MOLECULAR EVIDENCE FOR *Wolbachia* INFECTION IN *Prorops nasuta* (HYMENOPTERA: BETHYLIDAE), A PARASITOID WASP OF THE COFFEE BERRY BORER

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Wolbachia are common endosymbiotic bacteria of insects known for manipulating host reproduction in different ways. In this study we report *Wolbachia* infection in the parasitoid wasp *Prorops nasuta*, an insect introduced into the Americas for the biological control of the coffee berry borer *Hypothenemus hampei*. PCR screening for *Wolbachia* infection based on the endosymbiont marker genes *fstZ* and *wsp* resulted positive in wasps samples collected in coffee fields from Colombia and Brazil and obtained from a laboratory colony in Mexico. DNA sequence analyses of the *wsp* gene identified two different sequence clones that suggest multiple *Wolbachia* isolates infecting these wasp populations. Additionally, phylogenetic analysis of the *ftsZ* and *wsp* sequences located these *Wolbachia* isolates into supergroup A. Whether *Wolbachia* plays a role affecting reproduction in *P. nasuta* remains unclear. However, different scenarios of how *Wolbachia*-infection outcomes may affect insect-pest control using parasitoids are discussed.

Keywords: Endosymbiont, biological control, *Hypothenemus hampei.*

EVIDENCIA MOLECULAR DE INFECCIÓN POR *Wolbachia* EN *Prorops nasuta* (HYMENOPTERA: BETHYLIDAE), UNA AVISPA PARASITOIDE DE LA BROCA DEL CAFÉ

Wolbachia es una bacteria endosimbiótica común en insectos, conocida por manipular la reproducción del hospedante de diferentes maneras. En este estudio se reporta la infección por *Wolbachia* en la avispa parasitoide *Prorops nasuta*, un insecto introducido en las Américas para el control biológico de la broca del café *Hypothenemus hampei*. La detección por PCR para la infección por *Wolbachia* basada en los genes marcadores *fstZ* y *wsp* resultó positiva en muestras de avispas recolectadas en cafetales de Colombia y Brasil y obtenidas de una colonia de laboratorio en México. Los análisis de secuencia de ADN del gen *wsp* identificaron dos clones de secuencia diferentes que sugieren múltiples aislamientos de *Wolbachia* que infectan estas poblaciones de la avispa. Además, el análisis filogenético de las secuencias *ftsZ* y *wsp* localiza estos aislamientos de *Wolbachia* en el supergrupo A. Aún no está claro el papel que desempeña *Wolbachia* en la reproducción en *P. nasuta*. Sin embargo, se discuten diferentes escenarios de cómo la infección por *Wolbachia* puede afectar estrategias de biocontrol usando parasitoides.

Palabras clave: Endosimbionte, control biológico, *Hypothenemus hampei*.

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Wolbachia (Hertig) are maternally inherited bacterial endosymbionts of arthropods and nematodes that live predominantly inside the reproductive tissues of their host (Bandi, Trees, & Brattig, 2001; Werren, 1997). They are best known for manipulating host reproduction in ways that increase their own infection frequency in the coming host generations. Those reproductive manipulations include cytoplasmic incompatibility (CI) (fitness reduction caused when uninfected females mate with infected males or each sex infected by different strains), parthenogenesis (infected females produce only female offspring), male-killing (death of infected male embryos) or feminization (genetically programmed males are turned into females) (Werren *et al*. 2008). Recent estimations propose that *Wolbachia* infects half of the currently known insect species (about 52%) (Weinert *et al.*, 2015). Phylogenetic analyses of the genus *Wolbachia* have shown the existence of 17 major supergroups (A to Q) (Bandi, Anderson, Genchi, & Blaxter, 1998; Casiraghi, 2005; Gerth, Gansauge, Weigert, & Bleidorn, 2014; Glowska, Dragun-Damian, Dabert, & Gerth, 2015; Lo, Casiraghi, Salati, Bazzocchi, & Bandi, 2002; Ma *et al*., 2017; J. H. Werren *et al*., 1995). Most of the proposed *Wolbachia* supergroups infect arthopods, except supergroups C, D, J and L which have been found exclusively in nematodes (Haegeman *et al*., 2009; Koutsovoulos, Makepeace, Tanya, & Blaxter, 2014). However, among the *Wolbachia* spp. infecting arthropods, the majority belongs to supergoups A and B (Correa & Ballard, 2016; Glowska *et al*., 2015).

