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Abstract. Blood feeding tsetse flies (Diptera: Glossinidae) are among the most harmful insects in tropical sub-Saharan Africa as they are vectors for trypanosome parasites which can cause the Human African Trypanosomiasis (HAT) or sleeping sickness in humans and Animal African Trypanosomiasis (AAT) or nagana in livestock. These insects are considered a critical boundary for the economic development of African countries. Sustainable management of their populations can be achieved through the application of environmentally friendly techniques for vector control such as the sterile or incompatible insect techniques. Both are considered increasingly important components of area wide integrated pest management programs for certain key insect pest species. Reproductive parasites like *Spiroplasma* and *Wolbachia*, are endosymbiotic bacteria capable of inducing reproductive alterations to their insect-hosts, and they can be used as central components in such techniques. In this study, we investigated the presence of *Spiroplasma* and *Wolbachia* in a wild population of *Glossina tachinoides* from Burkina Faso with a 16S rRNA gene PCR approach. As a result, we found that approximately 50% of the population examined was infected with *Spiroplasma*, whereas *Wolbachia* was totally absent from our samples. Moreover, molecular genotyping was carried out through the MLST characterization of the *Spiroplasma* strain found in *G. tachinoides*. These findings provide useful information for enhancing the application of the previously mentioned techniques for controlling this devastating insect species.

Keywords: *Glossina tachinoides*; Sterile Insect Technique (SIT); Incompatible Insect Technique (IIT); 16S rRNA; MLST.

1 Introduction

Tsetse flies (Glossina spp) are considered a principal source of Trypanosoma spp which is the factor engendering Human African Trypanosomiasis (HAT) as well as animal African trypanosomiasis (AAT) [1]. They encompass a large set of important microbiotas, which can be acquired by means of both horizontal and vertical transmission, and affect the ability of the fly as a disease vector [2]. Furthermore, they are responsible for the elicitation of an array of different phenotypes to their hosts, including reproductive alterations such as cytoplasmic incompatibility [3].

Spiroplasma is a genus of endosymbiotic bacteria within the Mollicutes class, and the Tenericutes phylym [4, 5]. It has developed a large variety of symbiotic relations, affecting insect nutrition, ecology and evolution, protection against pathogens, as well as reproduction and sex determination [3]. It has been discovered that *Spiroplasma* protect *Drosophila neotestacea* from a nematode [6], the pea aphid (*Acyrthosiphon pisum*) from fungi [7], and *Drosophila hydei* from a parasitoid wasp [8]. Apart from that, *Spiroplasma* is able to cause infections in plants [9], it can develop disease in insects [10,11,12] as well as in crustaceans [13,14,15,16,17]. Furthermore, reproductive alterations like male-killing have been related to numerous species of *Spiroplasma* [18,19].

Wolbachia endosymbionts are considered a diverse group of intracellular endosymbionts that belong to the Alphaproteobacteria group. They are transmitted via the cytoplasm of eggs but also transferred horizontally among arthropods. *Wolbachia* can manipulate the reproduction functions of their hosts. For instance, they are able to induce cytoplasmic incompatibility, parthenogenesis, male-killing as well as feminization [20,21,22].

In order to control tsetse flies, the Sterile Insect Technique (SIT) has been applied successfully [23, 24] and as an alternative approach the Incompatible Insect Technique (IIT) is considered as well. More specifically, the Sterile Insect Technique (SIT) depends on the mass release of laboratory reared sterile flies, in order to mate with the wild population, resulting in infertile crosses which eventually lead to population control or pest elimination. Sterility is commonly induced by treatment with low doses of ionizing radiation [25]. IIT can be employed in order to enhance the efficacy of the SIT. The IIT was founded on *Wolbachia*-induced cytoplasmic incompatibility and aims to release incompatible males recurrently to mate with wild females, in order to remove the pest's population [26,27,28].

