransgenesis with recent advances in biotechnology and better understanding of evolutionary relationships between endosymbionts and their hosts (Beard *et al.*, 1998). Currently, at least in the laboratory condition, theories and protocols are well established to control insect pests by using the *Wolbachia* bacterium inducing cytoplasmic incompatibility (CI) where an uninfected female mated with an infected male produces no offspring (O'Neill *et al.*, 1997; Zabalou *et al.*, 2004).

Species of genus *Osmia* are important pollinators of plants of family Rosaceae. Some species of *Osmia* have been employed as pollinators for almonds, apples, pears, berry crops

(Vicens and Bosch, 2000; Park, 2008, pers. Comm.). The use of the genus takes place in Europe, USA, and Japan (Krunić *et al.*, 2005). Mass rearing of the species takes place in two main facilities in Sangju and Yecheon, North Kyung-sang province in Korea. However it is largely hampered by three main factors, first underdevelopment of rearing technique in protected environments, second pests such as competitors, predators, parasites in a broad sense, and lastly male biased sex ratio (Torchio and Tepedino, 1980; Bosch and Vicens, 2005; Krunić *et al.*, 2005)

In this study the sex ratio distorting endosymbionts e.g. *Wolbachia* and *Cardinium* were probed in the *Osmia* community. We report that *Osmia cornifrons* and a dipteran parasite are infected with *Wolbachia* and a parasitic mite species is infected with *Cardinium*. Partial 16S rRNA sequences of the bacteria suggest that the bacterial phylogenies are largely incongruent with their host's phylogenies. The results here will be a foundation of potential utilization of such endosymbionts in controlling pests in the *Osmia* community.

Materials and Methods

Specimen collection and identification

All specimens were provided by two collection facilities (Center for Agricultural Technology) in Sangju and Yecheon, North Kyung-sang province. These facilities installed bamboo nests in various locations near apple orchards and in forests from April to July in 2007. The bamboo nests were collected and preserved intact at the facilities until January 2008. The nests were open longitudinally and all pests such as nest competitors, predators, parasites were separated from *Osmia* species. Once the specimens were identified by their typological characters at NIAST, they were preserved in 70% EtOH (Sigma, USA) at -20°C.

Table 1. Reproductive anomalies induced by the two endosymbionts

Infection phenotype	<i>Wolbachia</i>	<i>Cardinium</i>
Cytoplasmic incompatibility	√	√
Feminization	√	√
Male killing	√	√
Parthenogenesis	√	√

* To whom correspondence should be addressed.
(Tel) 82-31-290-8545; (Fax) 82-31-290-8543
(E-mail) kjk1027@rda.go.kr

Table 2. Primers used to determine infection

Primers	Primer sequences (5'-3')	References
W-Spec f	CAT ACC TAT TCG AAG GGA TAG	<i>Wolbachia</i> FIBR (http://troi.cc.rochester.edu/~wolb/FIBR/)
W-Spec r	AGC TTC GAG TGA AAC CAA TTC	<i>Wolbachia</i> FIBR (http://troi.cc.rochester.edu/~wolb/FIBR/)
16S rRNA f (27)	AGA GTT TGA TCM TGG CTC AG	Weisburg <i>et al.</i> (1991)
16S rRNA r (1513)	ACG GYT ACC TTG TTA CGA CTT	Weisburg <i>et al.</i> (1991)
16S Wol f (176)	TTG TAG CCT GCT ATG GTA TAA YT	O'Neill <i>et al.</i> (1992)
16S Wol r (1012)	GAA TAG GTA TRA TTT YCA TGT	O'Neill <i>et al.</i> (1992)
Ch-f	TAC TGT AAG AAT AAG CAC CGG C	Zchori-Fein and Perlman (2004)
Ch-r	GTG GAT CAC TTA ACG CTT TCG	Zchori-Fein and Perlman (2004)
CLO-r1	GCCACTGTCTTCAAGCTCTACCAAC	Gotoh <i>et al.</i> (2007)

DNA extraction

Genomic DNA was extracted from each individual specimen using a commercial kit by following the manufacturer's instruction (QIAamp DNA mini kit, QIAGEN, Germany). When a specimen was longer than 5 mm, abdomen of the specimen was cut with a razor and was used to extract DNA. Other than that, DNA was extracted from the whole body. The genomic DNA was kept at -20°C until further analyses.