The wasp *Prorops nasuta* (Waterston) (Hymenoptera: Bethylidae) is an idiobiont solitary parasitoid that was introduced into the Americas as a biological control agent for the Coffee Berry Borer (CBB), *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae). *Prorops nasuta* was initially introduced from Uganda into Brazil in 1929 and reintroduced in several other coffee-growing countries in South and Central America during the 80's and 90's decades (Ferreira & Bueno, 1995; Heinrich, 1965; Infante, Mumford, & Baker, 2005; Infante *et al*., 2001). In Colombia, there were two wasp release events in 1991 and 1996 with insects imported from Ecuador (originally introduced from Kenya) and insects from Brazil, respectively. After these release events, *P. nasuta* was found in CBB-infested coffee crops in Colombia (Maldonado & Benavides, 2008; Rivera *et al.*, 2010; Morales *et al.*, 2011). In Mexico, *P. nasuta* was not established in the field after its introduction from Brazil in 1992 (Infante *et al.*, 2001).

Despite the importance of *P. nasuta* within biological control strategies for the CBB, surprisingly no study has focused on *Wolbachia* infection in this wasp. In parasitic waps, *Wolbachia* infection has been found to cause reproductive modifications such as CI and parthenogenesis (Cook & Butcher 1999), impose physiological cost to the hosts (Fleury, Vavre, Ris, Fouillet, & Boulétreau, 2000), play an important role in development of host ovaries (Dedeine, Boulétreau, & Vavre, 2005) and influence behavior and fitness on infected hosts (Dedeine *et al*., 2001; Kishani Farahani *et al*., 2015, 2017; Liu *et al*., 2018; van Nouhuys, Kohonen, & Duplouy, 2016). Nonetheless, in most of the cases there is not clear understanding of the effects of *Wolbachia* infection in the host biology. The understanding of endosymbiont-host symbiosis may be useful for improving strategies of insect-pest control through insect parasitoids. For example, *Wolbachia*-infections causing female-based sex distortion could be of interest since mostly or only females are produced and released in the field for pest control (Sivinski, 2013). Similarly, *Wolbachia*-infections causing CI must be taken into account to avoid mixing of incompatible parasitoid populations in mass-rearing or field-releases (Floate, Kyei-Poku, & Coghlin, 2006).

Here, we report the presence of *Wolbachia* infection in *P. nasuta* populations from Colombia and Brazil as well as wasps from a laboratory colony in Mexico. The molecular presented evidence indicates that all detected *P. nasuta*-infecting *Wolbachia* belongs to the supergroup A. Our findings also suggest that at least two different *Wolbachia* strains may be infecting *P. nasuta* in these populations.

MATERIALS AND METHODS

Insects and DNA isolation: *Prorops nasuta* samples from Colombia and Brazil were obtained from naturally CBB-infested coffee beans collected in coffee fields (Table 1). Wasps from Mexico were obtained from parasitized CBB reared under laboratory conditions for over 16 years. A *Drosophila melanogaster* (Meigen) wild strain naturally infected with *Wolbachia* was used as positive control for *Wolbachia* PCR detection. Pools of individuals of *P. nasuta* from each location were used for DNA isolation using the DNeasy Tissue Kit (Quiagen) according to manufacture protocol for animal tissue samples. Genomic DNA isolated was stored at -20°C.

PCR screening and DNA sequencing: The presence of *Wolbachia* in insect samples was determined by PCR amplification using the *Wolbachia*-specific *ftsZ* primers (*ftsZ*F: GTATGCCGATTGCAGAGCTTG and *ftsZ*R: GCCATGAGTATTCACTTGGCT) (Werren *et al.*, 1995) and *wsp* primers (*wsp*81F: TGGTCCAATAAGTGATGAAGAAAC and *wsp*691R: AAAAATTAAACGCTACTCCA) (Zhou *et al.*, 1998). PCR control reactions to test the quality of the DNA were carried out with 28s rDNA universal arthropod primers (28sF3633: TACCGTGAGGGAAAGTTGAAA and

Table 1. Samples of *Prorops nasuta* included in the *Wolbachia*-screening analysis.