The present study investigated the presence of *Spiroplasma* and *Wolbachia* in wild *Glossina tachinoides* from Burkina Faso employing a 16S rRNA screening approach. Moreover, the molecular characterization of representative positive individuals was performed by multi locus sequence typing (MLST) system. Five gene markers were used, *fruR, dnaA, ftsZ, parE and rpoB.*

2 Materials & Methods

2.1 DNA Extraction and PCR Amplification

A total of 63 adult *Glossina tachinoides* flies, originating from a wild population from Burkina Faso were collected and stored in absolute ethanol. Flies were surface sterilized with a 70% v/v ethanol solution and sterile deionized water. A CTAB-based protocol was used to isolate the DNA from the *Glossina tachinoides* flies. The 16S rRNA gene for *Spiroplasma* was amplified using the specific primer set 63F and TKSS. Molecular characterization of representative positive individuals was performed by amplifying five MLST gene markers (*fruR, dnaA, ftsZ, parE* and *rpoB*) with the use of specific primers (Table 1). PCR reactions were prepared with KAPA Taq PCR kit. Amplification was carried out in 25 ul reactions which was composed of: 2.5 ul of 10X KAPA buffer, 0.2 ul of dNTP solution (25 mM each), 0.4 ul of forward primer solution (25 uM), 0.4 ul of reverse primer solution (25 uM), 0.1 ul of KAPA Taq polymerase, 1 ul of template DNA and 20.4 ul of sterile deionized water. The initial stage of the PCR protocol was 3 minutes of denaturation at 95 ºC, coming before 35 cycles of 95 ºC for 30 seconds, the specific annealing temperature of each primer (Table 1) for 30 seconds, and 72 ºC for 1 or 1:30 minutes (see Table 1 for extension times). The reaction was finalized with 72 ºC for 3 minutes. PCR products were cleaned with a PEG precipitation protocol and sequencing reactions were prepared with ABI BigDye™ Terminator v3.1 Cycle Sequencing Kit. An ethanol/EDTA protocol, conforming to the manufacturer's directives (Applied Biosystems), was used to clean the products engendered by the sequencing reactions. Then, ABI PRISM 3500 Genetic Analyzer (Applied Biosystems) was used to analyze the cleaned reaction products [3,29].

Table 1. MLST primers used for *Spiroplasma* [3, 30].

Name / Reference	Primer Sequence 5'-3'	Gene region	Annealing temperature / extension time	Fragment size (bp)	References
63F TKSS	GCCTAATACATGCAAGTCGAAC TAGCCGTGGCTTTCTGGTAA	*16S rRNA Spiroplasma*	58 ºC / 1min	455	[3]
fru-f fru-r	GTCATAATTGCAATTGCTGG CAATGATTAAAGCGGAGGT	*fruR*	56 ºC / 1 min	398	[3]
SRdnaAF1 SRdnaAR1	GGAGAYTCTGGAYTAGGAAA CCYTCTAWYTTTCTRACATCA	*dnaA*	49 ºC / 1 min	515	[3]
FtsZF2 FtsZR3	TGAACAAGTCGCGTCAATAAA CCACCAGTAACATTAATAATAGCATCA	*ftsZ*	57 ºC / 1 min	774	[3]
ParEF2 ParER2	GGAAAATTTGGTGGTGATGG TGGCATTAATCATTACATTAATTTCT	*parE*	57 ºC / 1:30 min	1126	[3]
RpoBF1 RpoBR2	ATGGATCAAACAAATCCATTAGCAGA GCATGTAATTTATCATCAACCATGTGTG	*rpoB*	60 ºC / 1:30 min	1703	[3]
wspecF wspecR	YATACCTATTCGAAGGGATAG AGCTTCGAGTGAAACCAATTC	*16S rRNA Wolbachia*	54 ºC / 1 min	438	[29]

2.2 Phylogenetic Analysis

In the present study, all *Spiroplasma* gene sequences created were manually modified and visually fixed with SeqManII by DNAStar, and the alignment was performed with ClustalW [31], as executed in Geneious 7.0.2. The neighbor joining algorithm was used to infer the tree topology and evolutionary distances were calculated using the method of Jukes and Cantor. Phylogenetic trees were generated using Geneious 7.0.2. Multiple alignments were performed using MUSCLE algorithm while maintaining default algorithm parameters and the sequences were manually adjusted. Phylogenetic analyses were carried out for 16S rRNA, *rpoB, dnaA, parE, ftsZ* and *fruR* genes.