PCR screening and sequencing

Screening

All primers used for the experiments are listed in the Table 2. The W-Spec primer set was used only to determine the *Wolbachia* infection status of specimens (*Wolbachia* FIBR). Temperature profile for W-Spec diagnostic PCR is a 2 min pre-dwell at 94°C followed by 38 cycles of 30 sec at 94°C, 45 sec at 55°C, 1:30 min at 72°C and a post-dwell period of 10 min at 72°C. For *Cardinium* the Ch primer set was used to determine infection of the bacterium (Zchori-Fein and Perlman, 2004). Temperature profile for the PCR is a 2 min pre-dwell at 95°C followed by 31 cycles of 30 sec at 95°C,

30 sec at 57°C, 30 sec at 72°C and a post-dwell period of 5 min at 72°C. When a species was positive to either one of the bacteria, two more individuals of the species were PCR tested to confirm its infection status.

Sequencing

For *Wolbachia* nested PCR was performed following Weeks *et al.* (2003). The initial PCR was performed by using the 16S rDNA primer set (27f and 1513r) (Weisburg *et al.*, 1991). Temperature profile for the PCR is a 4 min pre-dwell at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 52°C, 1 min at 72°C and a post-dwell period of 5 min at 72°C. Then the *Wolbachia* specific PCR was followed by using the *Wolbachia* specific 16S rDNA primer set (176f and 1012r) taken from O'Neill *et al.* (1992). Two microliter of initial PCR product was used as template DNA in the *Wolbachia* specific PCR. Temperature profile for the PCR is a 4 min pre-dwell at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C and a post-dwell period of 5 min at 72°C.

For *Cardinium* the Chf and CLO-r1 primer combination was used to obtain 16S rDNA sequences (Duron *et al.*, 2008).

Table 3. Infection status of species in the *Osmia* community

Species	Family	Order	Collection	<i>Wolbachia</i>	<i>Cardinium</i>
<i>Osmia satoi</i>	Megachilidae	Hym.	Sangju		
<i>Osmia cornifrons</i>	Megachilidae	Hym.	Sangju	○	
<i>Osmia pedicornis</i>	Megachilidae	Hym.	Yecheon		
<i>Osmia taurus</i>	Megachilidae	Hym.	Yecheon		
Unidentified	Vespidae	Hym.	Sangju		
Unidentified	Vespidae	Hym.	Sangju		
<i>Oriencistrocerus drewseni</i>	Eumenidae	Hym.	Sangju		
<i>Nomada</i> sp.	Eumenidae	Hym.	Sangju		
<i>Nomada</i> sp.	Eumenidae	Hym.	Sangju		
<i>Nomada</i> sp.	Eumenidae	Hym.	Sangju		
Unidentified	?	Hym.	Sangju		
Unidentified	Apidae	Hym.	Sangju		
Unidentified	Apidae	Hym.	Sangju		
Unidentified	Tachinidae	Dip.	Sangju	○	
Unidentified	Dermastidae	Col.	Sangju		
Unidentified	Ptinidae	Col.	Sangju		
Mite sp.	?	Ast.	Sangju		○
Infection frequency (%)				11.7	5.9

Hym., Hymenoptera; Dip., Diptera; Col., Coleoptera; Ast., Astigmata

Temperature profile for the PCR is a 4 min pre-dwell at 94°C followed by 35 cycles of 30 sec at 94°C, 30 sec at 54°C, 1 min at 72°C, and a post-dwell period of 5 min at 72°C. In all PCRs the total volume was 25 µl containing 1× PCR buffer (TaKaRa, Japan), 0.5 U of EX *Taq* polymerase (TaKaRa), 0.05 mM of each dNTP (TaKaRa), 0.2 µM of each primer (Bioneer, Korea). All PCR reactions were performed on a thermocycler (Biometra, Germany).