Sample	Location	Population	Individuals*
BR02	Viçosa, Minas Gerais, Brazil	Wild	5
BR03	Viçosa, Minas Gerais, Brazil	Wild	5
MX03	ECOSUR, Chiapas, Mexico	Laboratory	5
MX05	ECOSUR, Chiapas, Mexico	Laboratory	5
CE03	Delicias, Jagua de Ibirico, Cesar, Colombia	Wild	1
NS24	Chinacota, Norte de Santander, Colombia	Wild	5
M03	El Trébol, Chinchiná, Caldas, Colombia	Wild	21
P03	Santa Ana, Palestina, Caldas, Colombia	Wild	2
Q34	Morelia Alta, Quimbaya, Quindío, Colomia	Wild	18
R10	Guadualito, Pereira, Risaralda, Colombia	Wild	7
A110	Yananchá, Ancuyá, Nariño, Colombia	Wild	8
S56	Bellavista, Sandoná, Nariño, Colombia	Wild	1
CN41	El Salado, Consacá, Nariño, Colombia	Wild	9

*Number of individuals included in the pooled DNA extraction

28sR4076: AGACTCCTTGGTCCGTGTTT). The samples were also screened for *Wolbachia* A and B supergroups using the specific A-*wsp* (*wsp*136AF: TGAAATTTTACCTCTTTTC and *wsp*691R) and B-*wsp* (*wsp*81F and *wsp*522BR: ACCAGCTTTTGCTTGATA) primers (Zhou *et al.*, 1998). All PCR amplifications were performed as described by Zhou *et al.* (1998). PCR products were visualized on agarose gels under UV light. Genomic DNA samples that failed to produce a DNA band with the 28s rDNA universal primers was discarded from the analysis. For DNA sequence confirmations, PCR products of the *ftsZ* gene from Colombia (sample R10) and *wsp* gene from Brazil (BR02), Mexico (MX03) and Colombia (R10) were cloned into the pGEM-T vector (Promega) and transformed in the One Shot® TOP10 chemically competent *E. coli* (Invitrogen). Recombinant plasmids were purified with the QIAprep Kit (Qiagen) and used for ABI automated sequencing with T7 and SP6 primers. Cloning of PCR products for sequencing was used since it allows the detection of multiple *Wolbachia* strains within a pooled DNA sample. DNA sequences were edited and assembled with CodonCode Aligner v1.6.3 software (CodonCode Corporation). Similarities with *ftsZ* and *wsp* genes reported previously were searched by BLASTn against NCBI databases.

Phylogenetic analysis: A 622 bp DNA fragment of *ftsZ* (positions 1 to 622) and a 561 bp DNA fragment of *wsp* (positions 25 to 585) sequences obtained in this study were aligned respectively with other *ftsZ* and *wsp* DNA sequences deposited previously at NCBI GenBank. Multiple sequence alignments were performed with ClustalW algorithm. Phylogenetic trees were inferred using maximum-likelihood (evolutionary model: GTR) with SeaView (Gouy *et al.*, 2010). Branch supports were estimated by the approximate likelihood ratio test (aLRT).

RESULTS

***Wolbachia* detection**: Thirteen samples of the parasitoid wasp *P. nasuta* obtained from Colombia, Brazil and Mexico and one sample of a *Wolbachia*-infected *D. melanogaster* were included for *Wolbachia* PCR screening using *Wolbachia*-specific *ftsZ* and *wsp* primers. All DNA samples of *P. nasuta* obtained from Colombia, Brazil and Mexico yielded positive for *Wolbachia* infection by PCR screening using the *ftsZ* (~770 bp) and *wsp* (~600 bp) genes (Figure 1A). The positive *D. melanogaster* control yielded also similar DNA bands. The quality of the DNA samples were confirmed by PCR amplification of the insect 28s rDNA gene (data not shown).