The sequences created in this study, 16S rRNA, *dnaA, fruR* and *parE* genes, have been submitted to GenBank under accession numbers MT238675-MT238677 and MT297696-MT297710.

3 Results

3.1 Prevalence of *Wolbachia* and *Spiroplasma* in Glossina tachinoides:

The presence of *Wolbachia* and *Spiroplasma* was investigated in a natural population of *G. tachinoides*. The number of individuals studied was 63 adult flies. A specific 16S rRNA gene PCR assay (Table 2) was used to test the flies. According to the screening data, it has been revealed that 44.5% of the *G. tachinoides* individual adults screened were infected with *Spiroplasma*. Furthermore, the prevalence in the male adults was 47% while in the females 42%. Interestingly, *Wolbachia* was not detected in all samples examined (Table 2).

Table 2. Prevalence of *Wolbachia* and *Spiroplasma* in *Glossina tachinoides* population from Burkina Faso, using 16S rRna gene-based PCR screening method.

Glossina species	***Country/ Area***	***Sex***	***Screened individuals***	***Spiroplasma positives (%)***	***Wolbachia positives***
Glossina tachinoides	Burkina Faso/ Folonzo	Male	51	24 (47%)	0
	Burkina Faso/ Folonzo	Female	12	5 (42%)	0

3.2 Multi Locus Typing Sequences Genotyping of the *G. tachinoides Spiroplasma* strain

According to the 16S rRNA gene dataset, the phylogenetic analysis illustrated that *Spiroplasma* strains present in *G. tachinoides* belong to Citri group (*Fig.1*). These results have been supported by the corresponding phylogenetic analysis made from a concatenated dataset of selected MLST loci (*Fig.2*). It is noteworthy that *Spiroplasma* strain infecting *G. tachinoides* is different from *Spiroplasma* strain present in *G. fuscipes fuscipes* with sequence polymorphisms observed in all tested loci. Four polymorphisms were noticed in 16S rRNA, three were detected in *dnaA*, two polymorphisms were identified in *ftsZ*, eight in *fruR*, and one polymorphism was found in both *rpoB* and *parE*. The two strains of *Spiroplasma* belong to the citri clade, which is mainly made out of plant pathogens. The majority of the pathogenic species of *Spiroplasma* are included in the Citri clade, with noticeable examples such as *S. kunkelii* which induces the corn stunt sickness [32], *S. phoeniceum* which infects periwinkle [33], as well as *S. penaei* which causes infection in Pacific white shrimp [34]. *S. insolitum* and *Spiroplasma* from *G. fuscipes fuscipes* are the nearest relatives of *Spiroplasma* strain from *Glossina tachinoides.*

