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VARIATIONS IN THE SENSITIVITY OF DIFFERENT PRIMERS FOR DETECTING *WOLBACHIA* IN *ANASTREPHA* (DIPTERA: TEPHRITIDAE)

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ABSTRACT

Wolbachia are endosymbiont bacteria of the family Rickettsiacea that are widespread in invertebrates and occur between 20% and 60% of Neotropical insects. These bacteria are responsible for reproductive phenomena such as cytoplasmic incompatibility, male killing, feminization and parthenogenesis. Supergroups A and B of *Wolbachia* are common in insects and can be identified using primers for 16S rDNA, *fts*Z and *wsp*; these primers vary in their ability to detect *Wolbachia*. The *fts*Z primer was the first primer used to detect *Wolbachia* in *Anastrepha* fruit flies. The primers for 16S rDNA, *fts*Z and *wsp* and the corresponding PCR conditions have been optimized to study the distribution of *Wolbachia* and their effect on the biology of *Anastrepha* in Brazil. In this work, we examined the ability of these primers to detect *Wolbachia* in *Anastrepha* populations from three regions in the State of São Paulo, southeastern Brazil. All of the samples were positive for *Wolbachia* supergroup A when screened with primers for 16S A rDNA and *wsp* A; the *wsp* B primer also gave a positive result, indicating cross-reactivity. The *fts*Z primer showed a poor ability to detect *Wolbachia* in *Anastrepha* and generated false negatives in 44.9% of the samples. These findings indicate that reliable PCR detection of *Wolbachia* requires the use of primers for 16S rDNA and *wsp* to avoid cross-reactions and false negatives, and that the *fts*Z primer needs to be redesigned to improve its selectivity.

Key words: *Anastrepha*, cross reaction, *fts*Z, *wsp*

INTRODUCTION

Wolbachia are intracellular obligatory bacteria of the family Rickettsiacea that occur in a wide range of arthropods and nematodes. These bacteria are maternally inherited by horizontal transmission (26, 29) and enhance their propagation by altering the reproductive system of their host in various ways, e.g., by cytoplasmic incompatibility, male killing, feminization and parthenogenesis (19, 24).

Molecular markers have been extensively used to detect *Wolbachia*. For example, primers for 16S rDNA (a ribosomal gene), *fts*Z (a regulatory gene of the bacterial cell cycle) and

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wsp (a gene for an cell membrane protein) have been used to study the phylogenetics of *Wolbachia* (2, 6, 18) and have led to the division of *Wolbachia* into eight taxonomic supergroups (A to H) (12, 13, 27). The initial studies in this field were done with 16S rDNA primers and identified two groups, A and B, that diverged about 50 million years ago and are widely distributed in insects (14, 27).

The *ftsZ* gene was extensively used by Werren and colleagues (27, 28) to detect *Wolbachia*, but several studies have demonstrated its low sensitivity (3, 9, 30, 31). This low sensitivity can lead to false negatives in bacterial detection when this primer is used alone, i.e., without another marker or technique to detect *Wolbachia*. The 16S and *wsp* primers have greater sensitivity for detecting *Wolbachia* (30, 31), although the *wsp* primer can generate false positives in discriminating between supergroups A and B (8). This lack of absolute specificity means that there is a need to use specific primers in the characterization of these bacteria.

Between 20% and 60% of Neotropical insects are infected with *Wolbachia* (28). Studies in different regions of Brazil have detected *Wolbachia* in fruit flies of the genus *Anastrepha* (3, 4, 17, 23). *Anastrepha* fruit flies are a major pest insect of Brazilian fruit crops because of the losses they cause to commercial fruit growers (5, 15). The presence of *Wolbachia* in *Anastrepha* is therefore of considerable interest since these bacteria may be exploited as biological controls of pest insects, as suggested by Bourtzis (1).

Coscrato (4) and Mascarenhas (17) showed that 16S and *wsp* primers varied in their ability to detect *Wolbachia* in different *Anastrepha* species; studies with other fruit fly genera, such as *Bactrocera* (8, 11, 25), *Rhagoletis cerasi* (20) and *Ceratitis capitata* (21), have reported similar findings. In this work, we examined the ability of 16S, *ftsZ* and *wsp* primers to detect *Wolbachia* in populations of *Anastrepha* from different regions of the State of São Paulo in southeastern Brazil.

