YOAN CAMILO GUZMAN SARMIENTO

INÍCIO DA RELAÇÃO *Wolbachia*-HOSPEDEIRO EM *Drosophila sturtevanti* E *Zaprionus indianus* (DIPTERA: DROSOPHILIDAE) NA REGIÃO NEOTROPICAL

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Magister Scientiae.*

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Dedico este trabalho a minha
filha Isabel Sophia

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RESUMO

Guzmán-Sarmiento, Yoan Camilo, M.Sc., Universidade Federal de Viçosa, fevereiro de 2017. **Início da Relação *Wolbachia*-hospedeiro em *Drosophila sturtevanti* e *Zaprionus indianus* (Diptera: Drosophilidae) na Região Neotropical**. Orientadora: Karla Suemy Clemente Yotoko. Coorientadores: Hermes Fonseca de Medeiros e Anésia Aparecida dos Santos

Wolbachia é um grupo de α-proteobactérias intracelulares, herdado verticalmente e está presente em artrópodos e nematódeos. É bem conhecido por favorecer sua própria transmissão, causando diferentes fenótipos reprodutivos que costumam aumentar o valor adaptativo das fêmeas infectadas. Em 2015, a equipe do Laboratório de Bioinformática e Evolução (UFV) iniciou a busca por espécies de Drosophilidae infectadas por *Wolbachia.* Das espécies infectadas, selecionamos duas para iniciar nossos estudos da relação *Wobachia*-hospedeiros. O primeiro capítulo desta dissertação trata da infecção encontrada em *Drosophila sturtevanti,* uma espécie nativa de ampla distribuição comumente encontrada em áreas de floresta e que já foi reportada como hospedeira de *Wolbachia*. O segundo capítulo trata da infecção inédita de *Zaprionus indianus,* uma invasora de origem africana, agora espalhada pelo continente americano. Encontramos prevalência de 100% de uma única linhagem em *D. sturtevanti*, idêntica a *wStv_MI*, previamente encontrada no Panama. Em *Z. indianus*, econtramos prevalências de ~30% em indivíduos coletados em três localidades distintas, com evidência de que estejam infectadas por pelo menos quatro cepas de *Wolbachia*. Enquanto o resultado do estudo com *D. sturtevanti* sugere um processo de homogeinização da infecção, o estudo com *Z. indianus* mostra uma dinâmica mais complexa, que pode incluir eventos frequentes de transmissão horizontal entre hospedeiros, dupla ou múltipla infecção no mesmo indivíduo e recombinação entre diferentes cepas de *Wolbachia*. Estes dois estudos levaram a novas perguntas, que já se sustentam como pontos de partida para o estudo da evolução da relação *Wolbachia*-Drosophilidae na região neotropical.

ABSTRACT

Guzmán-Sarmiento, Yoan Camilo, M.Sc., Universidade Federal de Viçosa, February, 2017. **Beginning of the *Wolbachia*-host relationship in *Drosophila sturtevanti* and *Zaprionus indianus* (Diptera: Drosophilidae) in Neotropics**. Advisor: Karla Suemy Clemente Yotoko. Co-advisors: Hermes Fonseca de Medeiros and Anésia Aparecida dos Santos.

Wolbachia is a group of intracellular α-proteobacteria which is inherited vertically and is extremely abundant in Arthropoda and Nematoda. It is well known for favoring its transmission by causing different reproductive phenotypes that increase the infected female fitness. In 2015, the group of the Laboratório de Bioinformática e Evolução (UFV) started to search for Drosophilidae species infected by *Wolbachia*. From the infected species, we selected two to start our studies of the relationship *Wolbachia*-hosts. The first chapter of this work addresses the infection found in *Drosophila sturtevanti*, a native species with a wide geographical range, found in forests and already related as a *Wolbachia*-host. The second chapter shows the first register of *Wolbachia* in *Zaprionus indianus*, an African invasive species that spread over Americas. In *D. sturtevanti*, we found a prevalence of 100% of a single *Wolbachia* strain, *wStv MI*, previously found in Panama. In *Z. indianus*, we found prevalences of ~30% in individuals from three different localities, with evidence of at least four different *Wolbachia* strains. While our result with *D. suturtevanti* suggests a homogenization process, the results with *Z. indianus* show a much more complex dynamics that could include numerous events of horizontal transfers among hosts, double of multiple infections in the same individual as well as recombination among different *Wolbachia* strains. The two studies led us to ask further questions, which are already starting points to the study of the evolution of the *Wolbachia*-host relationships in the Neotropical region.

SUMÁRIO

INTRODUCCIÓN

Wolbachia: ¿Qué es y cómo se estudia?

Wolbachia es un grupo de α-proteobacterias intracelulares, heredado de forma materna que se encuentra en artrópodos y nematodos (Werren 2003). Esta bacteria es uno de los endosimbiontes más abundante conocidos, pues estudios actuales estiman que por lo menos 50 % de los artrópodos estarían infectados por *Wolbachia* (Weinert et al. 2015). Para favorecer su propia transmisión, *Wolbachia* causa diferentes alteraciones en sus hospederos conocidas como fenotipos reproductivos, que suelen aumentan el valor adaptativo de las hembras infectadas (Werren et al. 2008; Correa and Ballard 2016). Entre los fenotipos reproductivos conocidos, se hallan Incompatibilidad Citoplasmática (IC), partenogénesis telitoquia, feminización de machos genéticos y muerte de embriones machos (Stouthamer et al. 1999; Werren et al. 2008). También se suman otras ventajas adaptativas como la protección antiviral (Martinez et al. 2012) y los aportes nutricionales (Nikoh et al. 2014). Por tanto, debido a su abundancia y los potenciales efectos ecológicos y evolutivos sobre sus hospederos, *Wolbachia* ha sido materia de investigación desde hace más de dos décadas.

Sistemas de clasificación de *Wolbachia*

Estudios filogenéticos que relacionan la variabilidad de hospederos e fenotipos inducidos por *Wolbachia* usaron inicialmente secuencias de rRNA

16S. A partir de estos datos, fueron establecidos siete grande supergrupos nombrados de A a H (Figura 1) (revisado por Werren et al. 2008)). En 1998, Zhou et al. propusieron el uso de la secuencia de gen *wsp* (*Wolbachia* Surface Protein), asociado a la respuesta inmune del hospedero, para separar linajes estrechamente relacionados de la bacteria. Sin embargo, posteriores trabajos demostraron conflictos en las filogenias reconstruidas a partir de la secuencia del *wsp* debido a la recombinación entre supergrupos en cuatro regiones hipervariables (HVRs) identificadas como HVR1, HVR2, HVR3 y HVR4 (Baldo et al. 2005). Patrones de recombinación también fueron hallados otros genes de *Wolbachia* como *ftsZ* y *gatB* (Baldo et al. 2006a). La metodología hoy utilizada de clasificación de *Wolbachia* fue propuesta por Baldo et al. (2006b), quienes formularon el MLST (Multi-Locus Sequence Typing).

La clasificación de *Wolbachia* por MLST cuenta con una extensa base de datos donde se almacenan secuencias que identifican las cepas y sus hospederos. En esta base de datos, cada secuencias diferente (alelo) de los loci (*gatB, coxA, hcpA, ftsZ, fbpA* y HVRs de *wsp*) es identificada con un numero entero, y el conjunto de estos numero forma el perfil de una cepa (Jolley and Maiden 2010). De esta forma, se obtiene un perfil alélico único para cada cepa que también recibe su clasificación en supergrupos (siempre que es posible). La identificación de las cepas infectantes es el primer paso para estudiar las relaciones ecológicas y evolutivas de la interacción *Wolbachia*-hospedero.

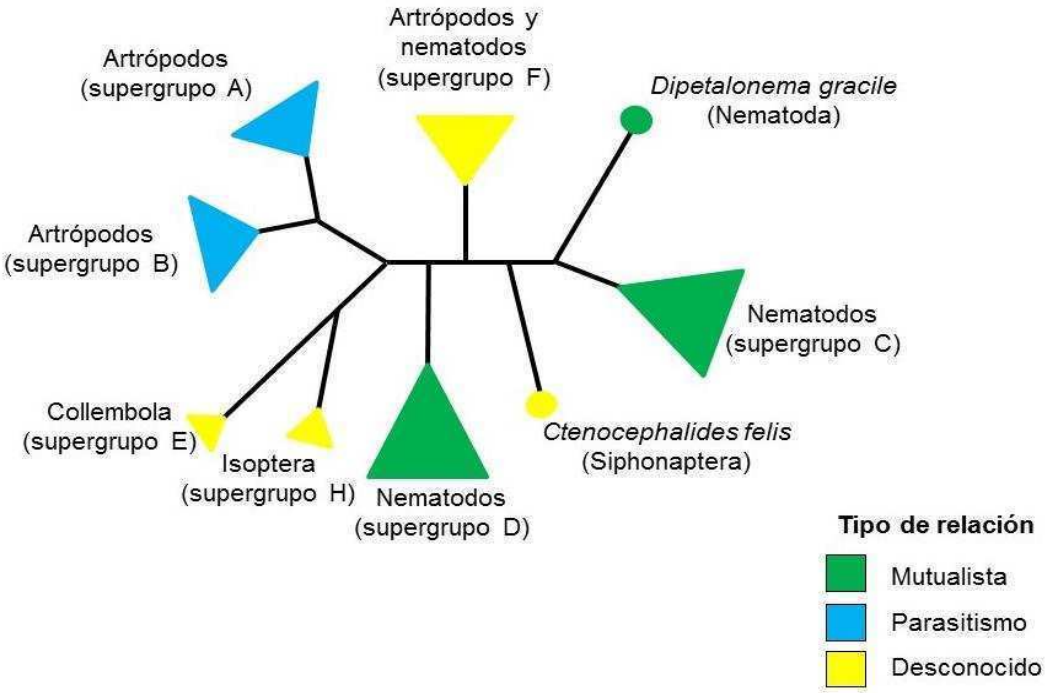

Figura 1. Árbol no enraizado de los principales supergrupos de *Wolbachia*. Mostrando los principales hospederos asociados y las relaciones que mantienen: mutualista, parasita o aún no determinada. (Werren et al. 2008).