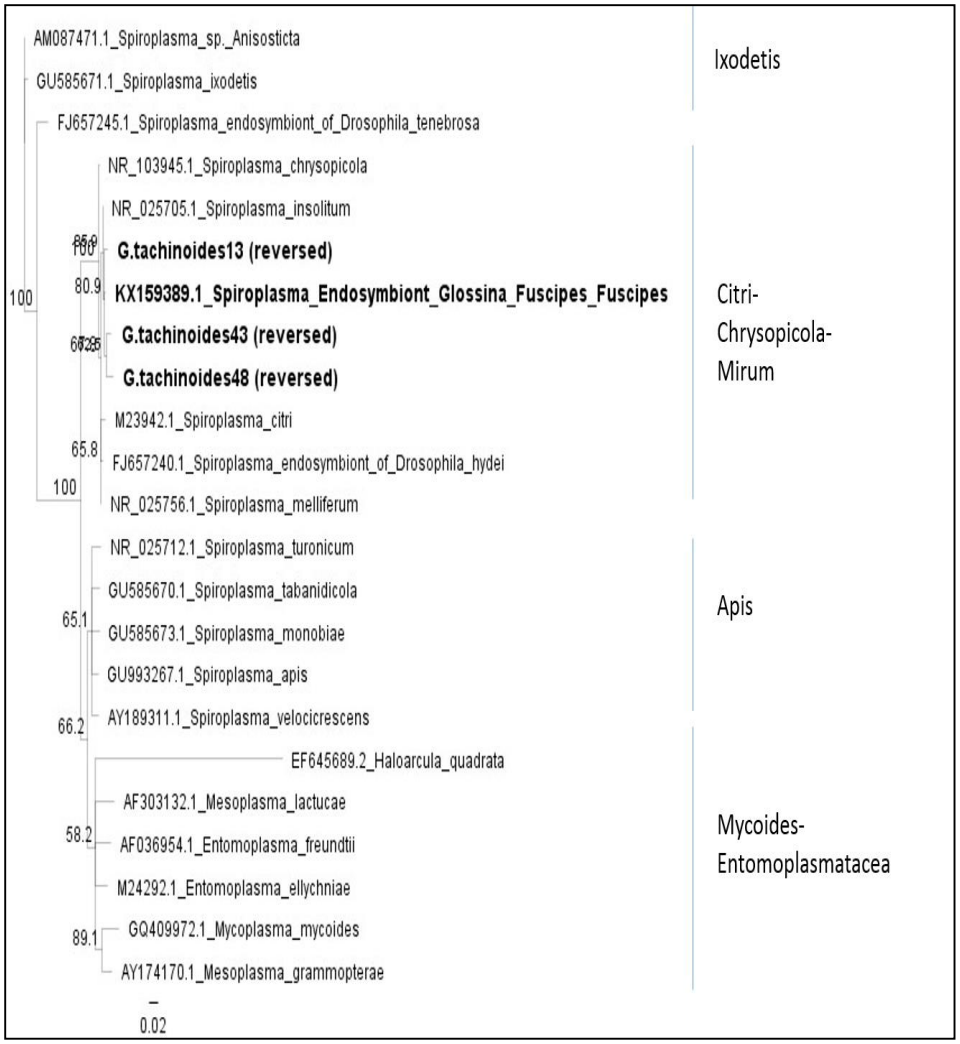

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequence. *Spiroplasma* from *G. tachinoides* and *G. fuscipes fuscipes* are indicated in bold letters. The numbers at each node represents bootstrap proportions based on 1000 replications. Evolutionary distances were calculated using the method of Jukes and Cantor, the topology was inferred using the neighbor joining method.