MATERIALS AND METHODS

Sixty-six fruit fly larvae collected from guava (*Psidium guajava*) and chapeu-do-sol (*Terminalia catappa*) fruits in three regions of the State of São Paulo, southeastern Brazil (Table 1), were stored in boxes with vermiculite and transported to the laboratory, where the larvae were allowed to develop to pupal stage. The pupae were subsequently removed and allowed to grow to the adult stage for species identification, after which they were stored in 70% ethanol at -20°C.

Table 1. Number of individuals, host plants, collection sites and geographic location of the fruit fly populations examined in this study. All collection sites were in the State of São Paulo.

Taxon	Species	No. of individuals	Host plant	Collection site	Geographic location
fraterculus	*A.* sp. 1 *aff. fraterculus*	11	Guava (*Psidium guajava*)	Jacareí	23° 17´S; 46° 01´W
	A. sp. 1 *aff. fraterculus*	19	Guava (*Psidium guajava*)	Serra Negra	22° 35´S; 46° 50´W
	A. sp.2 *aff. fraterculus*	21	Chapéu-do-sol (*Terminalia catappa*)	Caraguatatuba	23° 39´S; 45° 25´W
	A. sp. 3 *aff. fraterculus*	24	Chapéu-do-sol (*Terminalia catappa*)		

DNA was extracted from each fly abdomen using the protocol described by Jowett (10), with some modifications. Amplification reactions were done as described below.

The reaction mixture for the *wsp* A and B primers consisted of 50 ng of DNA, 10 X buffer (Invitrogen), 1.0 µl of 50 mM $MgCl_2$, 0.4 µl of dNTPs (10 µM each), 0.5 µl of forward (F) primer (20 µM), 0.5 µl of reverse (R) primer (20 µM), 0.5 µl of *Taq* DNA polymerase (5 U/µl) (Invitrogen) and water in a final volume of 20 µl. The amplification reaction consisted of one cycle of 1 min at 94°C, 1 min at 58°C and 2 min at 72°C, followed by 35 cycles of 15 s at 94°C, 1 min at 58°C and 2 min at 72°C, and one cycle of 15 s at 94°C, 1 min at 58°C and 7 min at 72°C. These conditions yielded PCR products of ~600 base pairs (bp) (2, 32).

The reaction mixture for the *fts*Z A and B primers (6) consisted of 50 ng of DNA, 10 X buffer (Invitrogen), 0.6 µl of 50 mM $MgCl_2$, 0.4 µl of dNTPs (10 µM each), 0.5 µl of F primer (10 µM), 0.5 µl of R primer (10 µM), 0.5 µl of *Taq* DNA polymerase (5 U/µl) (Invitrogen) and water in a final volume of 20 µl. The amplification reaction consisted of an initial 4 min incubation at 94ºC, followed by one cycle of 1 min at 58ºC and 2 min at 72ºC, 38 cycles of 15 s at 94ºC, 1 min of 58ºC and 2 min at 72ºC, one cycle of 15 s at 94ºC and 1 min at 58ºC, with a final extension of 7 min at 72ºC. These conditions yielded fragments of 1043-1055 bp.

The reaction mixture for the 16S A and B rDNA primers (18) consisted of 50 ng of DNA, 10X buffer (Invitrogen), 0.75 µl of 50 mM $MgCl_2$, 0.5 µl of dNTPs (10 µM each), 0.35 µl of F primer (20 µM), 0.35 µl of R primer (20 µM), 0.25 µl of *Taq* DNA polymerase (5 U/µl) (Invitrogen) and water in a final volume of 25 µl. The amplification reaction consisted initially of 2 min at 95ºC, 35 cycles of 30 s at 95ºC, 1 min at 55ºC and 1 min at 72ºC, with a final extension of 3 min at 72ºC. These conditions yielded fragments of ~259 bp.

In all cases, the PCR products were analyzed by electrophoresis in 1% agarose gels in Tris-borate EDTA buffer (TBE 1X) containing 1% ethidium bromide. After electrophoresis, the gels were examined in ultraviolet light and documented with an Eagle Eye II photodocumentation system (Stratagene).