***Wolbachia* sequence analysis**: DNA sequencing of the *P. nasuta Wolbachia* (wNas) *ftsZ* DNA fragment amplified from sample R10 (Colombia) resulted in a 773 bp sequence (GenBank accession: MF150853). A BLAST similarity search with wNas *ftsZ* sequence indicated a 99.72% nucleotide identity with the *ftsZ* gene encoded by a wAu strain (GenBank accession: LK055284) from *D. simulans* (Sutton, Harris, Parkhill, & Sinkins, 2014) reported at NCBI nr database. DNA sequencing for wNas *wsp* clones from Colombia (R10), Brazil (BR02) and Mexico (MX03) (GenBank accessions: MF150854 to MF150858) resulted in two *wsp* sequence clones (605 bp each); named here as *wsp*1 and *wsp*2. The wsp1 sequence clone was found in Colombia (*wsp*1R10, MF150854), Mexico (*wsp*1MX03, MF150855) and Brazil (*wsp*1BR02, MF150856). The latter only differ in a single base at position 214 (Figure 2). The *wsp*2 sequence clone was found in Mexico (wsp2MX03, MF150857) and Brazil (wsp2BR02, MF150858), and differs from *wsp*1 in 29 positions (Figure 2) The wNas *wsp*1 sequence clone showed 99% nucleotide identity with the *wsp* gene encoded by a wAnd strain (GenBank accession:

AB052667) from *Andricus mukaigawae* (Mukaigawa) (Hymenoptera; Cynipidae) (Abe & Miura, 2002). The wNas *wsp*2 sequence clone showed 100% nucleotide identity with the *wsp* gene encoded by a wPcurA1 strain (GenBank accession: AY878108) from *Pseudacteon curvatus* (Borgmeier) (Diptera: Phoridae) (Dedeine *et al.*, 2005).

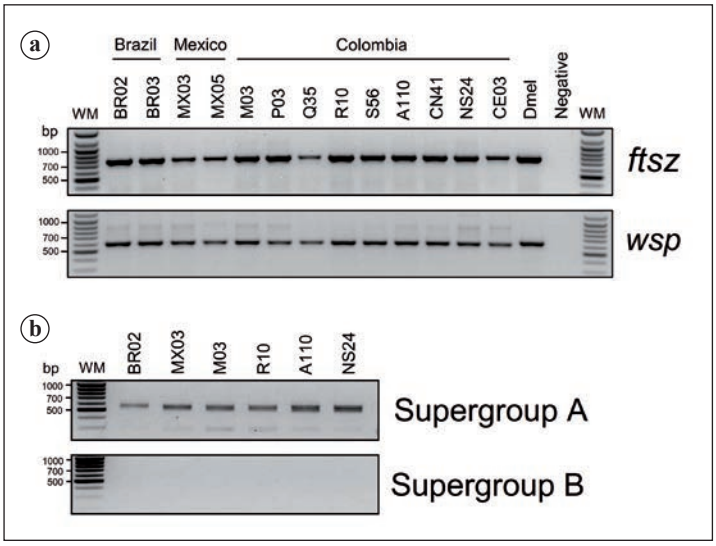

Figure 1. PCR screening for *Wolbachia* detection. (A) Molecular detection using PCR primers specific for *Wolbachia ftsZ* and *wsp* gene fragments in 13 *P. nasuta* DNA samples from Brazil, Mexico and Colombia. (B) Molecular screening of *Wolbachia* supergroups A and B on representative *P. nasuta* DNA samples from Brazil, Mexico and Colombia. Detailed information for each sample name are presented in Table 1.