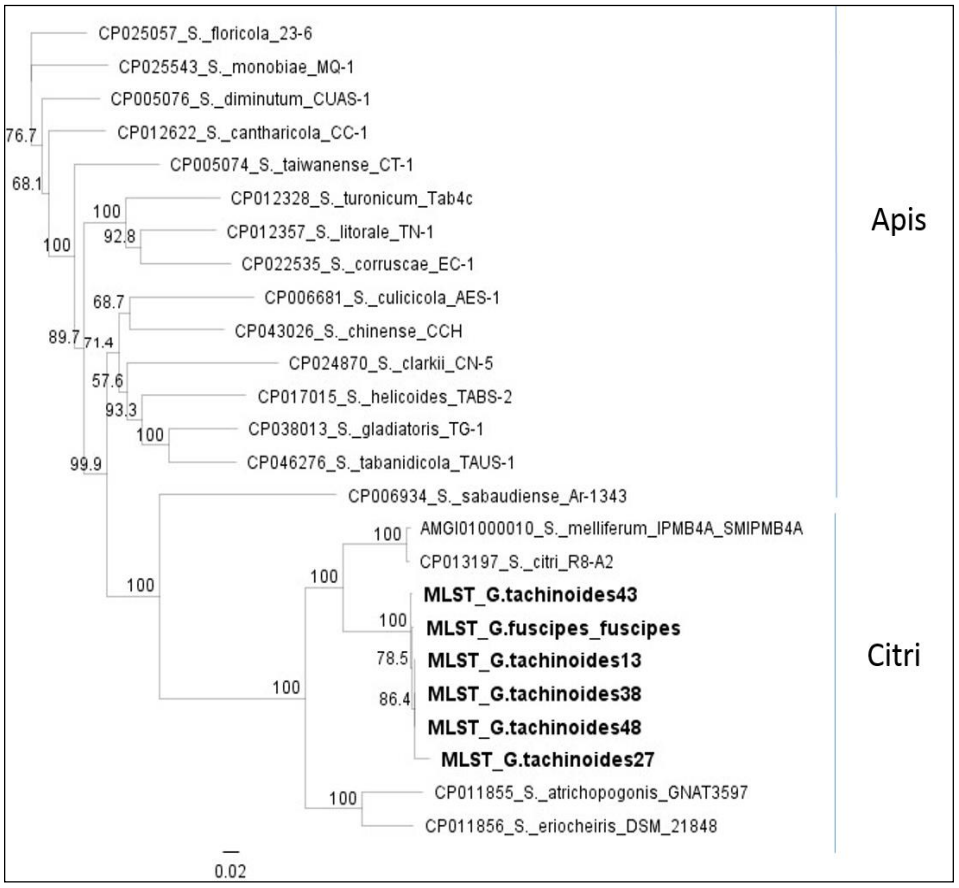

Fig. 2. Concatenated tree based on the MLST data for *SRdna*, *Fru-r* and *ParE* gene sequences. *Spiroplasma* from *G. tachinoides* are indicated in bold letters. The numbers at each node represents bootstrap proportions based on 1000 replications. Evolutionary distances were calculated using the method of Jukes and Cantor, the topology was inferred using the neighbor-joining method.

4 Conclusions & Perspectives

The present study revealed that *Spiroplasma* is present in *G. tachinoides*, whereas, *Wolbachia* was not detected. *Spiroplasma* 16S rRNA and MLST sequences identified in *G. tachinoides* samples grouped with the citri-chrysopicola-melliferum group. In a previous study, *Spiroplasma* was newly detected in both laboratory and wild population of *G. tachinoides* and *G. fuscipes fuscipes*, which belong to the palpalis sub-group. Besides, *Spiroplasma* has been identified in numerous samples from the wild population of *G. fuscipes fuscipes* and *G.tachinoides*. On the whole, this symbiont has a low titer infection (<1%). However, the abundance of *Spiroplasma* was relatively high (13.2%) in one whole fly of *G. tachinoides*. On the other hand, *Spiroplasma* density in *G. fuscipes fuscipes* has been evaluated in larval guts. Likewise, it has been appraised in guts and gonads of flies gathered at teneral stage and at adults aged 15 days. In fact, the density of *Spiroplasma* infecting larva guts of *Glossina fuscipes fuscipes* was considerably greater than the density of *spiroplasma* in guts of teneral stage and of adults aged 15 days. Regarding *Spiroplasma* in gonads of insects at teneral stage and adults aged 15 days, its density was lower in ovaries than in testes. However, *Spiroplasma* density was substantially superior in living females comparing to the ones prematurely dead which shows a potentially reciprocal link. Moreover, *Wolbachia* was not detected in *G. tachinoides* [3]. In order to further elucidate the role of *Spiroplasma* in *G. tachinoides* we plan to apply single cell genomics in order to decipher the genomic sequence of the *Spiroplasma* strain in *G. tachinoides*.