For sequencing, the PCR products were purified with GFX™ PCR DNA and gel band purification kits (Amersham Pharmacia Biotech), after which DNA (100 ng/µl) was mixed with 1.0 µl of buffer (1 M Tris-HCl, pH 9.0, containing 50 mM $MgCl_2$), 2.0 µl of Big Dye, 1.0 µl of primer (5 pmol/ul) and water in a final volume of 10 µl. Sequencing was done in a Genetic Analyzer 3100 (Applied Biosystems) sequencer. The sequences generated from these samples and the corresponding consensus sequences were assembled with phredPhrap/consed v. 14.0 and then used to search the National Center for Biotechnology Information (NCBI) database for homology with *Wolbachia* sequences; the searches were done using BLASTN (http://www.ncbi.nlm.nih.gov).

RESULTS AND DISCUSSION

Wolbachia was detected in all of the DNA fragments generated by the 16S and *wsp* primers in *Anastrepha* species 1, 2 and 3. The sequence of the DNA fragment amplified with the *wsp* primer showed 98% and 96% similarity with *Wolbachia* from *Anastrepha* sp.2 *aff. fraterculus* (EU116316.1) and *Wolbachia* from *Brugia pahangi (*AY527208.1), respectively. The results with the 16S rDNA and *wsp* primers allowed us to classify the bacteria as belonging to *Wolbachia* supergroup A. These results were similar to studies in other *Anastrepha* species (4, 17).

In some samples, the *wsp* B primer generated fragments that suggested the presence of supergroup B (figure 1A). However, this result was considered to be a false positive since no fragments were generated by the primer for 16S B rDNA (figure 1B). Kittayapong (11) and Ruang-Areerate (22) have previously shown that the *wsp* B primer yields false positive results for this *Wolbachia* supergroup because of cross-reactions; similar findings have been described by Coscrato (3) and Marcon (16). Together, these studies indicate that the

conclusive identification of *Wolbachia* supergroup A in *Anastrepha* requires the use of *wsp* and 16S rDNA primers.

