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Original Article

Yersinia massiliensis (Enterobacteriales: Enterobacteriaceae) in the host *Anaphes nitens* (Hymenoptera: Mymaridae): first report of association with insects

Yersinia massiliensis (Enterobacteriales: Enterobacteriaceae) no hospedeiro *Anaphes nitens* (Hymenoptera: Mymaridae): primeiro relato de associação com insetos

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Abstract

Endosymbiont bacteria can affect biological parameters and reduce the effectiveness of natural enemies in controlling the target insect. The objective of this work was to identify endosymbiont bacteria in *Anaphes nitens* (Girault, 1928) (Hymenoptera: Mymaridae), the main natural enemy used to manage *Gonipterus platensis* (Marelli, 1926) (Coleoptera: Curculionidae). Genomic DNA from six *A. nitens* populations was extracted and polymerase chain reactions (PCR) were performed with the primers to detect endosymbiont bacteria in this insect. The PCR products were amplified, sequenced, and compared with sequences deposited in the GenBank for the bacteria identification. All *A. nitens* populations had the bacterium *Yersinia massiliensis* (Enterobacteriales: Enterobacteriaceae). This bacterium was originally described as free-living, and it is associated with and composes part of the *A. nitens* microbiota. This is the first report of *Y. massiliensis* in an insect host.

Keywords: biological control, egg parasitoid, microbiota, molecular entomology.

Resumo

As bactérias endossimbiontes podem afetar os parâmetros biológicos e reduzirem a eficácia de inimigos naturais no controle do inseto alvo. O objetivo deste trabalho foi identificar bactérias endossimbiontes em *Anaphes nitens* (Girault, 1928) (Hymenoptera: Mymaridae), o principal inimigo natural usado no manejo de *Gonipterus platensis* (Marelli, 1926) (Coleoptera: Curculionidae). O DNA genômico de seis populações de *A. nitens* foi extraído e as reações em cadeia da polimerase (PCR) realizadas com os primers para detectar bactérias endossimbiontes neste inseto. Os produtos de PCR foram amplificados, sequenciados e comparados com as sequências depositadas no GenBank para identificação das bactérias. Todas as populações de *A. nitens* tinham a bactéria *Yersinia massiliensis* (Enterobacteriales: Enterobacteriaceae). Esta bactéria foi originalmente descrita como de vida livre e está associada e compõe parte da microbiota de *A. nitens*. Este é o primeiro relato de *Y. massiliensis* em um hospedeiro.

Palavras-chave: controle biológico, parasitoide de ovo, microbiota, entomologia molecular.

1. Introduction

The use of the egg parasitoid *Anaphes nitens* (Girault, 1928) (Hymenoptera: Mymaridae) is the main management strategy for the *Eucalyptus* spp. defoliator beetle, *Gonipterus platensis* (Marelli, 1926) (Coleoptera: Curculionidae) (Souza et al., 2016). This parasitoid is originally from Australia (Mapondera et al., 2012), and it has been successfully introduced in South Africa, Spain and California (United States of America) (Cordero-Rivera et al., 1999; Hanks et al., 2000) but with low efficiency in some regions of Portugal, Chile and Brazil (Reis et al., 2012; Gumovsky et al., 2015; Souza et al., 2016). The reasons for the low parasitism rates in these countries are not fully understood and some authors attribute it to the seasons, altitude or host-parasitoid incompatibility (Reis et al., 2012; Mapondera et al., 2012).

Secondary endosymbiont bacteria can reduce fitness (Zug and Hammerstein, 2018) and parasitism rates (Furihata et al., 2015), increase susceptibility to

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hyperparasitoids (Van Nouhuys et al., 2016) and alter the structure and composition of parasitoid communities (Rothacher et al., 2016; Monticelli et al., 2019). It is important to know the host microbiota to evaluate its interference in parasitoid efficiency in biological control programs (Almeida et al., 2010). However, there are no reports of endosymbiont bacteria in *A. nitens*.