Wolbachia en Drosophilideos: un modelo de estudio sesgado.

A pesar de los numerosos trabajos publicados con *Wolbachia,* preguntas fundamentales acerca de los mecanismos de la persistencia global y los efectos ecológicos y evolutivos siguen sin respuesta (Weeks et al. 2002). Además, grandes áreas geográficas y grupos taxonómicos de insectos permanecen sin explorar (Werren et al. 2008; Bennett et al. 2012). Existe también un gran sesgo en los estudios de *Wolbachia*: las especies de la región paleartica *Drosophila melanogaster* y *Drosophila simulans* (Diptera:Drosophilidae) son las que concentran la mayoría de trabajos

publicados (Miller and Riegler 2006), poniendo al margen la gran diversidad biológica de Drosophilidae en otras regiones geográficas.

Así mismo, hay un pequeño número de trabajos que exploran la prevalencia y los efectos de *Wolbachia* en drosophilideos Neotropicales. Bourtzis et al. (1996) demostraron la presencia de *Wolbachia* en 30 especies de drosofilideos, entre ellas solo seis especies Neotropicales en las cuales no se detectó *Wolbachia*. Más tarde, Miller and Riegler (2006) encontraran *Wolbachia* en ocho especies de *Drosophila* del grupo Saltans y dos del grupo Willistoni, ambas Neotropicales. Estudios posteriores se centraran en especies del grupo Willistoni, específicamente *Drosophila willistoni* (Müller et al. 2012; Müller et al. 2013) y *Drosophila paulistorum* (Miller et al. 2010).

El inicio de una nueva línea de investigación: *Wolbachia* en drosophilideos Neotropicales.

En 2015, el grupo del Laboratorio de Bioinformatica y Evolucion (Universidade Federal de Viçosa) inició un trabajo para buscar por las especies de Drosophilidae presentes en la región suroeste de Minas Gerais (Brasil), y, dentro de estas, cuales estaban infectadas por *Wolbachia*. Como resultados, construimos una lista de 39 especies confirmadas para la región, con 14 nuevos registros para el estado (Tabla 1) y evidencia de infección por *Wolbachia* en nueve especies. Esta búsqueda tiene el potencial de encontrar muchas otras especies, ya que históricamente los esfuerzos de muestreo se han concentrado en otros estados de Brasil (Gottschalk et al. 2008).

Tabla 1. Lista de Drosophilidae muestreadas en la región suroeste de Minas Gerais (Brasil) por el equipo del Laboratório de Bioinformática e Evolução en 2015.

Grupo	**Genero**	**Especie**	
Annulimana	*Drosophila*	*arauna**	Pavan and Nacrur, 1950
Calloptera	*Drosophila*	aff. *Schildi**	Malloch, 1924
	Drosophila	*quadrum**	(Wiedemann, 1830)
Coffeata	*Drosophila*	*fuscolineata*	Duda, 1925
Dreyfusi	*Drosophila*	*dreyfusi*	Duda, 1927
Guarani	*Drosophila*	*griseolineata*	Duda, 1927
	Drosophila	*guaru**	Dobzhansky and Pavan, 1943
Immigrans	*Drosophila*	*immigrans*	Sturtevant, 1921
Melanogaster	*Drosophila*	*melanogaster*^{w}	Meigen, 1830
	Drosophila	*simulans*^{w}	Sturtevant, 1919
Repleta	*Drosophila*	*mercatorum*	Patterson and Wheeler, 1942
Saltans	*Drosophila*	*neosaltans**^{w}	Pavan and Magalhaes in Pavan, 1950
	Drosophila	*prosaltans*^{w}	Duda, 1927
	Drosophila	*sturtevanti*^{w}	Duda, 1927
Tripunctata	*Drosophila*	*bandeirantorum*	Dobzhansky and Pavan, 1943
	Drosophila	*mediopicta*	Frota-Pessoa, 1954
	Drosophila	*mediopunctata*	Dobzhansky and Pavan, 1943
	Drosophila	*mediosignata**	Dobzhansky and Pavan, 1943
	Drosophila	*nappae*	Valente and Basso-da-Silva, 2004
	Drosophila	*paraguayensis*	Duda, 1927
	Drosophila	*roehrae**	Pipkin and Heed, 1964

Grupo	Genero	Especie	
	Drosophila	*trapeza*	Heed and Wheeler, 1957
	Drosophila	Aff. *tripunctata**	Loew, 1862
	Drosophila	*unipunctata*	Patterson and Mainland in Patterson, 1943
Willistoni	*Drosophila*	*capricorni*	Dobzhansky and Pavan, 1943
	Drosophila	*fumipennis*^{w}	Duda, 1925
	Drosophila	*malerkotliana*	Parshad and Paika, 1964
	Drosophila	*paulistorum*^{w}	Dobzhansky and Pavan in Burla et al., 1949
	Drosophila	*willistoni*^{w}	Sturtevant, 1916
Latifasciaeformis	*Scaptodrosophila*	*latifasciaeformis*	(Duda, 1940)
Vittiger	*Zaprionus*	*indianus*^{w}	Gupta, 1970
Dispar	*Zygotrica*	*dispar**	(Schiner, 1868)
Orbitalis	*Zygotrica*	*orbitalis**	(Sturtevant, 1916)
Vittimaculosa	*Zygotrica*	*vittinubila**	Burla, 1956
Not assigned to group	*Drosophila*	*suzukii** ^{w}	(Matsumura, 1931)
	Drosophila	*lutzii**	Sturtevant, 1916
	Drosophila	sp22.	Medeiros and Klaczko, 2004
	Hirtodrosophila	sp.	Duda, 1924
	Mycodrosophila	*projectans**	(Sturtevant, 1916)

* Nuevos registros para Minas Gerais; ^{w} especies donde se ha detectado la infección por *Wolbachia*

Dos de las especies infectadas fueron seleccionadas para iniciar nuestros estudios. La primera, *Drosophila sturtevanti* es una especie nativa de amplio rango de distribución y comúnmente encontrada en áreas de bosque.

En *D. stutervanti* ya había sido reportada la infección por *Wolbachia* en Panama (Miller and Riegler, 2006), pero esta infección fue caracterizada solamente por el gen wsp (sin las secuencias del MLST de *Wolbachia*). La segunda, *Zaprionus indianus,* es una especie invasora de origen africano que se expandió rápidamente en América y de la cual no se tenían reportes de infección de *Wolbachia*. De este modo esta disertación trae informaciones sobre la infección por *Wolbachia* en dos especies de hábitos ecológicos diferentes, ampliamente distribuidas y patrones de infección contrastantes. El primer capítulo muestra la infección generalizada de una cepa de *Wolbachia* en *D. sturtevanti* mientras el segundo capítulo evidencia la complejidad de la dinámica *Wolbachia*-hospedero en el primer reporte de *Wolbachia* en *Z. indianus*, con por lo menos cuatro cepas infectantes.

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CHAPTER 1

High-titer *Wolbachia* infection in a Brazilian population of *Drosophila sturtevanti*: evidence of a rapid spread of a recent acquired infection

ABSTRACT

Wolbachia is the most abundant endosymbiont in arthropods. It is a maternally inherited bacteria, well known to cause reproductive alterations in its hosts and confers some fitness benefits to infected females. In 2015, our research group found females of *D. sturtevanti* infected by Wolbachia in Viçosa MG, Brasil. This species previously presented intermediary prevalence of three strains of Wolbachia in samples collected Panama and Barbados from 1998, and no evidence of infection before 1996. We thus investigated whether the Brazilian population harbors one of the strains infecting the Central American *D. sturtevanti*. We found 100% of prevalence of a strain identical with the previously report strain *wStv* MI (from Panama). Our results suggest that this infection spread along the *D. sturtevanti* distribution probably due to increases in the fitness of the host.

Key words: *wStv*, *wsp*, MLST loci, Neotropical drosophilids.