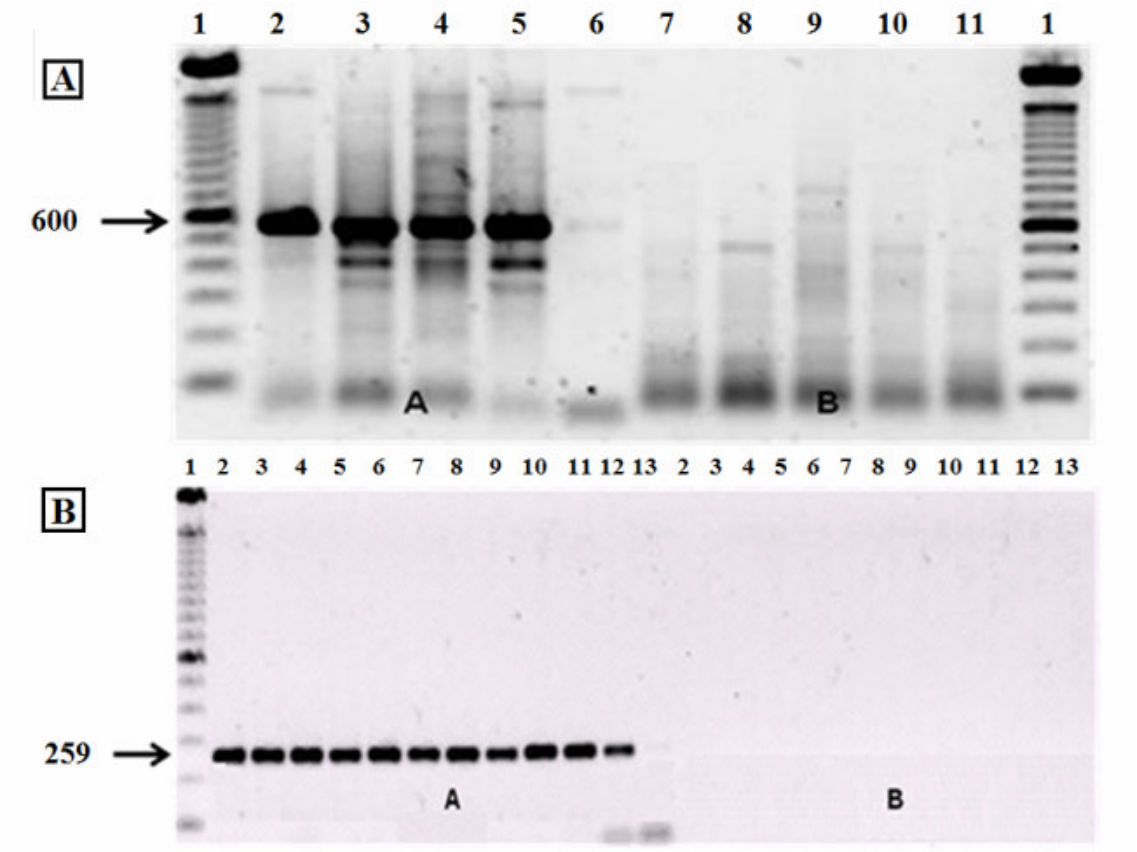

Figure 1. Electrophoresis of *Anastrepha* sp.1 samples in 1% agarose gels containing 1% ethidium bromide, after amplification. In (A), the primers used were *wsp* A in lanes 2A-6A and *wsp* B in lanes 7B-11B. In (B), the primers used were for 16S A rDNA in lanes 2A-13A and 16S B rDNA in lanes 2B-13B.

We also compared the results obtained for the *fts*Z primer with those for the 16S and *wsp* primers, particularly since data generated by the former primer have sometimes led to the misidentification of *Wolbachia*. No *Wolbachia* were detected in the 66 samples incubated with the *fts*Z primer, a finding in agreement with previous studies that have also used this primer to screen for these bacteria in other insects (7, 9, 30).

The *fts*Z gene is of particular importance because of the potential usefulness of its product for detecting *Wolbachia*, identifying supergroups and performing phylogenetic analyses. We therefore sought to optimize the PCR protocol for this primer by altering the concentrations of DNA, $MgCl_2$, dNTPs, primers and *Taq* DNA polymerase and quality of the DNA in order to detect *Wolbachia* in *Anastrepha* (Table 2). When the volume of DNA in the reaction was decreased to 3 µl (50 ng/ul), *Walbachia* was detected in 27% of *Anastrepha* sp.1 (Jacareí) samples (Table 3). Based on these results, this volume of DNA was used in subsequent reactions.

Table 2. Modifications in the reagent concentrations and volumes of the PCR reactions (*fts*Z I-VIII) used to detect *Wolbachia* in *Anastrepha* with the *fts*Z primer.

PCR reaction	Reagents	Concentration	Volume (µl)
*fts*Z I	DNA	50 ng/µl	3.00*
*fts*Z II	$MgCl_2$	50 mM	0.50*
	DNA	50 ng/µl	3.00
*fts*Z III	Primer F	8 µM*	0.50
	Primer R	8 µM*	0.50
	DNA	50 ng/µl	3.00*
*fts*Z IV	$MgCl_2$	50 mM	0.50*
	Primer F	8 µM*	0.50
	Primer R	8 µM*	0.50
	DNA	50 ng/µl	3.00
*fts*Z V	DNTP	10 mM	0.50*
	$MgCl_2$	50 mM	0.50*
	DNA	50 ng/µl	3.00*
*fts*Z VI	DNTP	10 mM	0.50*
	$MgCl_2$	50 mM	0.50*
	Primer F	8 µM*	0.50
	Primer R	8 µM*	0.50
	DNA	50 ng/µl	3.00*
*fts*Z VII	*Taq* DNA polymerase	1 unit	0.25*
	DNA	50 ng/µl	3.00
*fts*Z VIII	Fresh DNA*	50 ng/µl	5.00

*Alteration in PCR conditions (reagent concentration and/or volume).

Table 3. Efficiency of the *fts*Z primer in detecting *Wolbachia* in *Anastrepha* samples using the altered protocols (*fts*Z I-VIII) described in Table 2.