The objective of this work was to identify the presence of endosymbiont bacteria in the *A. nitens* host populations in Brazil.

2. Materials and Methods

Egg masses of *G. platensis* were collected in commercial *Eucalyptus* spp. plantations in the municipalities of Botucatu, Itararé, Itatinga, Lençóis Paulista and Pratânia in the state of São Paulo and Aracruz in the state of Espírito Santo, Brazil. They were kept in acrylic plates (5 cm diameter and 1 cm high) in biochemical oxygen demand (BOD) incubator chamber at 25 °C and a photoperiod of 12 hours until the emergence of *A. nitens* adults. The emerged insects were collected and preserved at 4 °C.

Genomic DNA from 50 adult insects per *A. nitens* population was extracted following the Chelex 100 protocol (Walsh et al., 1991). These insects were washed in 70% alcohol, macerated and homogenized in a solution of 80 μL of Chelex 100 resin (Bio-Rad Laboratories, USA) 10% and 8 μL of proteinase K (20 μg/mL) (Bioline, USA). Samples containing the DNA of the *A. nitens* populations were transferred to an Infinigen Thermal Cycler (model TC-96CG) and incubated at 95 °C for 20 minutes.

Egg capsules of *G. platensis* from laboratory rearing, up to 24 hours old and free of parasitism, were analyzed as the control. These capsules were immersed in distilled water for 1 hour, with a subsequent sampling of 35 eggs and the excrement layer of five egg capsules. The genomic DNA extraction procedure was the same as for *A. nitens* samples.

The 23S region was amplified with the primers of the secondary endosymbiont *Arsenophonus*, with polymerase chain reaction (PCR) 5'-CGTTTGATGAATTCATAGTCAAA-3 '(Forward) and 5' GGTCCTCCAGTTAGTGTTACCCAAC-3 '(Reverse) (Thao and Baumann, 2004). A PCR mix containing 12.5 μL of Taq DNA Polymerase (NeoBio), 7.5 μL water milliQ, 1.0 μL of each primer and 3.0 μL of DNA, totaling a volume of 25 μL, was used in each of the population samples. PCR reactions were performed under the following conditions: initial denaturation temperature at 95 °C for 2 min, followed by 30 cycles each of 95 °C for 30 sec, 58 °C for 30 sec, 72 °C for 1 min. and a final extension of 72 °C for 5 min. A molecular marker 100pb (Norgen) and 1% agarose gel, visualized in a UV Light Transilluminator (Major Science), were used.

PCR products were purified using a "PCR Purification Kit" (Cellco Biotec, Brazil). The amplified DNA fragments of the bacteria were sequenced by the Sanger DNA Automatic Sequencer (Model: ABI 3500-Applied Biosystems), at the Institute of Biotechnology (IBTEC/UNESP) in Botucatu and compared with the sequences deposited in GenBank, using the BLASTn program (Blast, 2020).

The nucleotide sequences obtained were analyzed using the software Geneious v11.1.5 and compared to a dataset composed of other accessions retrieved from the GenBank. The sequences were aligned using MAFFT v7.222 within the Geneious v.11.1.5 software, and phylogenetic analysis was performed using MrBayes 3.2.2 (Ronquist and Huelsenbeck, 2003). Two independent runs were conducted simultaneously using 10 million generations and excluding 25% from the resulting tree as burning. Phylogenetic tree was visualized, edited and rooted using FigTree v1.4.4 (FigTree, 2020).

3. Results

BLASTN analysis of the 600 bp amplicon resulted in high identity (98% identity with the accession number CP028487-1) with the bacterium *Yersinia massiliensis* (Enterobacteriales: Enterobacteriaceae).

This bacterium was found in all *A. nitens* populations and it was absent in *G. platensis* eggs and excrement layer of the egg capsules (Figure 1). Phylogenetic analysis grouped the sequences of this bacterium in a single sub-clade including samples from all localities (Figure 2).