INTRODUCTION

Wolbachia is a well-succeeded endosymbiont infecting more than 50% of Arthropoda species (Weinert et al. 2015). It is usually transmitted vertically, i.e., from the mother to the offspring through infected eggs, and produces different reproductive phenotypes in their hosts that tend to increase the fitness of infected females. In Drosophilidae, only two reproductive phenotypes were observed until now: the cytoplasmic incompatibility (CI) and the male killing (MK). CI corresponds to the sterility of crosses involving non-infected females and infected males while infected females produce viable offspring in crosses with infected and non-infected males (Hoffmann et al. 1986; Giordano et al. 1995; Bourtzis et al. 1996; Charlat et al. 2002; Miller et al. 2010; Richardson et al. 2016). MK is the death of genetic males (Dyer and Jaenike 2004; Richardson et al. 2016), although this phenotype appears rare within *Drosophila* (Montenegro et al. 2006; Sheeley and McAllister 2009). There are also infections that increase the fitness of their hosts without causing CI or MK (Hoffmann et al. 1996; Hamm et al. 2014), but, for example, protecting them against viral infections (Martinez et al. 2012; Chrostek et al. 2013).

Phylogenetic hypotheses of *Wolbachia* strains and their hosts are often discordant (O'Neill et al. 1992; Heath et al. 1999; Vavre et al. 1999; Werren and Windsor 2000), suggesting extensive *Wolbachia* horizontal transfers (HT) over time and offering an explanation for the broad range of hosts among terrestrial invertebrates (Boyle et al. 1993; Werren and Windsor 2000; Sintupachee et al. 2006; Ahmed et al. 2016). Specifically, *w*Mel and *w*Au, two

related *Wolbachia* strains originally described in *Drosophila melanogaster* Meigen, 1830 and *Drosophila Simulans* Sturtevant, 1919, respectively, had involved in HT between cosmopolitan and Neotropical (Miller and Riegler 2006; Müller et al. 2013), or Australian (Richardson et al. 2016) species, in both directions. Such HT events are evident in phylogenetic hypotheses showing the close relationships of the *Wolbachia* strains infecting different Drosophilidae hosts (e.g. Wallau et al. 2016).

Indeed, most Neotropical Drosophilidae studied so far are infected by *Wolbachia* strains closely related to strains associated with *D. melanogaster* (*w*Mel) and *D. simulans* (*w*Au, wRI, *w*Ha, *w*No, and *w*Ma). In 2006, Miller and Riegler presented two exceptions: some strains of *wStv*, infecting *Drosophila sturtevanti* Duda, 1927 and closely related with *w*Whi, described in the sand flies *Lutzomyia whitmani* Antunes & Coutinho, 1939, and *L. shannoni* (Dyar) (Ono et al. 2001); and *w*Fum, infecting *Drosophila fumipennis* Duda, 1925 and closely related to a strain infecting the fig wasp *Pegoscapus longiceps* Cameron, 1906 (Shoemaker et al. 2002).

In 2015, our research group started an effort to increase the understanding of the *Wolbachia* infections in Neotropical drosophilids. We thus did field samples of native drosophilids in our University campus. In our first attempt, we found females of *D. sturtevant*i infected by *Wolbachia* (positive PCR for the *wsp* gene - see methods). Given that this species is might involved in a recent HT of *Wolbachia* from sand flies in Panama (Miller and Riegler 2006), we wonder if these Brazilian flies carry the same or a different *Wolbachia* strain. We also measured the prevalence and characterized the infection using

the MLST typing (see methods). Our main goal is to investigate whether an infection from a distantly related host can spread over the new host distribution.

MATERIALS AND METHODS

Sampling and D. sturtevanti species status

The sampling was carried out in a small fragment of Atlantic Forest (Mata da Biologia) within the Campus of the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil (20° 45' 31.1" S, 42° 51' 42.7" W) using the traps proposed by Medeiros and Klaczko (1999) baited with fermented banana. We left the traps for 48 hours in the field. After collection, we sexed and identified the flies as morphospecies. The males were stored in 100 % ethanol while the females were put in culture medium (banana/barley medium) to obtain isofemale lines. The species status of collected *D. suturtevanti* was determined by using F1 male terminalia morphology and COI sequence bar coding.

DNA extraction

The total DNA (host + bacteria) was extracted from the F1 of isofemale lineages using the Wizard Genomic Extraction Kit (Promega A1120) following the manufacturer's instructions for animal tissue.

COI PCR, sequencing, and bar coding

We verified the DNA quality by amplification of the mitochondrial COI gene, with the primers COI 106F (5'-ATTCAGAATATGTTCAG-3') and COI 154R (5'-TTTAATTTTACCTGGATTTGG-3) (Personal communication Miller). The PCR had a final concentration of 5 X of amplification buffer, 1.0 mM of MgCl2, 0.25 mM of each dNTP, 0.4 µM of each primer, 1.0 U of Taq DNA polymerase and 300 ng of template DNA. It started with a denaturation step of 94 °C for 5', followed by 30 cycles of 30'' at 94 °C, 30'' at 55 °C and 1' at 72 °C; and a final extension step of 5' at 72 ºC. PCR products were inspected with 1.5% agarose gel electrophoresis using GelRed (Biotium) to confirm amplification and verify contamination. Only the positive samples for this PCR were used in the next steps.

Only the isofemale lines positive for COI and morphologically recognized as *D. sturtevanti* had their amplicons sequenced with a DNA sequencer Applied Biosystems 3730. The sequences were used to confirm the species status by comparing with other sequences previously obtained for *D. sturtevanti*.

Wolbachia Detection

We used the PCR amplification of the *wsp* (*Wolbachia* surface protein) gene with the primers *wsp*_F1 (5-'GTCCAATARSTGATGARGAAAC-3') and *wsp*_R1 (5-‘CYGCACCAAYAGYRCTRTAAA-3') (Baldo et al. 2006). This gene

is exclusive of *Wolbachia* and its amplification is widely accepted as a positive sign of the presence of this bacteria. The PCR was made up for 25 µl Total Volume, containing 12.5 µl of GoTaq Green Master Mix 2X (Promega, Madison, USA), 1 µl of each primer for a final concentration of 20 µM, 8.5 µl ultrapure water and 2 µl Of template DNA (Müller et al. 2013). It started with a denaturation step of 94 ° C for 2', followed by 37 cycles of 30'' at 94 °C, 45'' at 56 °C and 1'30'' at 72 °C; and a final extension step of 10' at 72 ºC. We adopted two negative controls: with ultrapure water and with uninfected individuals of a tetracycline treated lineage of *D. suturtevanti* kindly provided by Dr. Wolfgang Miller (Laboratory of Genome Dynamics, Department of Cell and Developmental Biology, Center for Anatomy and Cell Biology, Medical University of Vienna, Vienna, Austria). The PCR products (product size: 603 bp) were visualized on 1.5 % agarose gel Gel Red stained.

Prevalence of infection

We inferred the prevalence of *Wolbachia* in the *D. sturtevanti* population sampled in Viçosa comparing the number of isofemale lineages positive for the bacteria (inferred by the *wsp* positive PCRs) with the total number of isofemale lines established in our lab. We tested only one female per line. Be noted that this methodology might produce negative results in medium-to-low infection intensity (Müller et al. 2013).

Wolbachia strains characterization

The classification of *Wolbachia* strains is currently based on the analysis of the sequences of the *wsp* (*Wolbachia* Surface Protein) and MLST (Multi-Locus Sequence Typing) genes. The *wsp* is associated with the host's immune response so that it varies enough to separate closely related strains (Zhou et al. 1998). However, *wsp* has four hypervariable regions (HVRs), which show recombination among *Wolbachia* supergroups found in insects (Baldo et al. 2005). To solve the recombination problems between *Wolbachia* groups, Baldo et al. (2006) proposed the MLST, creating a unique profile for each *Wolbachia* strain based on five *Wolbachia* coding genes *(coxA, fbpA, ftsZ, gatB*, and *hcpA*).

We randomly drew two *wsp* PCR positive individuals (from different isolines) and sequenced the five MLS loci and four *wsp* HVRs for molecular typing. The PCR for each locus was made up according to the specifications of the *Wolbachia* MLST database (http://pubmlst.org/*Wolbachia*/info/amp_seq_single.shtml). The PCR products were sequenced with a DNA sequencer Applied Biosystems 3730. The resulting chromatograms were evaluated with the CodonCode software. We compared the obtained sequences with those deposited in GenBank and MLST databases using the BLAST (Basic Local Alignment Search Tool - https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990) and the Sequence query (*Wolbachia* locus/sequence definitions - https://pubmlst.org/perl/bigsdbbigsdb.pl?db=pubmlst_*Wolbachia*_seqdef&page=

sequenceQuery) (Baldo et al. 2006) algorithms, respectively.

RESULTS

Our sampling resulted in 10 isofemale lineages positively diagnosed as *Drosophila sturtevanti* by the evaluation of the male genitalia of F1 and the sequencing the COI of F1 females. These females were tested for the presence of the *wsp* sequence, and all of them resulted positive for the *wsp* PCR assays, giving us a prevalence of 100 % in this population.

The *wsp* sequence (the four HVRs sequences) match exactly with the *wStv* MI (accession numbers AY620215.1 and DQ412110.1), found in *D. sturtevanti* hosts. The first accession, described in Miller and Riegler (2006), was found in files collected in Maria Eugenia, Panama, while the another, described by Mateos et al. (2006), was found in isofemale lineages with no indications of local and date of collection. As already mentioned by Miller and Riegler (2006), the *wStv* MI (and the sequence found here) is also identical to the *wWhi* (accession numbers AF237885, and AF237886) isolated from the sand flies *L. whitmani*, and *L. shannoni* collected in Bahia, Brazil; and Puerto Boyaca, Colombia, respectively (Ono et al. 2001).