References

1. S. Aksoy, & B. L. Weiss. (2007). Symbiosis-Based Technological Advances to Improve Tsetse Glossina spp. SIT Application. In M. J. B. Vreysen, A. S. Robinson, & J. Hendrichs (Éds.), Area-Wide Control of Insect Pests (p. 137‑148). Springer Netherlands. https://doi.org/10.1007/978-1-4020-6059-5_12
2. Franck Jacob, Trésor T. Melachio, Guy R. Njitchouang, Geoffrey Gimonneau, Flobert Njiokou, Abate, L., Christen, R., Reveillaud, J., & Geiger, A. (2017). Intestinal Bacterial Communities of Trypanosome-Infected and Uninfected Glossina palpalis palpalis from Three Human African Trypanomiasis Foci in Cameroon. Frontiers in Microbiology, 8, 1464. https://doi.org/10.3389/fmicb.2017.01464
3. V. Doudoumis, F. Blow, A. Saridaki, A. Augustinos, N. A. Dyer, Goodhead, I., Solano, P., Rayaisse, J.-B., Takac, P., Mekonnen, S., Parker, A. G., Abd-Alla, A. M. M., Darby, A., Bourtzis, K., & Tsiamis, G. (2017). Challenging the Wigglesworthia, Sodalis, Wolbachia symbiosis dogma in tsetse flies : Spiroplasma is present in both laboratory and natural populations. Scientific Reports, 7(1), 4699. https://doi.org/10.1038/s41598-017-04740-3
4. Monika Enigl, & Peter Schausberger. (2007). Incidence of the endosymbionts Wolbachia, Cardinium and Spiroplasma in phytoseiid mites and associated prey. Experimental and Applied Acarology, 42(2), 75‑85. https://doi.org/10.1007/s10493-007-9080-3
5. Ludwig, W., Schleifer, K.-H., & Whitman, W. B. (s. d.). Revised road map to the phylum Firmicutes. 13.
6. J. Jaenike, R. Unckless, S. N. Cockburn, L. M. Boelio, & S. J. Perlman. (2010). Adaptation via Symbiosis : Recent Spread of a Drosophila Defensive Symbiont. Science, 329(5988), 212‑215. https://doi.org/10.1126/science.1188235
7. P. Łukasik, H. Guo, M. van Asch, J. Ferrari, & H. C. J. Godfray. (s. d.). Protection against a fungal pathogen conferred by the aphid facultative endosymbionts Rickettsia and Spiroplasma is expressed in multiple host genotypes and species and is not influenced by co-infection with another symbiont—Łukasik—2013—Journal of Evolutionary Biology—Wiley Online Library. Consulté 12 février 2020, à l'adresse https://onlinelibrary.wiley.com/doi/full/10.1111/jeb.12260
8. Jialei Xie, Igor Vilchez, & Mariana Mateos. (2010). Spiroplasma Bacteria Enhance Survival of Drosophila hydei Attacked by the Parasitic Wasp Leptopilina heterotoma. PLoS ONE, 5(8). https://doi.org/10.1371/journal.pone.0012149
9. Gail E. Gasparich. (2010). Spiroplasmas and phytoplasmas : Microbes associated with plant hosts. Biologicals, 38(2), 193‑203. https://doi.org/10.1016/j.biologicals.2009.11.007
10. C. Mouches, J.M. Bové, & J. Albisetti. (1984). Pathogenicity of Spiroplasma apis and other spiroplasmas for honey-bees in Southwestern France. Annales de l'Institut Pasteur / Microbiologie, 135(1), 151‑155. https://doi.org/10.1016/S0769-2609(84)80072-1
11. T. B. CLARK, R. F. WHITCOMB, J. G. TULLY, C. MOUCHES, C. SAILLARD, BOVé, J. M., WRÓBLEWSKI, H., CARLE, P., ROSE, D. L., HENEGAR, R. B., & WILLIAMSON, D. L. (1985). Spiroplasma melliferum, a New Species from the Honeybee (Apis mellifera). International Journal of Systematic and Evolutionary Microbiology, 35(3), 296‑308. https://doi.org/10.1099/00207713-35-3-296
12. Ian Humphery-Smith, Odile Grulet, Francette Le Goff, & Claude Chastel. (1991). Spiroplasma (Mollicutes : Spiroplasmataceae) Pathogenic for Aedes aegypti and Anopheles stephensi (Diptera: Culicidae). Journal of Medical Entomology, 28(2), 219‑222. https://doi.org/10.1093/jmedent/28.2.219
13. Wen Wang, Bohai Wen, Gail E. Gasparich, Ningning Zhu, Liwen Rong, Jianxiu Chen, & Zaikuan Xu. (2004). A spiroplasma associated with tremor disease in the Chinese mitten crab (Eriocheir sinensis). Microbiology, 150(9), 3035‑3040. https://doi.org/10.1099/mic.0.26664-0
14. Wen Wang, Wei Gu, Zhengfeng Ding, Yalai Ren, Jianxiu Chen, & Yayi Hou. (2005). A novel Spiroplasma pathogen causing systemic infection in the crayfish Procambarus clarkii (Crustacea : Decapod), in China. FEMS Microbiology Letters, 249(1), 131‑137. https://doi.org/10.1016/j.femsle.2005.06.005
15. Wen Wang, Wei Gu, Gail E. Gasparich, Keran Bi, Jiangtao Ou, Meng, Q., Liang, T., Feng, Q., Zhang, J., & Zhang, Y. (2011). Spiroplasma eriocheiris sp. Nov., associated