4. Discussion

The presence of the bacterium *Y. massiliensis* in all populations of *A. nitens* includes it in the composition of the microbiota of this insect host. The *Y. massiliensis* DNA sequencing from the primer developed for the endosymbiont *Arsenophonus* may be due to the amplification of the 23S gene region in a non-specific way (Rekha et al., 2006). *Yersinia massiliensis* was

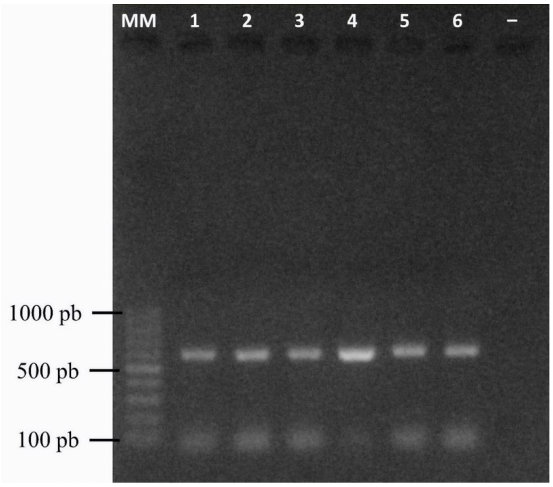

Figure 1. Agarose gel with polymerase chain reaction (PCR) products for *Yersinia massiliensis* (Enterobacteriales: Enterobacteriaceae) in individuals of different *Anaphes nitens* (Hymenoptera: Mymaridae) host populations. MM= molecular marker; 1= population of Aracruz, Espírito Santo, Brazil; 2= population of Botucatu, São Paulo, Brazil; 3= population of Itatinga, São Paulo, Brazil; 4= population of Lençóis Paulista, São Paulo, Brazil; 5= population of Pratânia, São Paulo, Brazil; 6= population of Itararé, São Paulo, Brazil.