The MLST database organizes the sequences by haplotypes containing entries for the four HVRs that define the *wsp* loci and each of the five loci of MLST (*gatB, coxA, hcpA, ftsZ* and *fbpA*). Each haplotype receives an ID number that contains, in turn, the identification number of the four HVRs, wsp and MLST loci (10 factors in total define the ID Wolbachia strain number).

Because recombinations among *Wolbachia* strains happen, it is possible to find the same MLST locus in different haplotypes. The *gatB* locus sequenced in this work, for example, is present in four different haplotypes (ids: 3, 91, 134 and 1795). Table 1 shows the ids associated with each of the five loci of MLST found here, their respective hosts, and local of collection (when available).

A rapid inspection on Table 1 reveals that most loci obtained here match with the id 91 (except for *coxA* 2), and the id 3 (except for *hcpA* 86), both found in hosts collected in Panama (Table 1). Comparing the different alleles, we found that the alleles 2 and 60 of *CoxA* (found in allele 3 and 91, respectively) differs by only one nucleotide (position 375) and does not change its correspondent amino acid. On the other hand, the alleles 26 and 86 (found in allele 3 and 91, respectively) differs by seven nucleotides (positions 105, 110, 156, 196, 199, 209, and 220), and four amino acids at the protein sequences (alignments not show).

Table 1. MLST alleles found in the *D. sturtevanti* studied here and their presence in other hosts, collected in other places, according to the MLST database. Each id corresponds to a different haplotype (a combination of the five MLST alleles and the four *wsp* HVRs) deposited in the database.

Allele	id	Host	Country
gatB 23	3	*Acromis sparsa*	Panama
	91	Drosophilidae	Panama
	134	*Odontomachus clarus*	USA
	1795	n/a	n/a
coxA 2	3	*Acromis sparsa*	Panama
	12	*Aedes albopictus*	Thailand
	108	*Metapone madagascaria*	Madagastar
	113	*Anoplolepis gracillipes*	Philippines
	119	*Lophomyermex* sp.	Thailand
	122	*Rhytidopoera metallica*	Australia
	127	*Pheidole* sp.	Thailand
	134	*Odontomachus clarus*	USA
	144	*Crematogaster* sp.	Thailand
	337	*Rhagoletis pomonela*	USA
	338	*Rhagoletis pomonela*	USA
	339	*Rhagoletis pomonela*	USA
hcpA 86	91	Drosophilidae	Panama
	1795	n/a	n/a
ftsZ 21	3	*Acromis sparsa*	Panama
	91	Drosophilidae	Panama
fbpA 26	3	*Acromis sparsa*	Panama
	91	Drosophilidae	Panama
	929	n/a	n/a
	1517	n/a	n/a

The MLST database also contains numbers that identified the four HVRs of the wsp sequence and these numbers define the wsp loci that also have a unique number. Our sequence has the alleles 54, 28, 62, and 60, for HVR1, HVR2, HVR3, and HVR4, respectively, which are identical to the alleles of the *Wolbachia* strain ID 91 drescrived in an drosophilid species and entirely different from the *Wolbachia* strain ID 3 that presents the alleles 4, 4, 5, and 4.

Table 2 - Comparison of the allele composition of the *Wolbachia* infection found in the *D. sturtevanti* population studied here and those of the ids 3 and 91 of the MLST database.

Id	***gatB***	***coxA***	***hcpA***	***ftsZ***	***fbpA***
This study	23	2	86	21	26
3	23	2	26	21	26
91	23	60	86	21	26

DISCUSSION

The *D. sturtevanti* population collected on the campus of the Universidade Federal de Viçosa harbors one of the Panamanian *Wolbachia* strains, *wStv* MI, described by Miller and Riegler (2006), with a prevalence estimated at 100%. This work provides the first characterization of the MLST loci for the infection of *D. sturtevanti*. All loci presented here were already found in other hosts, but the haplotype (the combination of loci) is new and differs by only one substitution point from the haplotype found in a Drosophilidae (not identified) from Panama.

In a recent opinion article, (Sahoo 2016) reviewed some valuable insights regarding the process of *Wolbachia* infection in a given species and highlighted that a recent *Wolbachia* acquisition is maintained in a low frequency until a critical threshold value (~ 8.0 %; Turelli and Hoffmann 1995). After that, the infection tends to spread rapidly in the host population (Hoffmann et al. 1990; Champion de Crespigny et al. 2005). Therefore, the infection prevalence tends to be either low or high and Sahoo (2016) concluded that intermediate frequency only exists for short time spans.

Indeed, the rare registers in the literature regarding the association between *Wolbachia* and *D. sturtevanti* allow us to infer that *Wolbachia* colonized these flies recently. And that the infection remained at a low frequency for some time, enhanced to an intermediary frequency and now is widely spread along the host distribution. Bourtzis et al. (1996) searched for *Wolbachia* in 30 Drosophilidae species (including an isofemale lineage of *D. sturtevanti*) using PCR assays based on *dna*A and 16*S* rRNA primers specific for *Wolbachia*. They found no evidence of infection in *D. sturtevanti*, which might mean that such lineage was non-infected or had a low titer infection, not detectable by PCR. Ten years later, Miller and Riegler (2006) found tree closely related strains from three different populations in Panama: *wStv* MI, *wStv* Pan6, and *wStv* SG. They searched for *Wolbachia* in isofemale lines from eight localities of Panama and Barbados and found it in four localities, two collected in 1998 and other two collected in 1999. Still in 2006, Mateos et al. tested seven *D. sturtevanti* isofemales and found only two infected by *wStv* MI, the same found by Riegler and Miller (2006). Unfortunately, both Bourtzis et al. (1996)

and Mateos et al. (2006) did not provide information on where and when their lineages were collected.

In 2015, we found the *wStv* MI in a Brazilian population. The fact that the *wsp* sequence is identical to *wStv* (from a Panamanian population) suggests that this infection not only spread rapidly in its original population but along the species distribution, considering that the collection points are at least 5,000 km apart. Moreover, the fact that we initiated only ten isofemale lineages from our sample and all of them resulted positive for *Wolbachia* suggests that the infection is high titer in this population. Besides the collections in Viçosa, we also collected few individuals of *D. sturtevanti* in two other cities around (the first in Juiz de Fora, at 170 km from Viçosa, and the second in Araponga, at 40 km from Viçosa). We did not initiate isofemale lineages, nor calculated the prevalence, but tested only two individuals from each of these localities for the presence of *Wolbachia* and found positive PCRs for *wsp*. We sequenced these *wsp* and found that they are identical with the *wStv* MI. These preliminary results reinforce the hypothesis that this infection is widely spread along the *D. sturtevanti* distribution.

Our data suggest that, Wolbachia was horizontally transferred from other hosts (presumably from Panamanian sand flies, as suggested by Miller and Riegler, 2006) in some point in time before 1988, since these authors found two infected lineages collected in 1998. After that, the infection spread along the *D. sturtevanti* distribution and today is fixed in the population studied here. Therefore, we can infer that an infection from a distantly related host can spread over the new host distribution.

This report marks the beginning of our studies involving the evolution of *Wolbachia* and Neotropical drosophilids. Given the high titter and the broad distribution of the *wStv* infection, we need to test whether the crosses between infected and non-infected lineages produce CI in *D. sturtevanti*, which could explain the rapid spread of the bacteria along the host's distribution.

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CHAPTER 2

Zaprionus indianus (Diptera: Drosophilidae) and its new Wolbachia strains: the begining of a host-parasite relationship.

ABSTRACT

Zaprionus indianus, an African species invaded America at the ends of the 1990s and rapidly spread over the continent, with a significant genetic variation. In 2015, we found, for the first time, individuals of this species infected with *Wolbachia*, an endosymbiont that causes reproductive alterations in its hosts and has the potential to increase the fitness of infected females. We asked ourselves if the infection spread over the distribution of the fly or if it remained locally concentrated. We thus sampled more individuals, from three distant localities: Viçosa, MG (where we found the first infected flies), Brasília, DF and Altamira, PA. We found a prevalence of about 30 % in each locality and showed evidence of at least four *Wolbachia* strains infecting the individuals sampled in the three areas. Giving that *Z. indianus* is a new *Wolbachia*-host, we found evidence of frequently horizontal transfers from other hosts to this fly, suggesting that horizontal transfers are a common phenomenon on ecological time scales. The outcomes of interaction *Wolbachia -Z. indianus* are difficult to predict, and complementary studies that should be done along time, will be necessary to investigate the ecological and evolutionary consequences of such interaction.