with mortality in the Chinese mitten crab, Eriocheir sinensis. International Journal of Systematic and Evolutionary Microbiology, 61(4), 703‑708. https://doi.org/10.1099/ijs.0.020529-0

16. Linda M. Nunan, Donald V. Lightner, Marietta A. Oduori, & Gail E. Gasparich. (2005). Spiroplasma penaei sp. Nov., associated with mortalities in Penaeus vannamei, Pacific white shrimp. International Journal of Systematic and Evolutionary Microbiology, 55(6), 2317‑2322. https://doi.org/10.1099/ijs.0.63555-0
17. Liang, T., Li, X., Du, J., Yao, W., Sun, G., Dong, X., Liu, Z., Ou, J., Meng, Q., Gu, W., & Wang, W. (2011). Identification and isolation of a spiroplasma pathogen from diseased freshwater prawns, Macrobrachium rosenbergii, in China : A new freshwater crustacean host. Aquaculture, 318(1‑2), 1‑6. https://doi.org/10.1016/j.aquaculture.2011.03.018
18. Zhengfeng Ding, Jie Du, Jiangtao Ou, Wenjie Li, Ting Wu, Xiu, Y., Meng, Q., Ren, Q., Gu, W., Xue, H., Tang, J., & Wang, W. (2012). Classification of circulating hemocytes from the red swamp crayfish Procambarus clarkii and their susceptibility to the novel pathogen Spiroplasma eriocheiris in vitro. Aquaculture, 356‑357, 371‑380. https://doi.org/10.1016/j.aquaculture.2012.04.042
19. Hisashi Anbutsu, & Takema Fukatsu. (2003). Population Dynamics of Male-Killing and Non-Male-Killing Spiroplasmas in Drosophila melanogaster. Applied and Environmental Microbiology, 69(3), 1428‑1434. https://doi.org/10.1128/AEM.69.3.1428-1434.2003
20. John H. Werren. (1997). Biology of Wolbachia. Annual Review of Entomology, 42(1), 587‑609. https://doi.org/10.1146/annurev.ento.42.1.587
21. A. M. F. Aguiar, & F. A. Ilharco. (2001). Aphids (Homoptera : Aphidoidea) from Maderia Island [Portugal]: new records and corrections. Boletín de Sanidad Vegetal. Plagas (España). http://agris.fao.org/agris-search/search.do?recordID=ES2002000465
22. Aggeliki Saridaki, & Kostas Bourtzis. (2010). Wolbachia : More than just a bug in insects genitals. Current Opinion in Microbiology, 13(1), 67‑72. https://doi.org/10.1016/j.mib.2009.11.005
23. Abd-Alla, A. M. M., Bergoin, M., Parker, A. G., Maniania, N. K., Vlak, J. M., Bourtzis, K., Boucias, D. G., & Aksoy, S. (2013). Improving Sterile Insect Technique (SIT) for tsetse flies through research on their symbionts and pathogens. Journal of Invertebrate Pathology, 112, S2‑S10. https://doi.org/10.1016/j.jip.2012.07.009
24. Bourtzis, K., Lees, R. S., Hendrichs, J., & Vreysen, M. J. B. (2016). More than one rabbit out of the hat : Radiation, transgenic and symbiont-based approaches for sustainable management of mosquito and tsetse fly populations. Acta Tropica, 157, 115‑130. https://doi.org/10.1016/j.actatropica.2016.01.009