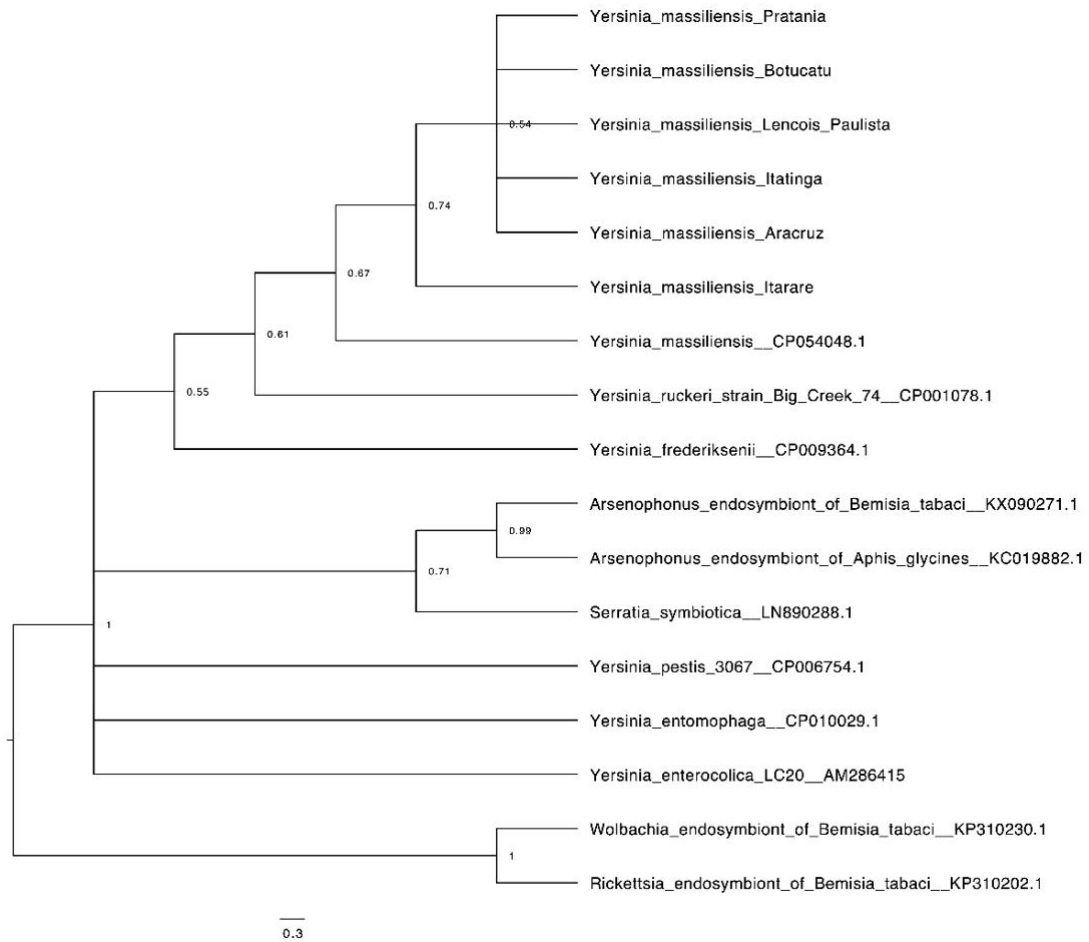

Figure 2. Phylogenetic analysis for *Yersinia massiliensis* symbiont samples from different *Anaphes nitens* populations (Hymenoptera: Mymaridae).

originally described as a freshwater free-living gram-negative coccobacillus (Merhej et al., 2008) and has been found in aquatic and food environments in Brazil and Argentina (Souza et al., 2011) as non-pathogenic (Thomas et al., 2018).

Some bacteria in the Enterobacteriaceae family may be free-living bacteria or secondary endosymbionts (Renoz et al., 2019), as the genus *Hamiltonella*, *Regiella*, *Serratia* and *Yersinia* (Kikuchi, 2009). The composition of the insect microbiota varies with the host, from a few dozens in Lepidoptera (Paniagua Voirol et al., 2018) to hundreds of species in termites (Brune and Dietrich, 2015). Insects can acquire free-living bacteria and may establish mutual relations with them (Sudakaran et al., 2017). The host diet is the main impact factor in the available resources for the microbiota and, consequently, defines the microorganism species surviving in its interior (Foster et al., 2017).

The genus *Yersinia* currently contains 15 species, *Y. enterocolica*, *Y. pseudotuberculosis* and *Y. pestis* are pathogenic to humans and some vertebrates, *Y. entomophaga* is pathogenic to insects, and the others are considered non-pathogenic opportunistic bacteria (Merhej et al., 2008; Hurst et al., 2011; Souza et al., 2011, Reuter et al., 2014). *Yersinia massiliensis* was found in *A. nitens* collected in areas with a high parasitism rate (State of Espirito Santo) or low parasitism rate (State of São Paulo) (Souza et al., 2016) by this natural enemy on *G. platensis*. Therefore, it is unlikely to be the cause of parasitism differences in Brazil.

Endosymbiont bacteria interactions should be studied case by case, as may range from positive to negative depending on the host, as observed by *Wolbachia,* which increased parasitism of *Asobara japonica* Belokobylskij (Hymenoptera: Braconidae) but reduced fecundity of *Leptopilina heterotoma* Thomson (Hymenoptera: Figitidae) (Fleury et al., 2000; Furihata et al., 2015). In *Trichogramma atopovirilia* (Hymenoptera: Trichogrammatidae), *Wolbachia* induce parthenogenesis and its infection does not show negative effects (Almeida et al., 2010; Almeida and Stouthamer, 2017).

The association of *Y. massiliensis* with the parasitoid *A. nitens* represents the first report of this bacterium in an insect host.