Phylogenetic analysis

The 16S rRNA sequences of the bacteria were retrieved from GenBank and aligned manually using Bioedit version 7.0. The neighbor-joining phylogenies without gaps were constructed using MEGA version 4.0.2 with 500 replicates of bootstrap to compute standard error of distance estimates (Tamura *et al.*, 2007). The Jukes-Cantor model was used to calculate the nucleotide substitution distances (Jukes and Cantor, 1969).

Results

Species composition in the *Osmia* community

The community consists of 17 species of 4 orders. There are 13 hymenopteran species including nest competitors

and an unidentified parasitoid. Others include a parasitic dipteran insect, two predatory coleopteran insects and a parasitic mite. The four *Osmia* species have an important role in apple pollination. On the other hand other hymenopteran insects compete for nests except the unidentified parasitoid (Table 3). Vespidae and eumenid species are also predators of other insects. However they are not categorized as predators in this study, because they do not hunt *Osmia* species but compete for nest sites. According to a survey there are 20 species in 8 orders associated in the *Osmia* community in Japan (Maeta, 1978). It is expected that more species have yet to be found in the Korean *Osmia* community.

Incidence of sex ratio distorting microorganisms

The results show that *O. cornifrons* and the parasitic fly species are infected with *Wolbachia* (GenBank accession no. EU930865 and EU930864, respectively). On the other hand *Cardinium* is detected only from the mite species (Genbank accession no. EU930867). There is no specimen that harbored both bacteria (Table 3). Incidence of the bacteria is lower than the results from the previous surveys (Werren and Windsor, 2000; Weeks *et al.*, 2003; Zchori-Fein and Perlman, 2004; Duron *et al.*, 2008).