Protocols	Samples				Total
	***A*.sp.1 (Jac)**	***A*.sp.1 (SN)**	***A*.sp.2 (Cag)**	***A*.sp.3 (Cag)**	
*fts*Z I	27%	-	-	-	4%
*fts*Z II	-	-	-	-	-
*fts*Z III	-	-	-	-	-
*fts*Z IV	-	-	-	-	-
*fts*Z V	-	-	-	-	-
*fts*Z VI	-	-	-	-	-
*fts*Z VII	63.3%	42.1%	40.0%	45.0%	44.9%
*fts*Z VIII	55%	-	n	n	20.2%

Collection sites: Jac – Jacareí; SN – Serra Negra; Cag – Caraguatatuba.
(-): absence of fragment.
(n): not tested with the protocol indicated.

No *Wolbachia* were detected with protocols *fts*Z II, III, IV, V and VI (Table 2), indicating that the alterations incorporated in these reactions did not improve the efficiency of detection. In reaction *fts*Z VII in which the volume of *Taq* DNA polymerase was changed and the number of cycles was increased from 35 to 40 (in order to enhance the number of DNA fragments) (Table 2), *Wolbachia* was detected in 44.9% of the samples (Table 3). However, this protocol showed poor reproducibility for the same sample analyzed at different times (Figure 2A, B). Similar findings were reported by Jeyaprakash and Hoy (9) for other arthropod species and these authors proposed that DNA present in the reaction could interfere with *Taq* DNA polymerase activity to generate false negatives.

Werren and Windsor (30) observed that the quality of DNA was a determinant factor in the successful detection of *Wolbachia* with the *fts*Z primer and recommended that only newly extracted DNA be used for the PCR, i.e., one should avoid using DNA stored at -20°C. To examine the influence of DNA quality on the detection of *Wolbachia* with the *fts*Z primer we extracted DNA from *Anastrepha* sp.1 (Jacareí and Serra Negra) and used it in protocol VIII, along with the *wsp* and 16S rDNA primers. In these conditions, *Walbachia* was detected in 20.2% of the samples screened with the *fts*Z primer (Table 3), whereas all of the samples tested with the *wsp* and 16S rDNA primers were positive for the bacteria. These findings confirm the low sensitivity of the *fts*Z primer in detecting *Wolbachia* in *Anastrepha*, despite the alterations in the extraction and amplification protocols.

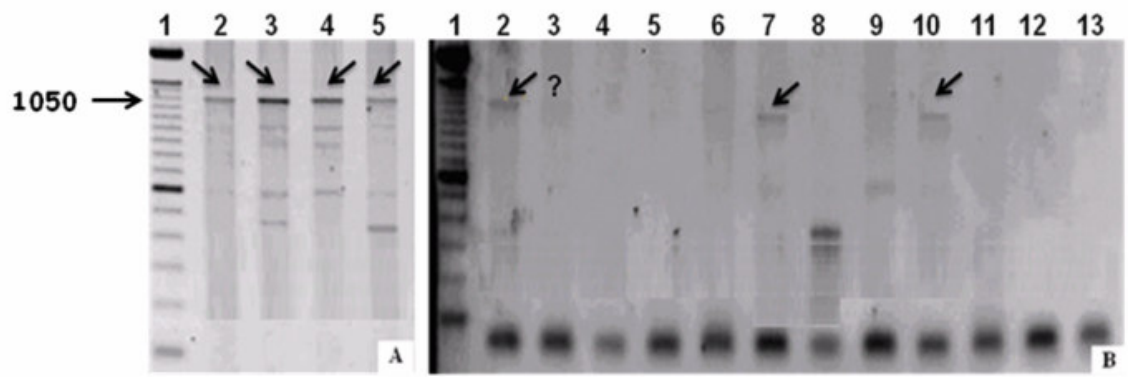

Figure 2. Electrophoresis of *Anastrepha* sp. 1 (Jacareí) samples in 1% agarose gels containing 1% ethidium bromide, after amplification with the primers *fts*Z (A) and *fts*Z (B). The *Anastrepha* sp.1 samples are in lanes 2-5 in (A) and lanes 2-13 in (B). Lane 1 - 100 bp ladder. Arrows indicate the presence of fragments. ? = presence of fragment in column B3 uncertain.