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References

ALMEIDA, R.P. and STOUTHAMER, R., 2017. Phylogeny of the *Trichogramma* endosymbiont *Wolbachia*, an alpha-proteobacteria (Rickettsiae). *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 78, no. 3, pp. 421-428. http://dx.doi.org/10.1590/1519-6984.166671. PMid:29160361.

ALMEIDA, R.P., VAN LENTEREN, J.C. and STOUTHAMER, R., 2010. Does *Wolbachia* infection affect *Trichogramma atopovirilia* behaviour? *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 70, no. 2, pp. 435-442. http://dx.doi.org/10.1590/S1519-69842010005000016. PMid:20379652.

BLAST - Basic local alignment search tool. 2020. [viewed 4 April 2019]. Available from: https://blast.ncbi.nlm.nih.gov/Blast.cgi.

BRUNE, A. and DIETRICH, C., 2015. The gut microbiota of termites: digesting the diversity in the light of ecology and evolution. *Annual Review of Microbiology*, vol. 69, no. 1, pp. 145-166. http://dx.doi.org/10.1146/annurev-micro-092412-155715. PMid:26195303.

CORDERO-RIVERA, A., SANTOLAMAZZA-CARBONE, S. and ANDRÉS, J.A., 1999. Life cycle and biological control of the *Eucalyptus* snout beetle (Coleoptera, Curculionidae) by *Anaphes nitens* (Hymenoptera, Mymaridae) in north-west Spain. *Agricultural and Forest Entomology*, vol. 1, no. 2, pp. 103-109. http://dx.doi.org/10.1046/j.1461-9563.1999.00016.x.

FLEURY, F., VAVRE, F., RIS, N., FOUILLET, P. and BOULÉTREAU, M., 2000. Physiological cost induced by the maternally-transmitted endosymbiont *Wolbachia* in the *Drosophila* parasitoid *Leptopilina heterotoma*. *Parasitology*, vol. 121, no. 5, pp. 493-500. http://dx.doi.org/10.1017/S0031182099006599. PMid:11128800.

FIGTREE - Molecular Evolution, Phylogenetics and Epidemiology. 2020. [viewed 20 May 2019]. Available from: http://tree.bio.ed.ac.uk/software/figtree/.

FOSTER, K.R., SCHLUTER, J., COYTE, K.Z. and RAKOFF-NAHOUM, S., 2017. The evolution of the host microbiome as an ecosystem on a leash. *Nature*, vol. 548, no. 7665, pp. 43-51. http://dx.doi.org/10.1038/nature23292. PMid:28770836.

FURIHATA, S., HIRATA, M., MATSUMOTO, H. and HAYAKAWA, Y., 2015. Bacteria endosymbiont, *Wolbachia*, promotes parasitism of the parasitoid wasp *Asobara japonica*. *PLoS ONE*, vol. 10, no. 10, pp. e0140914. http://dx.doi.org/10.1371/journal.pone.0140914. PMid:26492411.

GUMOVSKY, A., LITTLE, D., ROTHMANN, S., JAQUES, L. and MAYORGA, S.E., 2015. Re-description and first host and biology records of *Entedon magnificus* (Girault & Dodd) (Hymenoptera, Eulophidae), a natural enemy of *Gonipterus* weevils (Coleoptera, Curculionidae), a pest of *Eucalyptus* trees. *Zootaxa*, vol. 3957, no. 5, pp. 577-584. http://dx.doi.org/10.11646/zootaxa.3957.5.6. PMid:26249097.

HANKS, L.M., MILLAR, J.G., PAINE, T.D. and CAMPBELL, C.D., 2000. Classical biological control of the Australian weevil *Gonipterus scutellatus* (Coleoptera: Curculionidae) in California. *Environmental Entomology*, vol. 29, no. 2, pp. 369-375. http://dx.doi.org/10.1093/ee/29.2.369.