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                          10A        20G        30A        40T        50A        60T        70T        80G        90T       100G
PnasWsp1_MX03     1 TGGTCCAATAAGTGATGAAGAAACTAGTTACTATATTCGTTTGCAATACAACGGTGAAATTTTACCTCTTTTCACAAAAGTTGATGGTATTACCTATAAGA 101
PnasWsp1_R10      1 ............................................................................................... 101
PnasWsp1_BR02     1 ............................................................................................... 101
PnasWsp2_MX03     1 ..........................C.....CG.....................................A....A......GAA.....A. 101
PnasWsp2BR02      1 ..........................C.....CG.....................................A....A......GAA.....A. 101

                         110A       120G       130A       140C       150G       160T       170A       180T       190T       200G
PnasWsp1_MX03   102 AAGACAATAGTGATTGCAGTCCATTAAAAGCGTCTTTTCTAGCTGGTGGTGGTGCGTTTGGTTACAAAATGGACGACATCAGGGTTGATGTTGAAGGAGTT 202
PnasWsp1_R10    102 ............................................................................................... 202
PnasWsp1_BR02   102 ............................................................................................... 202
PnasWsp2_MX03   102 .G.C..CAGACAT.CAT.A...........A......A..................................................................... 202
PnasWsp2BR02    102 .G.C..CAGACAT.CAT.A...........A......A..................................................................... 202

                         210A       220A       230A       240G       250A       260G       270T       280T       290G       300A
PnasWsp1_MX03   203 TATTCATACCTAAACAAAAATAATGTTACAGATGCAAGATTTACGCCAGATACTATTGCAGACAGTGTAACAGCAATTTCAGGGCTAGTTAACGTTTATTA 303
PnasWsp1_R10    203 ............................................................................................... 303
PnasWsp1_BR02   203 ...........G............................................T.............................................. 303
PnasWsp2_MX03   203 .......................G.............A.....................T.............................................. 303
PnasWsp2BR02    203 .......................G.............A.....................T.............................................. 303

                         310A       320G       330T       340T       350G       360C       370T       380T       390C       400T
PnasWsp1_MX03   304 CGATATAGCAATTGAAGATATGCCTATCACTCCATATGTTGGTGTTGGTGTTGGTGCAGCGTATATTAGCACTCCTTTGAAAGACGCTGTGAATGATCAAA 404
PnasWsp1_R10    304 ............................................................................................... 404
PnasWsp1_BR02   304 ............................................................................................... 404
PnasWsp2_MX03   304 ............................................................................................... 404
PnasWsp2BR02    304 ............................................................................................... 404

                         410A       420T       430A       440G       450A       460T       470A       480C       490T       500T
PnasWsp1_MX03   405 AAAGTAAATTTGGTTTTGCTGGTCAAGTAAAAGCTGGTGTTAGTTATGATGTAACTCCAGAAGTCAAACTTTATGCTGGAGCTCGTTATTTCGGTTCTTAT 505
PnasWsp1_R10    405 ............................................................................................... 505
PnasWsp1_BR02   405 ............................................................................................... 505
PnasWsp2_MX03   405 ............................................................................................... 505
PnasWsp2BR02    405 ............................................................................................... 505

                         510C       520T       530G       540G       550A       560C       570C       580A       590G       600A
PnasWsp1_MX03   506 GGTGCTAATTTCGATAAAAGCGGTGGTGAGAAAGGCAAAGGAGGATATACAGTCCTTTACAGCACTGTTGGTGCAGAAGCTGGAGTAGCGTTTAATTTTT 605
PnasWsp1_R10    506 ............................................................................................... 605
PnasWsp1_BR02   506 ............................................................................................... 605
PnasWsp2_MX03   506 ..........................A.....C.............................................................. 605
PnasWsp2BR02    506 ..........................A.....C.............................................................. 605
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Figure 2. Clustal DNA sequence alignment of *wsp* sequence clones detected in *P. nasuta*. Using the wsp1 sequence clone from sample MX03 as reference, the polymorphic nucleotide positions are shown among the different *wsp* clones sequenced in this study. The conserved nucleotide positions are represented by dots (.).

***Wolbachia* classification**: PCR analysis to identify the *Wolbachia* groups showed that all infections in *P. nasuta* belongs to the supergroup A, as it was detected by the amplification of a DNA fragment of ~550 pb using the A-*wsp* specific primers in samples from Colombia, Brazil and Mexico (Figure 1B). The B-*wsp* specific primer combination failed to amplify the expected DNA band. The phylogenetic analysis based on *fstZ* and *wsp* sequences also placed the wNas *Wolbachia* isolates into the supergroup A clade (Figure 3A). Additionally, each wNas *wsp* sequence clone was clustered in distinct subclades, closely related to *Wolbachia* strains wAnd and wRi, respectively (Figure 3B).