Together, the results of this study indicate that the most efficient way of detecting *Wolbachia* in *Anastrepha*, and of identifying the relevant supergroup and making phylogenetic inferences, is through the combined use of 16S rDNA and *wsp* primers. The 16S rDNA primer can be used by itself to detect *Wolbachia* and identify supergroups. However, since this primer is for preserved gene it is inappropriate for phylogenetic and population analyses. This limitation can be overcome by concomitant use of the *wsp* primer, which by itself is inappropriate for identifying *Wolbachia* supergroups A and B. The variability of *wsp* makes primers of this gene particularly useful for phylogenetic and population analyses.

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REFERENCES

1. Bourtzis, K. (2008). *Wolbachia*-based technologies for insect pest population control. *Adv. Exp. Med. Biol.* 627, 104-113.
2. Braig, H.R.; Zhou, W.; Dobson, S.; O'Neil, S.L. (1998). Cloning and characterization of the gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia*. *J. Bacteriol*. 180, 2373-2378.
3. Coscrato, V.E. (2006). Detecção e filogenia da bactéria endossimbionte *Wolbachia* em espécies de moscas-das-frutas do gênero *Anastrepha* e *Ceratitis* (Diptera: Tephritidae). Botucatu – S.P., Brazil. 144 p. (PhD Dissertation. Instituto de Biociências. UNESP).
4. Coscrato, V.E.; Braz, A.S.; Perondini, A.L.P.; Selivon, D.; Marino, C.L. (2009). *Wolbachia* in *Anastrepha* fruit flies (Diptera: Tephritidae). *Curr. Microbiol.* 59, 295-301.
5. Duarte, A.L. and Malavasi, A. (2000). Tratamentos quarentenários. *In*: Malavasi, A. and Zucchi, R.A. (eds). Moscas-das-frutas de importância econômica no Brasil (conhecimento básico e aplicado). FAPESP-Holos, Ribeirão Preto, Brazil, p.187-192.
6. Holden, P.R.; Brookfield, J.F.Y.; Jones, P. (1993). Cloning and characterization of an *ftsZ* homologue from a bacterial symbiont of *Drosophila melanogaster*. *Mol. Gen. Genet*. 240, 213-220.
7. Hong, X.Y.; Gotoh, T.; Noda, H. (2002). Sensitivity comparison of PCR *primers* for detecting *Wolbachia* in spider mites. *Appl. Entomol. Zool*. 37, 379-383.
8. Jamnongluk, W.; Kittayapong, P.; Baimai, V.; O'Neill. S.L. (2002). *Wolbachia* infections of tephritid fruit flies: molecular evidence for five distinct strains in a single host species. *Curr. Microbiol*. 45, 255-260.
9. Jeyaprakash, A. and Hoy, M.A. (2000). Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. *Insect Mol. Biol*. 9, 393-405.
10. Jowett, T. (1986). Preparation of nucleic acids. In: Roberts, D.B. (ed). *Drosophila*: a practical approach. Oxford University Press, Oxford. p. 275-286.
11. Kittayapong, P.; Milne, J.R.; Tigvattananont, S.; Baimai, V. (2000). Distribution of the reproduction-modifying bacteria, *Wolbachia*, in natural populations of tephritid fruit flies in Thailand. *Sci. Asia*. 26, 93-103.
12. Lo, N.; Casiraghi, M.; Salati, E.; Bazzocchi, C.; Bandi, C. (2002). How many *Wolbachia* supergroups exist? *Mol. Biol. Evol*. 19, 341-346.
13. Lo, N.; Paraskevopoulos, C.; Bourtzis, K.; O'Neill, S.L.; Werren, J.H.; Bordenstein, S.R.; Bandi, C. (2007). Taxonomic status of the intracellular bacterium *Wolbachia pipientis*. *Int. J. Syst. Evol. Microbiol*. 57, 654-657.
14. Lukenhaus, J.F. (1990). Regulation of cell division in *E. coli*. *Trends Genet*. 6, 22-25.
15. Malavasi, A.; Zucchi, R.A.; Sugayama, R.L. (2000). Biogeografia, *In*: Malavasi, A. and Zucchi, R.A. (eds). Moscas-das-frutas de importância econômica no Brasil (conhecimento básico e aplicado). FAPESP-Holos, Ribeirão Preto, Brazil, p. 93-98.
16. Marcon, H.S. (2009). Identificação da bactéria endossimbionte *Wolbachia* em populações de moscas-das-frutas do complexo *Anastrepha fraterculus* (Diptera: Tephritidae). Botucatu – S.P., Brazil. 110 p. (MSc Dissertation. Instituto de Biociências. UNESP).
17. Mascarenhas, R.O. (2007). Endossimbionte *Wolbachia* em moscas-das-frustas do gênero *Anastrepha* (Thephritidae) e em vespas parasitóides (Braconidae) associadas. São Paulo – S.P., Brazil. 80 p. (MSc Dissertation. Instituto de Biociências. USP).
18. O'Neil, S.L.; Giordano, R.; Colbert, A.M.; Karr, T.L.; Robertson, H.M. (1992). 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc. Natl. Acad. Sci*. USA 89, 2699-2702.
19. O'Neil, S.L.; Hoffmann, A.A.; Werren, J.H. (1997). Influential passengers: inherited microorganisms and arthropod reproduction. Oxford University Press, New York, 214 p.
20. Riegler, M. and Stauffer, C. 2002. *Wolbachia* infections and superinfections in cytoplasmically incompatible populations of the European cherry fruit fly *Rhagoletis cerasi* (Diptera, Tephritidae). *Mol. Ecol.* 11, 2425-2434.
21. Rocha, L.S.; Mascarenhas, R.O.; Perondini, A.L.P.; Selivon, D. (2005). Occurrence of *Wolbachia* in Brazilian samples of *Ceratitis capitata* (Wiedmann) (Diptera: Tephritidae). *Neotrop. Entomol*. 34, 1013-1015.
22. Ruang-Areerate, T.; Kittayapong, P.; Baimai, V.; O'Neill, S.L. (2003). Molecular phylogeny of *Wolbachia* endosymbionts in Southeast Asian mosquitoes (Diptera: Culicidae) based on *wsp* gene sequences. *J. Med. Entomol.* 40, 1-5.
23. Selivon, D.; Perondini, A.L.P.; Ribeiro, A.F.; Marino, C.L.; Lima, M.M.A.; Coscrato, V.E. (2002). *Wolbachia* endosymbiont in a species of *Anastrepha fraterculus* complex (Diptera: Tephritidae). *Invertebr. Reprod. Dev*. 42, 121-127.
24. Stouthamer, R.; Breewer, J.A.J.; Hurst, G.D.D. (1999). *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. *Annu. Rev. Microbiol*. 53, 71-102.
25. Sun, X.; Li, Z.H.; Liang, G.Q.; Chen, H.J.; Chen, N.Z. (2004). Detection of *Wolbachia* in *Bactrocera (B.) dorsalis* (Hendel) of China. *Plant Quarant*. 18, 321-323.