HURST, M.R., BECHER, S.A., YOUNG, S.D., NELSON, T.L. and GLARE, T.R., 2011. *Yersinia entomophaga* sp. nov., isolated from the New Zealand grass grub *Costelytra zealandica*. *International Journal of Systematic and Evolutionary Microbiology*, vol. 61, no. 4, pp. 844-849. http://dx.doi.org/10.1099/ijs.0.024406-0. PMid:20495033.

KIKUCHI, Y., 2009. Endosymbiotic bacteria in insects: their diversity and culturability. *Microbes and Environments*, vol. 24, no. 3, pp. 195-204. http://dx.doi.org/10.1264/jsme2.ME09140S. PMid:21566374.

MAPONDERA, T.S., BURGESS, T., MATSUKI, M. and OBERPRIELER, R.G., 2012. Identification and molecular phylogenetics of the cryptic species of the *Gonipterus scutellatus* complex (Coleoptera: Curculionidae: Gonipterini). *Australian Journal of Entomology*, vol. 51, no. 3, pp. 175-188. http://dx.doi.org/10.1111/j.1440-6055.2011.00853.x.

MERHEJ, V., ADÉKAMBI, T., PAGNIER, I., RAOULT, D. and DRANCOURT, M., 2008. *Yersinia massiliensis* sp. nov., isolated from fresh water. *International Journal of Systematic and Evolutionary Microbiology*, vol. 58, no. 4, pp. 779-784. http://dx.doi.org/10.1099/ijs.0.65219-0. PMid:18398169.

MONTICELLI, L.S., OUTREMAN, Y., FRAGO, E. and DESNEUX, N., 2019. Impact of host endosymbionts on parasitoid host range-from mechanisms to communities. *Current Opinion in Insect Science*, vol. 32, no. 1, pp. 77-82. http://dx.doi.org/10.1016/j.cois.2018.11.005. PMid:31113635.

PANIAGUA VOIROL, L.R., FRAGO, E., KALTENPOTH, M., HILKER, M. and FATOUROS, N.E., 2018. Bacterial symbionts in Lepidoptera: their diversity, transmission, and impact on the host. *Frontiers in Microbiology*, vol. 9, pp. 556. http://dx.doi.org/10.3389/fmicb.2018.00556. PMid:29636736.

REIS, A.R., FERREIRA, L., TOMÉ, M., ARAUJO, C. and BRANCO, M., 2012. Efficiency of biological control of *Gonipterus platensis* (Coleoptera: Curculionidae) by *Anaphes nitens* (Hymenoptera: Mymaridae) in cold areas of the Iberian Peninsula: Implications for defoliation and wood production in *Eucalyptus globulus*. *Forest Ecology and Management*, vol. 270, pp. 216-222. http://dx.doi.org/10.1016/j.foreco.2012.01.038.

REKHA, R., RIZVI, M.A. and JAISHREE, P., 2006. Designing and validation of genus-specific primers for human gut flora study. *Electronic Journal of Biotechnology*, vol. 9, no. 5, pp. 1. http://dx.doi.org/10.2225/vol9-issue5-fulltext-2.

RENOZ, F., PONS, I., VANDERPOORTEN, A., BATAILLE, G., NOEL, C., FORAY, V., PIERSON, V. and HANCE, T., 2019. Evidence for hut-associated *Serratia symbiotica* in wild aphids and ants provides new perspectives on the evolution of bacterial mutualism in insects. *Microbial Ecology*, vol. 78, no. 1, pp. 159-169. http://dx.doi.org/10.1007/s00248-018-1265-2. PMid:30276419.