DISCUSSION

From the 17 *Wolbachia* supergroups (supergroup A to Q) designated so far (Werren *et al.*, 1995; Zhou *et al.*, 1998), supergroups A and B are mostly present in insects (Correa & Ballard, 2016; Glowska *et al*., 2015). In this study, the PCR-screening and phylogenetic analyses based on *wsp* gene fragments supported the

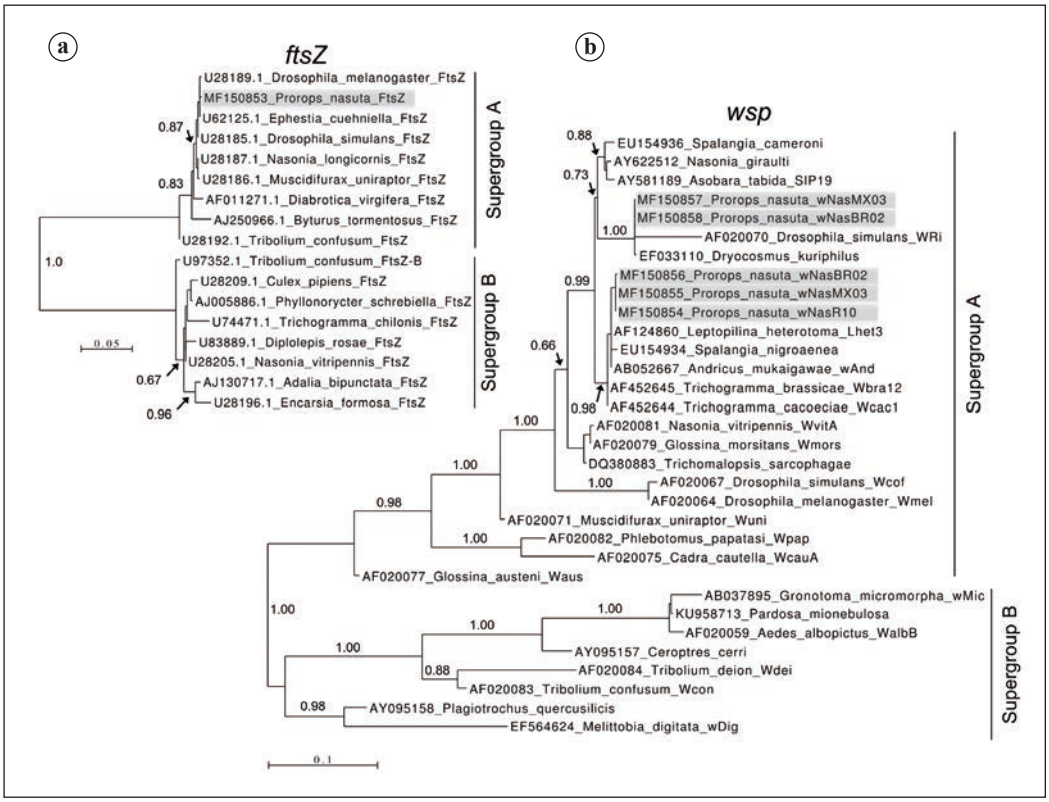

Figure 3. Phylogenetic gene trees for *Prorops nasuta*-infecting *Wolbachia* isolates. Maximum-likelihood trees based on *ftsZ* (A) and *wsp* (B) sequences, including representative of several *Wolbachia* strains withing supergroups A and B. Branch supports were estimated using approximate likelihood-ratio test (aLRT). The *Wolbachia* isolates sequenced in this study are highlighted in grey. GeneBank accession numbers of *Wolbachia ftsZ* and *wsp* sequences precede the host insect species names.