Key words: *wsp*, MLST loci, phylogenetic inference, horizontal transfer, recombination

INTRODUCTION

An African Drosophilidae, *Zaprionus indianus* (Gupta, 1970), now considered as a cosmopolitan species (David et al. 2006), invaded the Brazilian territory at the end of the 1990s. Vilela (1999) registered its presence in São Paulo, but the species rapidly spread through Americas (Commar et al. 2012). Such colonization success is attributed to the high amount of phenotypic plasticity and genetic variability introduced in Brazil (Loh and Bitner-Mathé 2005). Indeed, Mattos Machado et al. (2005) found the same level of allozyme polymorphism in Brazil and Africa while David et al. (2006) and Ananina et al. (2007) found this same pattern using morphology and chromosomal data, respectively. These studies found no genetic structure in *Z. indianus* populations and Yassin et al. (2008) showed that the propagules that invaded Brazil came from the ancestral African populations, and not from the populations that invaded Asia.

Aiming to better understand the relationships between *Wolbachia* and their hosts in the Neotropics, we initiated, in 2015, an exploratory study regarding the Drosophilidae diversity in Viçosa, MG to investigate which species harbor the bacteria. During this work, we found, for the first time, the presence of *Wolbachia* in *Z. indianus* (Almeida et al. 2015). Bouiges et al. (2013), studying the mitochondrial variability of nine species of *Zaprionus* in Africa, contrasted the high polymorphism found in *Z. indianus* with the low variability found in *Z. sepsoides*, known to be infected by *Wolbachia* (Cordaux

et al. 2008) and suggested that *Wolbachia* does not infect those *Z. indianus* populations.

Wolbachia is a well-succeeded endosymbiont infecting more than 50% of Arthropoda species (Weinert et al. 2015). It is usually transmitted vertically, i.e., from the mother to the offspring through infected eggs, and produces different reproductive phenotypes, such as cytoplasmic incompatibility (CI) (Hoffmann et al. 1986; Giordano et al. 1995; Bourtzis et al. 1996; Charlat et al. 2002; Miller et al. 2010b; Richardson et al. 2016) and male killing (MK) (Dyer and Jaenike 2004; Montenegro et al. 2006; Sheeley and McAllister 2009; Richardson et al. 2016) in Drosophilidae hosts. Such reproductive phenotypes tend to increase the fitness of infected females affecting the populational dynamics of the hosts (Werren 2003; Werren et al. 2008). Different *Wolbachia* strains provoke different reproductive phenotypes in different hosts (Zhou et al. 1998), making the identification of the *Wolbachia* strain(s) the first step in studies focused on the evolution of parasite-hosts dynamics.

Giving that *Z. indianus* is a new *Wolbachia*-host, we are facing a unique chance to study the evolution of the host-parasite relationship from the beginning. Providing that *Z. indianus* has a high dispersion capacity and that *Wolbachia* tends to increase the fitness of infected hosts, we hypothesized that this infection is already spread through *Z. indianus* geographic distribution in Brazil. To test this hypothesis, we (i) investigated if specimens collected are infected, (ii) calculated the infection prevalence in populations, and (iii) identified the *Wolbachia* strain (s) infecting them.

MATERIALS AND METHODS

Fly Sampling

In addition to increasing the sampling in Viçosa, MG (20 ° 45' 36.2" S, 42 ° 52' 14.6" W), we collected *Z. indianus* in Brasília, DF (15° 49' 34.8" S, 48° 04' 08.9" W) and Altamira, PA (3 ° 12' 42.2 " S, 52 ° 12'44.9" W) (Fig. 1) using the fermented banana-baited traps proposed by Medeiros and Klaczko (1999). After collection, we determined the species status of *Z. indianus* by the presence of two white longitudinal stripes across the head and thorax (Fig. 2), a highlighted characteristic of *Zaprionus* species (Markow and O'Grady 2005). The files were then stored in absolute alcohol at -4 ° C.

Figure 1. *Z. indianus* sampling sites in Brazil.

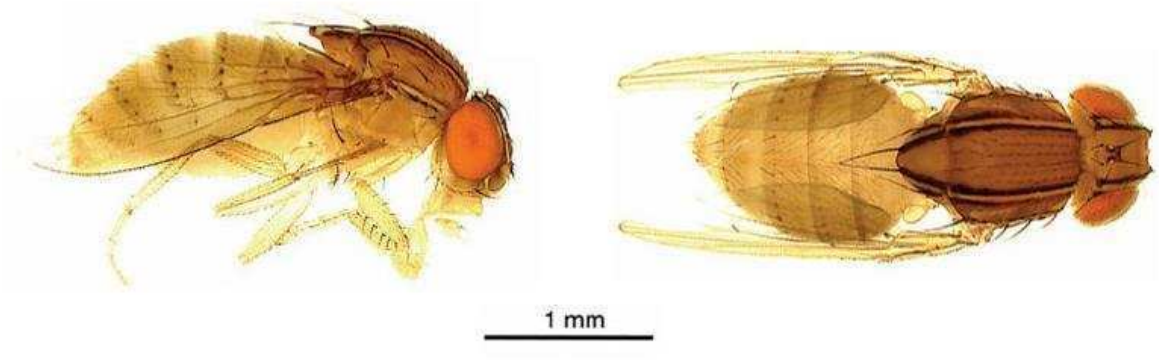

Figure 2. *Z. indianus* and its white longitudinal stripes across the head and thorax. A. lateral view; B.dorsal view. Scale bar = 1 mm (Kacsoh et al. 2014)

DNA extraction

The total DNA (host + bacteria) was extracted from females using the Wizard Genomic Extraction Kit (Promega A1120) following the manufacturer's instructions for animal tissue.

We verified the DNA quality by amplification of the mitochondrial COII gene, with the primers TL2-J-3037 (5′-ATGGCAGATTAGTGCAATGG-3′) and TK-N-3785 (5′- GTTTAAGAG ACCAGTACTTG-3) (Yassin et al. 2008). The PCR had a final concentration of 5 X of amplification buffer, 2.5 mM of $MgCl_2$, 0.25 mM of each dNTP, 0.4 µM of each primer, 1.0 U of Taq DNA polymerase and 300 ng of template DNA. It started with a denaturation step of 94 °C for 5', followed by 35 cycles of 30" at 94 °C, 30" at 55 °C and 1' at 72 °C; and a final extension step of 5' at 72 ºC. PCR products were inspected with 1.5% agarose gel, GelRed (Biotium) stained. Only the positive samples for this PCR were used in the next steps.

Wolbachia detection

We used the PCR amplification of the *wsp* (*Wolbachia surface protein*) gene with the primers *wsp*_F1 (5-'GTCCAATARSTGATGARGAAAC-3') and *wsp*_R1 (5-‘CYGCACCAAYAGYRCTRTAAA-3') (Baldo et al. 2006b). This gene is exclusive of *Wolbachia* and its amplification accepted as a positive sign of the presence of this bacteria.

The PCR was made up for 25µl Total Volume, containing 12.5µl of GoTaq Green Master Mix 2X (GoTaq DNA Polymerase supplied in 2X Reaction Buffer, 400µM of each dNTP (Promega, Madison, USA), 1ul of each primer, 2µl of template DNA and ultrapure water up to the final volume (Müller et al. 2013). It started with a denaturation step of 94 ° C for 2', followed by 37 cycles of 30'' at 94 °C, 45'' at 56 °C and 1'30'' at 72 °C; and a final extension step of 10' at 72 ºC. We adopted two negative controls: ultrapure water and the DNA of uninfected individuals of a tetracycline treated lineage of *D. simulans* kindly provided by Dr. Wolfgang Miller (Laboratory of Genome Dynamics, Department of Cell and Developmental Biology, Center for Anatomy and Cell Biology, Medical University of Vienna, Vienna, Austria). The PCR products were visualized on 1.5 % agarose gel, Gel Red stained.

Prevalence of infection

We inferred the prevalence of *Wolbachia* in the *Z. indianus* populations from Altamira, Brasília, and Viçosa by the amplification of the *wsp* gene in 50, 52 and 40 females respectively.

Wolbachia strains characterization

The first aim to classify *Wolbachia* strains used the 16S rRNA sequences, and established seven supergroups (A-H). A and B are common in insects, C and D in filarial nematodes, E in Collembola, F in insects and nematodes and H in Isoptera (reviewed by Werren et al. 2008). In 1998, Zhou et al. proposed that the *Wolbachia* Surface Protein (*wsp*) gene, associated with the host's immune response, varies enough to separate closely related lineages and classify the bacteria in their supergroups. However, *wsp* has four hypervariable regions (HVRs), which show recombination among the *Wolbachia* supergroups found in insects (Baldo et al. 2005). Giving that, Baldo et al. (2006) proposed a Multi-Locus Sequence Typing (MLST), based on five loci (*gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA*) sequences as well as the HVRs of the *wsp*. The *Wolbachia* MLST database currently contains 470 allelic profiles of 1826 isolates (Last Updated: 2016-09-25). The database assigned an id number for each distinct sequence of the four HVRs and the five MLST loci. Each profile corresponds to a distinct combination of these id numbers.

Whenever possible, the database classifies each HVR or MLST allele in one of the seven supergroups and provides the host of the deposited sequence (Jolley and Maiden 2010).