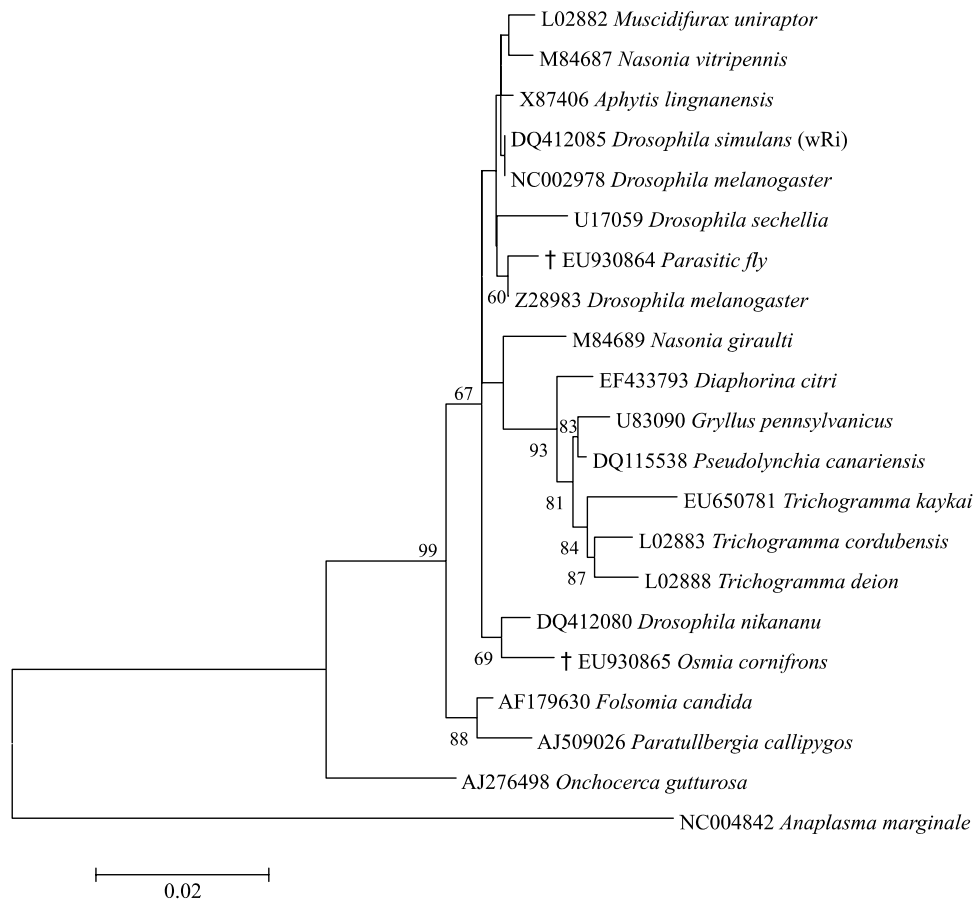


Fig. 1. Neighbor-joining tree based on partial 16S rRNA sequences of *Wolbachia* bacterium from various hosts. GenBank accession no. and host names appear from which each *Wolbachia* strain is isolated. † indicates that the sequences are from this study. Bootstrap support is indicated on each branch ($\geq 60\%$ only). The closely related *Anaplasma marginale* was used as the outgroup.

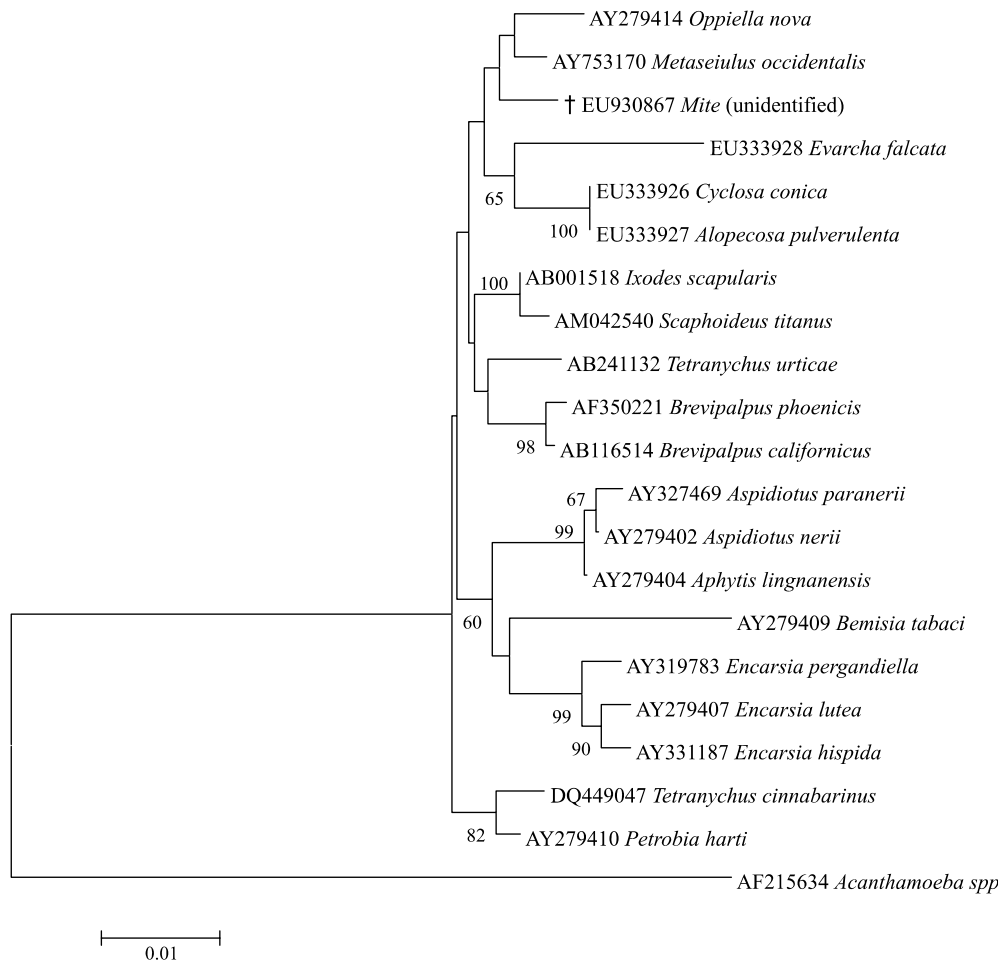


Fig. 2. Neighbor-joining tree based on partial 16S rRNA sequences of *Cardinium* bacterium from various hosts. GenBank accession no. and host names appear from which each *Cardinium* strain is isolated. † indicates that the sequence is from this study. Bootstrap support is indicated on each branch ($\geq 60\%$ only). The closely related *Acanthamoeba* was used as the outgroup.