REUTER, S., CONNOR, T.R., BARQUIST, L., WALKER, D., FELTWELL, T., HARRIS, S.R., FOOKES, M., HALL, M.E., PETTY, N.K., FUCHS, T.M., CORANDER, J., DUFOUR, M., RINGWOOD, T., SAVIN, C., BOUCHIER, C., MARTIN, L., MIETTINEN, M., SHUBIN, M., RIEHM, J.M., LAUKKANEN-NINIOS, R., SIHVONEN, L.M., SIITONEN, A., SKURNIK, M., FALCÃO, J.P., FUKUSHIMA, H., SCHOLZ, H.C., PRENTICE, M.B., WREN, B.W., PARKHILL, J., CARNIEL, E., ACHTMAN, M., MCNALLY, A. and THOMSON, N.R., 2014. Parallel independent evolution of pathogenicity within the genus *Yersinia*. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 18, pp. 6768-6773. http://dx.doi.org/10.1073/pnas.1317161111. PMid:24753568.

RONQUIST, F. and HUELSENBECK, J.P., 2003. MrBayes 3: bayesian phylogenetic inference under mixed models. *Bioinformatics*, vol. 19, no. 12, pp. 1572-1574. http://dx.doi.org/10.1093/bioinformatics/btg180. PMid:12912839.

ROTHACHER, L., FERRER-SUAY, M. and VORBURGER, C., 2016. Bacterial endosymbionts protect aphids in the field and alter parasitoid community composition. *Ecology*, vol. 97, no. 7, pp. 1712-1723. http://dx.doi.org/10.1890/15-2022.1. PMid:27859175.

SOUZA, N.M., JUNQUEIRA, L.R., WILCKEN, C.F., SOLIMAN, E.P., CAMARGO, M.B., NICKELE, M.A. and BARBOSA, L.R., 2016. *Ressurgência de uma antiga ameaça: Gorgulho-do-eucalipto Gonipterus platensis (Coleoptera: Curculionidae)*. Piracicaba: Instituto de Pesquisas e Estudos Florestais, 20 p. Circular Técnica, no. 209.

SOUZA, R.A., FALCÃO, D.P. and FALCÃO, J.P., 2011. Emended description of *Yersinia massiliensis*. *International Journal of Systematic and Evolutionary Microbiology*, vol. 61, no. 5, pp. 1094-1097. http://dx.doi.org/10.1099/ijs.0.021840-0. PMid:20525812.

SUDAKARAN, S., KOST, C. and KALTENPOTH, M., 2017. Symbiont acquisition and replacement as a source of ecological innovation. *Trends in Microbiology*, vol. 25, no. 5, pp. 375-390. http://dx.doi.org/10.1016/j.tim.2017.02.014. PMid:28336178.

THAO, M.L. and BAUMANN, P., 2004. Evidence for multiple acquisition of *Arsenophonus* by whitefly species (Stenorrhyncha: aleyrodidae). *Current Microbiology*, vol. 48, no. 2, pp. 140-144. http://dx.doi.org/10.1007/s00284-003-4157-7. PMid:15057483.

THOMAS, M.C., ARLING, V., GOJI, N., JANZEN, T.W., DUCEPPE, M., MATHEWS, A., CARRILLO, C. and AMOAKO, K.K., 2018. First complete genome sequence of *Yersinia massiliensis*. *Genome Announcements*, vol. 6, no. 20, e00416-18. http://dx.doi.org/10.1128/genomeA.00416-18. PMid:29773629.

VAN NOUHUYS, S., KOHONEN, M. and DUPLOUY, A., 2016. *Wolbachia* increases the susceptibility of a parasitoid wasp to hyperparasitism. *The Journal of Experimental Biology*, vol. 219, no. 19, pp. 2984-2990. http://dx.doi.org/10.1242/jeb.140699. PMid:27707863.

WALSH, P., METZGER, D. and HIGUCHI, R., 1991. Chelex-100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *BioTechniques*, vol. 10, no. 4, pp. 506-513. PMid:1867860.

ZUG, R. and HAMMERSTEIN, P., 2018. Evolution of reproductive parasites with direct fitness benefits. *Heredity*, vol. 120, no. 3, pp. 266-281. http://dx.doi.org/10.1038/s41437-017-0022-5. PMid:29234159.