position of the *P. nasuta*-infecting *Wolbachia* isolates into the supergroup A. According to Zhou *et al.* (1998), *Wolbachia* strains in supergroups A and B can be assigned to 12 groups (Mel, AlbA, Mors, Uni, Riv, Haw, Pap, Aus, Con, Dei, Oru and Pip), and based on a *wsp* gene sequence fragment, members of the same *Wolbachia* group must share at least 97.5% sequence similarity. The wNas *wsp*1 and *wsp*2 sequence clones share 95.0% sequence similarity and are clustered in distant phylogenetic positions inside the supergroup A (Figure 3. B). These observations may suggest the possibility that at least two different *Wolbachia* strains could be infecting the *P. nasuta* populations. Further molecular genotyping using the *Wolbachia* multilocus sequence typing (MLST) (Baldo *et al.*, 2006) will be necessary for better characterization of the *P. nasuta*-infecting *Wolbachia* isolates. Since DNA extraction was performed on pools of wasps in this study, we could not obtain the frequency of *Wolbachia* infection per location sampled here. Future estimations of infection frequencies will be useful to establish the distribution and prevalence of *Wolbachia* infection in wasp field-populations and may help to better understand its role in the *P. nasuta* biology.

Horizontal transmission of *Wolbachia* across phylogenetically distant species has been proposed as one possible natural mechanisms for the widespread infection in insects (Heath *et al.*, 1999). This idea is supported by the discovery of closely related *Wolbachia* isolates in a number of host-parasitoid associations (Vavre *et al.*, 1999; Noda *et al.*, 2001; Li *et al.*, 2013). For example, host-parasitoid horizontal transmission was strongly suggested when the majority of *Wolbachia* strains isolated from two parasitoids species (the braconid wasp *Macrocentrus cingulum* and the tachinid fly *Lydella grisescens*) were genetically closely related (≥ 99% *wsp* sequence similarity) to those detected in their host (the Lepidoptera corn borer Ostrinia furnacalis) (Li *et al.*, 2013). *Wolbachia* infection has already been reported in *H. hampei* in samples from Africa, Asia and the Americas (Vega, Benavides, Stuart, & O'Neill, 2002; Mariño, Verle Rodrigues, & Bayman, 2017). The wNas *wsp* clones obtained in this study share 93-94% similarity with the *wsp* sequences obtained from the CBB-infecting *Wolbachia* strains within the supergroup A (GenBank accessions KX436087 to KX436090) (Mariño, Verle Rodrigues, & Bayman, 2017) and 80-81% similarity with the CBB-infecting *Wolbachia* strains within the supergroup B (GenBank accession AF389084) (Vega, Benavides, Stuart, & O'Neill, 2002). Whether or not *P. nasuta* was able to obtain the *Wolbachia* infection from their host through horizontal transmission is an open question that could be answered using larger numbers of *P. nasuta* samples and a most robust DNA sequence analysis.

Since *Wolbachia* can manipulate host reproduction causing cytoplasmic incompatibility and other sex-ratio distorting effects in Hymenopterans (Cook & Butcher 1999), the evidence of *Wolbachia*-infection in *P. nasuta* presented here bring new questions about the possible reproductive effects on natural *P. nasuta* wasp populations. Whether *Wolbachia* plays a role affecting reproduction or fitness in *P. nasuta* remains unclear. The possibility that infection with different *Wolbachia* strains may cause CI among *P. nasuta* populations could be important when designing parasitoid mass-rearing or mass-release strategies. CI would have negative impacts since mixing of these populations may retard population growth rate in mass-rearing or if looking for augmentation of local wasp populations. This plausible scenario should arouse greater scientific interest to understand the effect of *Wolbachia*-infection on *P. nasuta* biology.

CONCLUSIONS

This study reports the first molecular evidence of *Wolbachia* infection in the parasitoid wasp *P. nasuta* in laboratory and field populations. All *P. nasuta*-infecting *Wolbachia* isolates detected in this study belongs to supergroup A. Our findings suggest the possibility of multiple *Wolbachia* strains infecting different *P. nasuta* populations. Further investigations will be necessary to understand the outcome of this endosymbiont-wasp interaction and their implications for the use of *P. nasuta* in biocontrol strategies.

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