25. Jorge Hendrichs, & Alan Robinson. (s. d.). Sterile Insect Technique. Sterile Insect Technique, 5.
26. Stouthamer, R., Breeuwer, J. A. J., & Hurst, G. D. D. (1999). Wolbachia Pipientis : Microbial Manipulator of Arthropod Reproduction. Annual Review of Microbiology, 53(1), 71‑102. https://doi.org/10.1146/annurev.micro.53.1.71
27. Steven P Sinkins. (2004). Wolbachia and cytoplasmic incompatibility in mosquitoes. Insect Biochemistry and Molecular Biology, 34(7), 723‑729. https://doi.org/10.1016/j.ibmb.2004.03.025
28. Ioannidis Panagiotis, & Kostas Bourtzis. (2007). Insect symbionts and applications : The paradigm of cytoplasmic incompatibility-inducing Wolbachia. Entomological Research, 37(3), 125‑138. https://doi.org/10.1111/j.1748-5967.2007.00102.x
29. Elias D. Asimakis, Vangelis Doudoumis, Ashok B. Hadapad, Ramesh S. Hire, Costas Batargias, Niu, C., Khan, M., Bourtzis, K., & Tsiamis, G. (2019). Detection and characterization of bacterial endosymbionts inSoutheast Asian tephritid fruit fly populations. BMC Microbiology, 19(S1), 290. https://doi.org/10.1186/s12866-019-1653-x
30. Doudoumis, V., Tsiamis, G., Wamwiri, F., Brelsfoard, C., Alam, U., Aksoy, E., Dalaperas, S., Abd-Alla, A., Ouma, J., Takac, P., Aksoy, S., & Bourtzis, K. (2012). Detection and characterization of Wolbachia infections in laboratory and natural populations of different species of tsetse flies (genus Glossina). BMC Microbiology, 12(Suppl 1), S3. https://doi.org/10.1186/1471-2180-12-S1-S3
31. Thompson, J. D., Higgins, D. G., & Gibson, T. J. (s. d.). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. 8.

32. Whitcomb, R. F., Chen, T. A., Williamson, D. L., Liao, C., Tully, J. G., Bove, J. M., Mouches, C., Rose, D. L., Coan, M. E., & Clark, T. B. (1986). Spiroplasma kunkelii sp. nov.: Characterization of the Etiological Agent of Corn Stunt Disease. International Journal of Systematic Bacteriology, 36(2), 170‑178. https://doi.org/10.1099/00207713-36-2-170
33. Saillard, C., Vignault, J. C., Bove, J. M., Raie, A., Tully, J. G., Williamson, D. L., Fos, A., Garnier, M., Gadeau, A., Carle, P., & Whitcomb, R. F. (1987). Spiroplasma phoeniceum sp. Nov., a New Plant-Pathogenic Species from Syria. International Journal of Systematic Bacteriology, 37(2), 106‑115. https://doi.org/10.1099/00207713-37-2-106
34. Linda M. Nunan, Donald V. Lightner, Marietta A. Oduori, & Gail E. Gasparich. (2005). Spiroplasma penaei sp. Nov., associated with mortalities in Penaeus vannamei, Pacific white shrimp. International Journal of Systematic and Evolutionary Microbiology, 55(6), 2317‑2322. https://doi.org/10.1099/ijs.0.63555-0