For the strain characterisation in this work, we randomly drew two *wsp* PCR positive individuals from each collection locality (Viçosa, MG – Vic_04 and Vic_05; Brasília, DF – Bra_36 and Bra_52; and Altamira, PA – Alt_01 and Alt_10) and sequenced the five MLST loci and the *wsp* for molecular typing. The PCR for each locus was made up according to the specifications of the *Wolbachia* MLST database (http://pubmlst.org/*Wolbachia*/info/amp_seq_single.shtml). The PCR products were purified with an exosap protocol (Amersham) and sequenced with a DNA sequencer Applied Biosystems 3730. The resulting chromatograms were evaluated with the CodonCode software, and those sequences showing double peaks in the chromatogram were eliminated from the analysis. We compared the obtained sequences with those deposited in the MLST database using the Sequence Query (*Wolbachia* locus/sequence definitions - https://pubmlst.org/perl/bigsdbbigsdb.pl?db=pubmlst_*Wolbachia*_seqdef&page= sequenceQuery) (Baldo et al. 2006b).

Phylogenetic Inferences

Alignment

All MLST sequences are potentially coding, so we made the alignments taking into account the reading frame of each gene, using the Muscle algorithm (Edgar 2004), available in the Mega software version 6.0 (Tamura et al. 2013). For the phylogenetic analysis, we added to each gene alignment sequences from complete *Wolbachia* genomes already identified by supergroups (A, B, D or F – Table1), as well as the sequences obtained for the *Wolbachia* strain found in *D. sturtevanti* (Chapter 1 of this dissertation).

Table 1: Complete *Wolbachia* genomes which sequences of MLST and *wsp* were included in the phylogenetic trees.

Strain	Access #	Host	Supergroup
wMel	NC_002978	*Drosophila melanogaster*	A
wInc_Cu	NZ_CP011148	*Drosophila incompta*	A
wHa	NC_021089	*Drosophila simulans*	A
wRi	NC_012416	*Drosophila suzukii*	A
wPip	NC_010981	*Culex quinquefasciatus*	B
wNo	NC_021084	*Drosophila simulans*	B
Bmal_D	NC_006833	*Brugia malayi* (Nematoda)	D
Clec_F	NZ_AP013028	*Cimex lectularius*	F

Phylogenetic analysis

We inferred separated phylogenetic relationships of the different *loci* of the *Wolbachia* strains under study using 10 million Markov Chain steps performed by MrBayes version 3.2.6 (Huelsenbeck and Ronquist 2001; Huelsenbeck et al. 2001) through of CIPRES Science Gateway version 3.3 (Miller et al. 2010a). We estimated the nucleotide substitution model of each locus using the software Partitionfinder 2 (Lanfear et al. 2016), with the "greedy" algorithm (Lanfear et al. 2012) and the Akaike information criterion (AICc) for estimating a separate substitution model for each of the three-codon positions with information about heterogeneity rate across sites (Lanfear et al. 2016).

RESULTS

Wolbachia Prevalence in *Z. indianus*

We found infected individuals in the three sampled localities. The prevalence of *Wolbachia* was calculated in 36% in Altamira, 30% in Brasília and 37% in Viçosa (Figure 3).

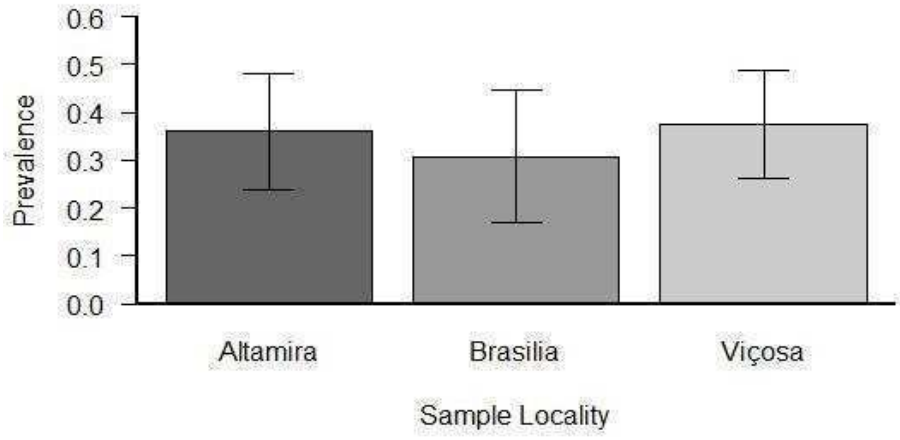

Figure 3 Prevalence of *Wolbachia* in specimens of *Z. indianus* sampled in three different localities in Brazil. The presence of the bacteria was diagnosed by the amplification of the *wsp* locus. The vertical bars correspond to the confidence interval based on the binomial distribution.

Wolbachia strain characterization

Table 2 shows the allele identities of the sequences studied here. Most alleles were marked as “new,” meaning that no sequences in the *Wolbachia* MLST database are identical to them. The two individuals screened in Viçosa presented the same alleles for all loci. From them, only two were already cataloged in the *Wolbachia* MLST database: the allele 3 for the HVR3, found

in several insect orders; and allele 102 for the HVR4, found in other three insect species (Table 3).

In Brasilia, we found evidence of at least two independent strains of *Wolbachia*. Table 2 shows different alleles for the four HVRs of the *wsp*. Bra_36 presents the allele 16 for the complete *wsp*, shared with *Wolbachia* strains of other Drosophilidae; and Bra_52 presents the allele 89 for this same locus, shared with a non-identified Drosophilid sampled in Panama (see table 3). As this sequence is also identical to the sequence found in *D. sturtuevanti* in chapter 1 (Stv_ch1), it obligated us to repeat the PCR and the sequencing using a new set of reagents in other laboratory to discard the possibility of contamination. For the MLST loci, only the allele 23 of the locus *coxA* and the allele 23 of *fbpA* (both shared with other *Drosophila*) could be identified in BSB_36. The *fbpA* of Bra_52 is a new allele, and the sequences of the other MLST loci cannot be obtained, most because of double peaks in the electropherogram (Figura 4), or difficulties in the PCR that resulted from the absence of amplification or bands larger or smaller than expected for the fragment.

In Altamira, all HVRs of the *wsp* sequence of the two individuals (Alt_01 and Alt_10) were identical, but only the allele HVR3 3 (the same found in Viçosa) could be identified. For the MLST loci, we obtained high quality sequences only for the individual Alt_01, and identified the alleles *coxA* 2 and and *ftsZ* 21, both shared with other orders of insects (Table 3). Moreover, five alleles of Alt_01 are new (HVR1, HVR2, HVR4 and *fbpA*); *gatB* cannot be

evaluated because resulted in a wrong sequence; and *hcpA* produced a PCR band larger than expected.

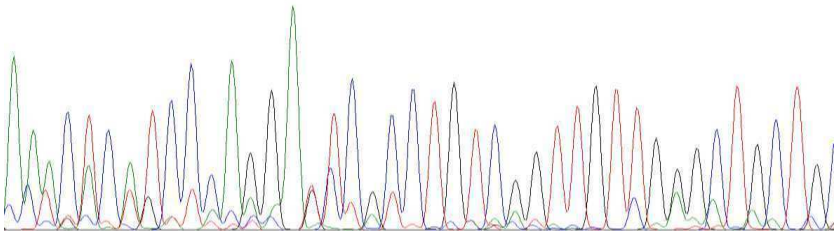

Figure 4 Example of electropherogram with double (or multiple) peaks.

Table 2. *wsp* and MLST allele identities found in individuals of *Z. indianus* collected in Viçosa, MG, Brasília, DF and Altamira, PA.

Locus	Vic_04	Vic_05	Bra_36	Bra_52	Alt_01	Alt_10
hvr1	new	new	11	54	new	new
hvr2	new	new	13	28	new	new
hvr3	3	3	15	62	3	3
hvr4	102	102	14	60	new	New
wsp	new	new	16	89	new	New
gatB	new	new	n/a (a)	n/a (a)	n/a (b)	n/a (b)
coxA	new	new	23	n/a (c)	2	n/a (c)
hcpA	new	new	n/a (d)	n/a (a)	n/a (d)	n/a (c)
ftsZ	new	new	n/a (e)	n/a (c)	21	n/a (c)
fbpA	new	new	23	new	new	n/a (c)

(a) Double peaks; (b) wrong sequence; (c) No PCR; (d) large PCR band; (e) small PCR band;

Phylogenetic Relationships

Figure 5 shows the phylogenetic inferences based on the MLST loci and the complete *wsp* sequence including all alleles found in this work (Table 2),

as well as the alleles from the sequences listed in Table 1 and the sequences obtained from de *Wolbachia* strain found in *D. sturtevanti* (chapter 1). All Viçosa's alleles grouped with the sequences assigned as the Supergroup B, wNo and wPip (Fig 5-F; Fig 6A-D). Most of these clusters present high posterior probability (PP = 1.0), with exception of two high variable regions of *wsp*, HVR3 and HVR4 (Fig 6C and D), when this cluster present PP = 0,82 and 0,93, respectively.

For Brasilia and Altamira, we obtained the *wsp* sequence, but only a few reliable sequences for the MLST loci (table 2). Moreover, these sequences present conflicting results regarding the Supergroup assignation of the strains. In the *coxA* tree (Fig 5A), there is a well-supported cluster containing all the sequences of the Supergroup A, the sequences Bra_36 (grouped with wRI, PP = 0,88), and Alt_01 (grouped with the *D. sturtevanti*'s strain sequence - Stv_ch1. 1, PP = 1).