26. Vavre, F.; Fleury, F.; Lepetit, D.; Fouillet, P.; Bouletreau, M. (1999). Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. Mol. Biol. Evol. 16, 1711-1723.
27. Werren, J.H.; Windsor, D.; Guo, L.R. (1995a). Distribution of *Wolbachia* among neotropical arthropods. Proc. R. Soc. Lond. Ser. B: Biol. Sci. 262, 197-204.
28. Werren, J.H.; Zhang, W.; Guo, L.R. (1995b). Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. Proc. R. Soc. Lond. Ser. B: Biol. Sci. 261, 55-63.
29. Werren, J.H. (1997). Biology of *Wolbachia*. Annu. Rev. Entomol. 42, 587-609.
30. Werren, J.H., and Windsor, D.M. (2000). *Wolbachia* infection frequencies in insects: evidence of a global equilibrium? Proc. R. Soc. Lond. Ser. B: Biol. Sci. 267, 1277-1285.
31. Xiao-Yue, H.; Gotoh, T.; Noda, H. (2002). Sensitivity comparison of PCR *primers* for detecting *Wolbachia* in spider mites. Appl. Entomol. Zool. 37, 379-383.
32. Zhou, W.; Rousset, F.; O'Neill, S.L. (1998). Phylogeny and PCR based classification of *Wolbachia* strains using *wsp* gene sequences. Proc. R. Soc. Lond. Ser. B: Biol. Sci. 265, 509-515.