Phylogenetic analyses

As seen in other phylogenetic analyses of the bacteria, there is no evidence of co-cladogenesis between hosts and the bacterial strains. This implies that even though there was no horizontal transfer of the bacteria within the community, horizontal transmission occurred between phylogenetically distant hosts (Fig. 1 and 2).

Discussion

Probing sex ratio distorting symbiotic microorganisms should be the very first step to understand coevolution of such organisms with arthropods that have had huge impacts on their hosts' reproductive biology and immune system. They are widespread in arthropod hosts. It may be at global equilibrium around 25~40% of infection frequency at least in the case of *Wolbachia*, even though the meta analysis study by Hilgenboecker *et al.* (2007) suggests even higher infection frequency. In our survey infection frequency of *Wolbachia* is lower than expected. It does not imply that *Wolbachia* infection frequency should be low in Korea. It

rather means that this is most likely caused by the limited sampling within the *Osmia* community. In most of other infection surveys specimens were randomly sampled (Werren *et al.*, 1995; Weeks *et al.*, 2003; Zchori-Fein and Perlman, 2004; Duron *et al.*, 2008). More infection cases are expected with random sampling method in the future survey.

Osmia species usually show male biased sex ratio (Torchio and Tepedino, 1980; Park, 2008, pers comm). *Wolbachia* infection induces female biased sex ratio except CI, since the bacterium has evolved to increase the chance of being vertically transmitted via cytoplasm (Stouthamer *et al.*, 1999; Jeong and Suh, 2008). Therefore *Wolbachia* may not be involved in producing more males in the species. Instead the male biased sex ratio may be due to balanced resource investments to both sexes by producing more male offspring under the assumption that the species are panmictic as predicted by Fisher (1930).

There is little information on the parasitic fly and the mite species. In other dipteran species *Wolbachia* infection induces either CI or male killing (O'Neill *et al.*, 1997). Therefore it is probable that *Wolbachia* may induce a similar

effect in the fly species. Strains of the *Cardinium* bacterium induce feminization and CI in mites (Weeks *et al.*, 2001; Gotoh *et al.*, 2007). In the mite *Cardinium* may induce CI rather than feminization, since both sexes are found in the mite. This speculation is supported by the fact that two mite species associated with *Osmia* species in Japan show 1:1 sex ratio with complicated reproductive strategies (Qu *et al.*, 2003). However we do not exclude asymptotic infection of the bacterium.

This study is conducted to probe sex ratio distorting microorganisms as potential biological control agents in the *Osmia* community. Based on the results here, future study should determine what effects the infection of the bacteria has on their hosts' reproduction. Furthermore we will be able to establish new strategies to control pests by utilizing such endosymbionts.

Acknowledgements

The authors thank the centers for agricultural technology in Sangju and Yecheon, Kyungsang North province for providing specimens. Two anonymous reviewers are deeply appreciated for their useful comments.

This study is supported by Rural Development Administration (RDA) fund, Republic of Korea (LS0704).