The *fbpA* tree (Fig. 5B), also contains a well-supported Supergroup A cluster (PP = 0,99), containing Bra_36 and Stv_ch1. However, Alt_01 could not be assigned on Supergroup A. Indeed, Alt_01 cannot be confirmed in any group represented in the tree. This tree also contains the other sequence from Brasilia, Bra_52, that clustered with the sequences assigned as the Supergroup B (PP = 1).

In the *ftsZ* tree (Fig. 5C), Alt_01 grouped with Stv_ch1 (PP = 0.97), but these alleles were positioned out of the Supergroup A, clustering together (PP = 0,99) with sequences assigned to the Supergroup B. The *gatB* tree (Fig. 5D)

shows the Stv_ch1 sequence clustering with sequences of group A, which was also showed by the hpcA tree (Fig. 5E).

The whole *wsp* tree (Fig. 5F) put the sequences of Altamira (Alt_01 and Alt_10) within the sequences of Supergroup B (PP = 1.0), and positioned Bra_36 within the Supergroup A. Bra_52 clustered with Stv_ch1 (PP = 1.0), in a position that does not allow us to assign them in any Supergroup represented in our tree.

Figure 6 shows the phylogenetic relationships based on each of the four hypervariable regions of the *wsp* (HVRs). As found in the whole tree (Fig. 5F), the four trees clustered Bra_36 with wRi, and Bra_52 with Stv_ch1. The cluster Bra_36-wR1 seems to belong to Supergroup B in the HVR1 tree (Fig. 6A) (PP = 0.9), to supergroup A in the HVR3 tree (Fig. 6C) (PP = 1.0), and cannot be assigned to any Supergroup in HVRs 2 and 4 (Fig. 6B and D). The cluster Bra_52-Stv_ch11 clustered whith the sequences of Supergroup A in HVR3 (Fig. 6B) (PP = 1.0) and could not be assigned to any Supergroup in the other trees. In HVR1 (Fig 6A), it grouped with the sequences of Altamira (PP = 1.0), but this cluster cannot be assigned to any Supergroup. Also, in HVR2 (Fig. 6B), Alt_01 and Alt_10 cannot be allocated in any Supergroup, but in HVR3 and 4 (Fig. 6C and D) they seem connected with sequences of Supergroup B (PP = 0,88 and 0,93, respectively).

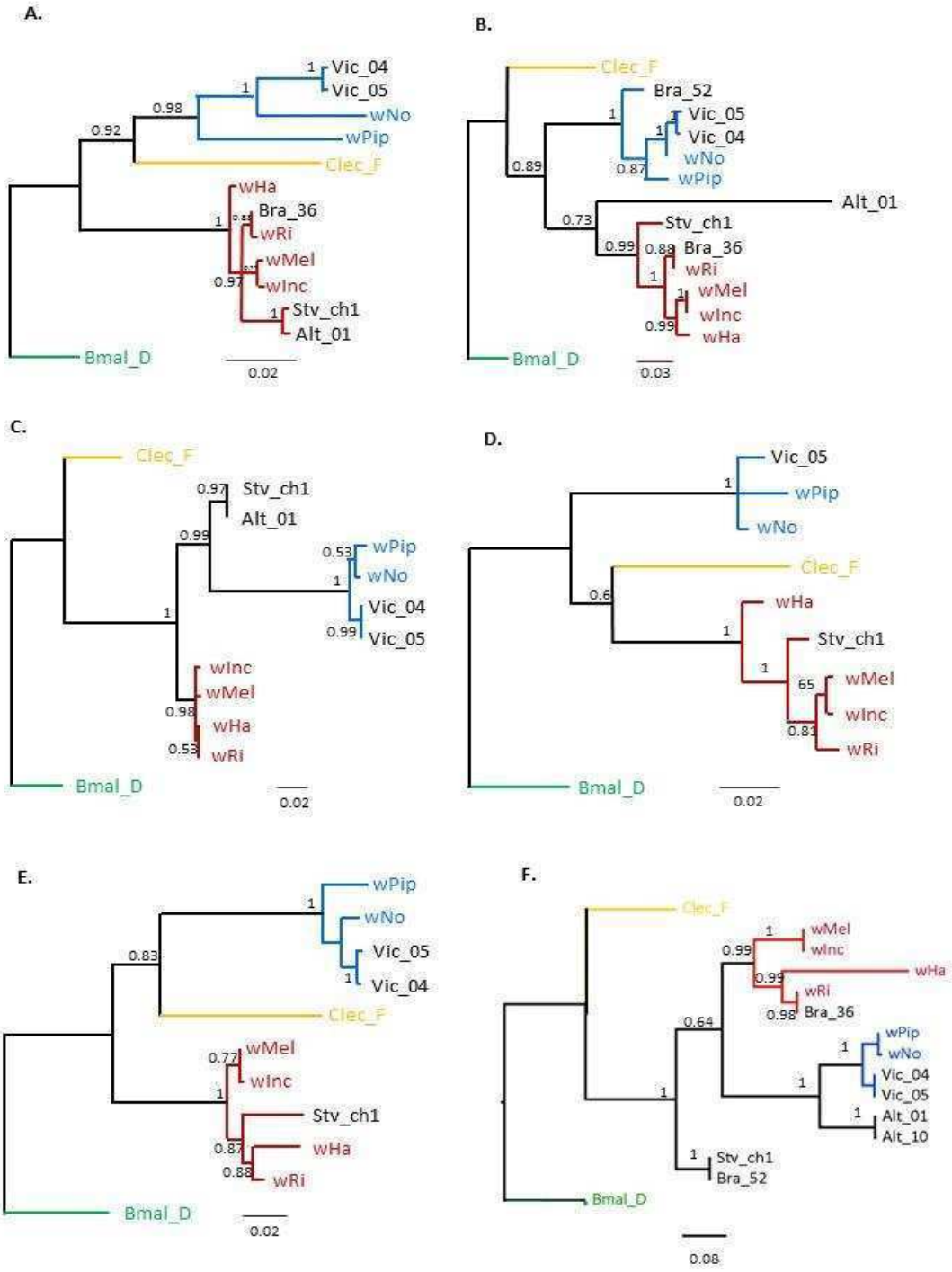

Figure 5 Bayesian inferences (10 million Markov Chain steps - 25% burned out) of the five loci of MLST: *CoxA* (A), *fbpA* (B), *ftsZ* (C), *gatB* (D), *hcpA* (E); and the complete *wsp* sequences (F) The numbers besides internal nodes are the posterior probability of them. Branches in red represent Supergroup A assignation; in blue, Supergroup B, in yellow, Supergrpoup F and in green Supergroup D.

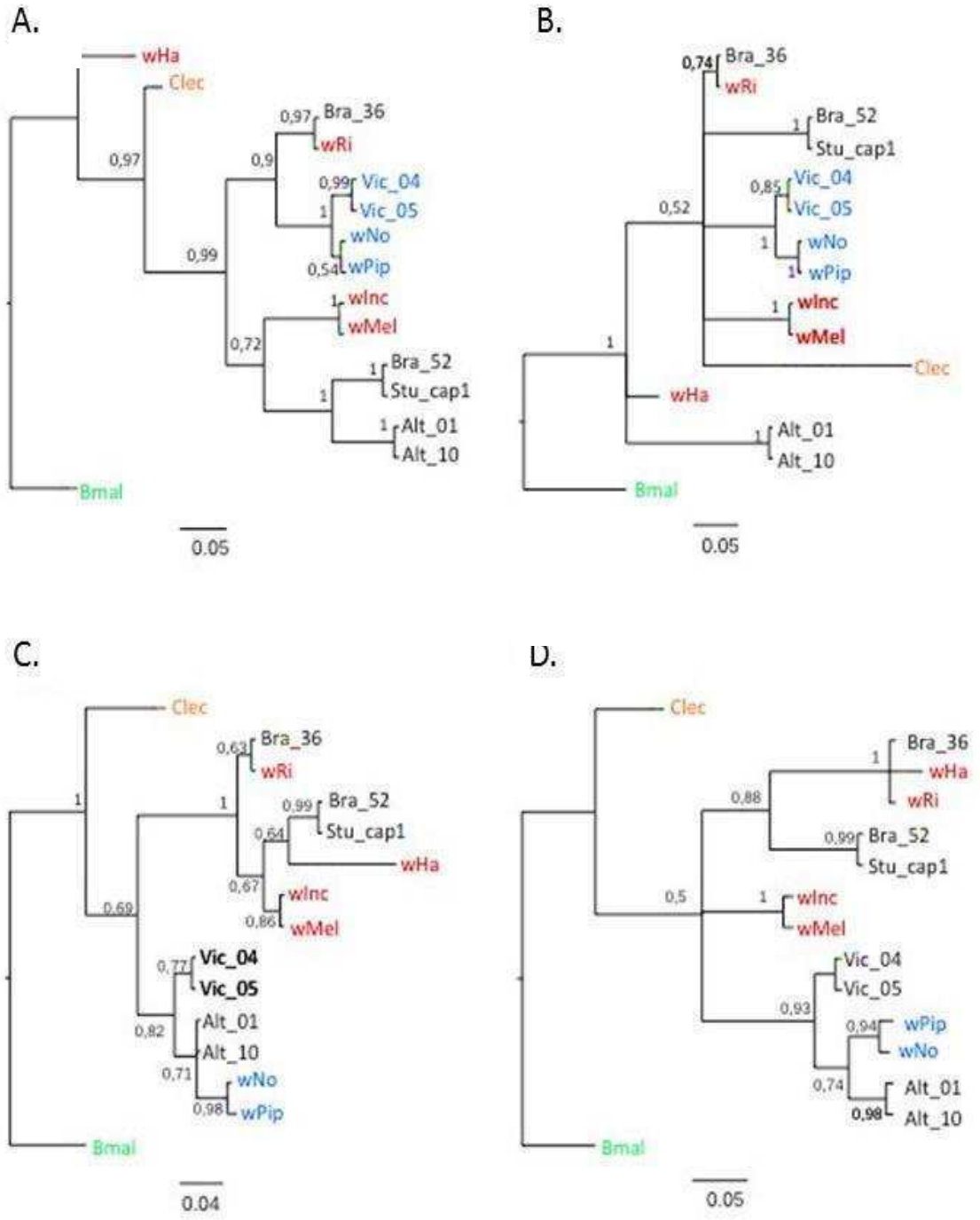

Figure 6 Bayesian inferences (10 million Markov Chain steps - 25% burned out) of the four hypervariable regions of *wsp*: HVR1 (A), HVR2 (B), HVR3 (C), and HVR4 (D). The numbers besides internal nodes are the posterior probability of them. Terminal nodes in red red belong to Supergroup A; in blue, to Supergroup B, in orange, to Supergroup F; and in green, to Supergroup D.

DISCUSSION

As far as we know, this study provides the first relate of *Wolbachia* infection in *Z. indianus*. As this fly is a fruit pest (Castro and Valente 2001; Bernardi et al. 2016) and *Wolbachia* is a potential pest controller (Zabalou et al. 2004; Rocha et al. 2005; Guidolin and Cônsoli 2013), we assumed that other researchers had already searched for *Wolbachia* in *Z. indianus*, but did not publish their negative results. Therefore, we assumed that *Z. indianus* is a new *Wolbachia*-host.

After finding *Wolbachia* in *Z. indianus* collected in Viçosa, and considering the high dispersion capacity of the fly (Mattos Machado et al. 2005; David et al. 2006), we tested if the bacteria already spread along the recently colonized territory of the fly using samples of two other localities: Brasília (950 km far from Viçosa) and Altamira (2850 and 1900 km far from Viçosa and Brasilia, respectively). The three localities showed a similar prevalence (~30%) of infection, which led us to hypothesize a process of fixing a strain of *Wolbachia* throughout the distribution of the fly. When *Wolbachia* increases the fitness of the infected females (by CI or MK), the prevalence tends to increase in the population (Sahoo 2016). In fact, we showed, in chapter 1, a prevalence of 100% in a *D. sturtevanti* population sampled in 2015, after a period when the *Wolbachia* was absent or in low titer (Bourtzis et al. 1996) and another period when it was intermediary (Mateos et al. 2006; Miller and Riegler 2006) in other distant populations.

However, the sequencing of the *wsp* and MLST loci revealed a different evolutionary process in the *Wolbachia-Z. indianus* relationship. We found evidence of at least four different *Wolbachia* strains infecting the individuals of the three sample sites, two of them found at the same sample point in Brasília, DF (see Figures 5B and G). Also, some sequences obtained for Brasilia and Altamira show double peaks, preventing their use in the phylogenetic inferences (see Table 2) and suggesting the presence of at least two different sequences (meaning two different strains) in the same individual.

The distinct sequences found in Brasilia are not only different but seem to belong to different supergroups. The *fbpA* locus (Fig. 5-B) suggests that Bra_36 belongs to the Supergroup A and Bra_52 belongs to supergroup B while the complete *wsp* sequence maintained Bra_36 in Supergroup A and positioned Bra_52 out of the Supergroups represented in the tree. Perrot-Minnot et al. (1996) also found different strains in the same host lineage and showed that subgroup A and B are bidirectionally incompatible. Further studies using cloning sequences and RT-PCR will be necessary to know if the sequences presenting double peaks correspond to individuals infected by two strains, as also found by Rousset and Solignac 1995 and Perrot-Minnot et al. 1996. Narita et al. (2007) showed that individuals infected with one lineage of *Wolbachia* present CI whereas individuals double infected exhibit feminization of the offspring.

The pattern found in Altamira is still more blurred: although the two specimens present identical sequences for the *wsp*, we recovered sequences

for *coxA*, *fbpA* and *ftsZ* only for Atm_01. For Atm_10, these sequences present double peaks, as well as the other MLST loci for both individuals. Moreover, Alt_01 grouped with the strain found in *D. sturtevanti* (Chapter 1) in Cox_A and *ftsZ*, but not in *fbpA* and *wsp*. In *fbpA*, the Alt_01 sequence does not group with any other, and in *wsp*, it groups with the sequences of Viçosa and sequences of Supergroup B.

To explain this blurred pattern, we have at least two alternatives: the first is the presence of double infections in both individuals, and Alt_01 shows one or other copy in different MLST loci. The second predicts double (or multiple) infections and recombination, probably in an ancestor host (Werren et al. 2001; Baldo et al. 2005; Baldo et al. 2006a). Recombination could explain both the ambiguous position of Alt_01 in different MLST loci and the position of Alt_01 and Alt_10 in the trees based on the four HVRs of the *wsp* sequence (Figure 7). Baldo et al. (2006a) noted that the DNA exchange across *Wolbachia* lineages results in a remarkable phylogenetic conflict, invalidating attempts to reconstruct *Wolbachia* strains relationships due to the chimeric nature of the genomes.

Our results suggest that *Z. indianus* was infected by at least four *Wolbachia* strain in a short period: less than 20 years considering the introduction of the species in Brazil, in 1999, and the large mitochondrial variability found in African *Z. indianus* populations (Bouiges et al. 2013). These four infections must occurred through lateral transference from other hosts and show that it is a common phenomenon on ecological time scales, contrary to

the suggestion of Correa and Ballard (2016), who stated that most of the horizontal transferences of *Wolbachia* among hosts occurred over evolutionary time. The long-term efects of these infections in *Z. indianus* are difficult to predict, and complementary studies are necessary to know if one of these strains will be dominant in a short or mid-term, or if different infections cause CI or MK in *Z. indianus*.

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CONCLUSIÓN GENERAL

Este trabajo muestra dos patrones contrastantes en infecciones potencialmente recientes de W*olbachia*. El capítulo 1 trata de la infección en *D. sturtevanti*, especie que no fue estudiada en cuanto a la presencia de la bacteria hasta el año 1996, cuando no fueron encontradas evidencias de infección (Bourtzis et al. 1996). En 2006, Miller y Riegler detectaron la infección por tres linajes distintas de *Wolbachia* en muestras colectados a partir del 1998, en Panamá y Barbados, con baja prevalencia. Nueve años después, reportamos 100% de prevalencia de una de las cepas halladas en 2006 en Panamá (*wStv* Mi). Los resultados sugieren una homogeneización de la infección, probablemente a lo largo del rango de dispersión de *D. sturtevanti*. Es probable que *wStv* Mi conceda algún tipo de beneficio a su hospedero, facilitando su dispersión. Dentro de los tipos de beneficio, figuran protección ante enemigos naturales que facilita la dispersión de cepas que causan incompatibilidad citoplasmática (CI) (Fenton et al. 2011) o incremento en la longevidad del nuevo hospedero (Veneti et al. 2012). Esta clase de beneficios en hospederos recién infectados pueden promover la dispersión de *Wolbachia*, aunque inicialmente as infecciones presenten baja prevalencia (Zug and Hammerstein 2015).

En contraste, el Capítulo 2 mostro lo dinámico que puede llegar a ser la colonización inicial de W*olbachia* en un hospedero. La detección de por lo menos cuatro cepas de *Wolbachia*, con nuevos alelos en la mayoría de los loci estudiados, es prueba de la facilidad con se transmite de forma horizontal y

deja muy clara la intensa taxa de recombinación entre cepas, características que han favorecido la adaptación y colonización de nuevos hospederos (Baldo et al. 2006). Nuestros datos permiten además inferir infecciones dobles o múltiples, lo que evidencia la variabilidad inexplorada de cepas de *Wolbachia* presentes en el Neotropico. Este capítulo relata tan solo una foto en la dinámica espacio temporal de la historia *Wolbachia- Z. indianus* en la que aún no conocemos el desenlace, que tendrá efectos impredecibles en medio y largo plazos.

Estos dos estudios plantean nuevas interrogaciones que ya se sustentan como puntos de partida para el desarrollo de futuras investigaciones: ¿Cuál es el efecto de *Wolbachia* en el fitness de *D. sturtervanti*? ¿La infección está de hecho fixada en America? Por otro lado, ¿cuáles son las consecuencias de las infecciones por W*olbachia* en poblaciones de *Z. indianus*? ¿Es posible mantener varias cepas en una población? o ¿alguna de ellas se va a extinguir?, o ¿todas? Una nueva línea de investigación se inicia con el objetivo de revelar las posibles consecuencias ecológicas y evolutivas de la infección de W*olbachia* en el Neotropico tomando como modelo los drosofilideos

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