References

- Beard, C.B., R.V. Durvasula, and F.F. Richards. 1998. Bacterial symbiosis in arthropods and the control of disease transmission. *Emerg. Infect. Dis.* 4, 581-591.
- Bosch, J. and N. Vicens. 2005. Sex allocation in the solitary bee *Osmia cornuta*: do females behave in agreement with Fisher's theory? *Behav. Ecol. Sociobiol.* 59, 124-132.
- Duron, O., G.D.D. Hurst, E.A. Hornett, J.A. Josling, and J. Engels-tadtter. 2008. High incidence of the maternally inherited bacterium *Cardinium* in spiders. *Mol. Ecol.* 17, 1427-1437.
- Fisher, R.A. 1930. The genetical theory of natural selection. Clarendon, Oxford, UK.
- Gotoh, T., H. Noda, and S. Ito. 2007. *Cardinium* symbionts cause cytoplasmic incompatibility in spider mites. *Heredity* 98, 13-20.
- Hilgenboecker, K., P. Hammerstein, P. Schlattmann, A. Telschow, and J.H. Werren. 2008. How many species are infected with *Wolbachia*?-a statistical analysis of current data. *FEMS Microbiol. Lett.* 281, 215-220.
- Jeong, G. and E. Suh. 2008. *Wolbachia* induced reproductive anomalies and their future applications. *Entomol. Res.* 38, 41-48.
- Jukes, T.H. and E.E. Cantor. 1969. Evolution of protein molecules, p. 21-132. In H.N. Munro (ed.), *Mammalian Protein Metabolism*. Vol. 3. Academic Press, New York, USA.
- Krunic, M., L. Stanisavljevic, M. Pinzauti, and A. Felicioli. 2005. The accompanying fauna of *Osmia cornuta* and *Osmia rufa* and effective measures of protection. *Bull. Insectol.* 58, 141-152.
- Maeta, Y. 1978. Comparative studies on the biology of the bees of the genus *Osmia* of Japan, with special reference to their managements for pollinations of crops (Hymenoptera: Megachilidae) (in Japanese with abstract in English). *Bull. Tohoku. Nat. Agric. Exp. Sta.* 57, 117-145.
- O'Neill, S.L., R. Giordano, A.M.E. Colbert, T.L. Karr, and H.M. Robertson. 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc. Natl. Acad. Sci. USA* 80, 2699-2702.
- O'Neill, S.L., A.A. Hoffman, and J.H. Werren. 1997. Influential passengers-inherited microorganisms and arthropod reproduction. Oxford University Press, Oxford, UK.
- Qu, D., Y. Maeta, K.J. Nakatsuka, K. Kitamura, and M. Goubara. 2003. Reproductive strategy in the two species of cleptoparasitic astigmatid mites, *Chaetodactylus nipponicus* and *Tortonia* sp. (Acari: Chaetodactylidae and Suidasiidae), infesting *Osmia cornifrons* (Hymenoptera: Megachilidae) II, life history, phoretic positions, development and reproductivity. *Jpn. J. Ent.* 6, 55-73.
- Stouthamer, R., J.A.J. Breeuwer, and G.D.D. Hurst. 1999. *Wolbachia pipientis*: Microbial manipulator of arthropod reproduction. *Annu. Rev. Microbiol.* 53, 71-102.
- Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA 4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596-1599.
- Torchio, P.F. and V.J. Tepedino. 1980. Sex ratio, body size and seasonality in a solitary bee, *Osmia lignaria propinqua* Cresson (Hymenoptera: Megachilidae). *Evolution* 34, 993-1003.
- Vicens, N. and J. Bosch. 2000. Nest site orientation and relocation of population of the orchard pollinator *Osmia cornuta* (Hymenoptera: Megachilidae). *Environ. Entomol.* 29, 69-75.
- Weeks, A.R., F. Marec, and J.A.J. Breeuwer. 2001. A mite species that consists entirely of haploid females. *Science* 292, 2479-2482.
- Weeks, A.R., R. Velten, and R. Stouthamer. 2003. Incidence of a new sex-ratio-distorting endosymbiotic bacterium among arthropods. *Proc. R. Soc. Lond. B.* 270, 1857-1865.
- Weisburg, W.G., S.M. Barns, D.A. Pelletier, and D.J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173, 697-703.
- Werren, J.H. and D.M. Windsor. 2000. *Wolbachia* infection frequencies in insects: evidence of a global equilibrium? *Proc. R. Soc. Lond. B.* 267, 1277-1285.
- Werren, J.H., D.M. Windsor, and L. Guo. 1995. Distribution of *Wolbachia* among neotropical arthropods. *Proc. R. Soc. Lond. B.* 262, 197-204.
- Zabalou, S., M. Riegler, M. Theodorakopoulou, C. Stauffer, C. Savakis, and K. Bourtizis. 2004. *Wolbachia*-induced cytoplasmic incompatibility as a means for insect pest population control. *Proc. Natl. Acad. Sci. USA* 101, 15042-15045.
- Zchori-Fein, E. and S.J. Perlman. 2004. Distribution of the bacterial symbiont *Cardinium* in arthropods. *Mol. Ecol.* 13, 2009-2016.