Wolbachia Occurrence in Planthopper (Hemiptera: Delphacidae) Vectors of Cereal Viruses in Argentina

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ABSTRACT Maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) are the most important cereal crops for the Argentinean economy and are affected by several diseases. Different planthopper species transmit causal agents of some of those diseases, including Mal de Río Cuarto virus, barley yellow striate mosaic virus, and the recently proposed maize yellow striate virus. Many planthopper species are sap feeders and therefore are expected to host bacteria providing essential nutrients lacking in the diet. Previous studies have evidenced that some of these bacterial symbionts are involved in the virus transmission. *Wolbachia* is a group of obligate intracellular bacteria infecting numerous arthropod species and causing reproductive alterations in their hosts. These bacteria have been detected in planthopper species, considered rice pests in various regions of the world. To date, *Wolbachia* infection status of planthopper species of Argentina is unknown. Amplification by PCR and sequencing of *16S rDNA*, *wsp*- and *ftsZ*-specific genes demonstrated *Wolbachia* infection in *Caenodelphax teapae* (Fowler), *Delphacodes kuscheli* Fennah, *Pyrophagus tigrinus* Remes Lenicov & Varela, *Tagosodes orizicolus* (Muir), and *Toya propinqua* (Fieber). This is the first report of *Wolbachia* in delphacid vectors of viruses affecting maize and wheat. An understanding of the bacterial diversity harbored by these insect vectors could lead to new options for future management of diseases of economically important crops in a developing country.

RESUMEN El maíz (*Zea mays* L.) y el trigo (*Triticum aestivum* L.) son los cereales de mayor importancia para la economía de Argentina y son afectados por varias enfermedades. Varias especies de chicharritas transmiten los agentes causales de algunas de esas enfermedades, incluyendo al Mal de Río Cuarto virus, barley yellow striate mosaic virus y el recientemente propuesto maize yellow striate virus. Muchas chicharritas se alimentan de savia y por ello se espera que contengan bacterias que les provean los nutrientes esenciales, ausentes en la dieta. Algunos de esos simbiontes bacterianos han sido relacionados con la transmisión de virus. *Wolbachia* es un grupo de bacterias intracelulares obligadas que infectan a numerosas especies de artrópodos causándoles alteraciones reproductivas. Estas bacterias han sido detectadas en especies de chicharritas consideradas plagas del arroz en varias regiones del mundo. Actualmente se desconoce el estado de infección con *Wolbachia* de las especies de delfácidos de Argentina. La amplificación por PCR y la secuenciación de los genes específicos *16S rDNA*, *wsp* y *ftsZ*, demostraron la infección con *Wolbachia* en *Caenodelphax teapae* (Fowler), *Delphacodes kuscheli* Fennah, *Pyrophagus tigrinus* Remes Lenicov & Varela, *Tagosodes orizicolus* (Muir) y *Toya propinqua* (Fieber). Este es el primer reporte de *Wolbachia* en delfácidos vectores de virosis que afectan al maíz y al trigo. El hecho de conocer la diversidad bacteriana que vive dentro de estos insectos vectores podría dar lugar a nuevas opciones para el futuro manejo de enfermedades de cultivos de importancia económica para un país en desarrollo.

KEY WORDS *Wolbachia*, planthopper, vector, virus

Maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) are the most important cereal crops for the Argentinean economy. The *World Market and Trade Reports* (U.S. Department of Agriculture [USDA]) ranked Argentina as the fifth-largest global maize producer (26 million tons), and the third-largest exporter. As wheat producer, this country is ranked as the thirteenth-largest global producer (12.5 million tons), and the eighth-largest exporter (USDA 2014).

Insect pests causing direct damage, through lesions or injuries on plants during feeding and oviposition, or indirect damage by transmitting different pathogens, are one of the factors affecting wheat and maize crop yield. In Argentina, several delphacid insect species were mentioned as important vectors of viruses affecting maize and wheat. Among the species listed as

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vectors of Mal de Río Cuarto virus (MRCV) are *Delphacodes kuscheli* Fennah (Remes Lenicov et al. 1985), *Caenodelphax teapae* (Fowler) (Velazquez 2010), *Chionomus haywardi* (Muir) (Velazquez et al. 2003), *Toya propinqua* (Fieber) (Velazquez 2010), *Pyrophagus tigrinus* Remes Lenicov & Varela (Velazquez et al. 2006), *Peregrinus maidis* (Ashmead) (Virla et al. 2004), and *Tagosodes orizicolus* (Muir) (Mattio et al. 2008). *D. kuscheli*, *P. tigrinus*, *Ch. haywardi*, and *T. propinqua* have also been identified as vectors of barley yellow striate mosaic virus (BYSMV; Dumón et al. 2011) and *Pe. maidis* as vector of the recently proposed maize yellow striate virus (Maurino et al. 2012). Currently, the only applied method for vector management is the chemical control, which poses potential risks to the environment.

Weeds and volunteer plants, where these insects find refuge, are virus reservoirs that play an important role in the disease epidemiology. Given the close virus–host–vector relationship, research should be directed toward understanding the interactions between viruses and insect vectors in order to identify key points in the transmission of the pathogen. Accordingly, it is well known that delphacids, sap-feeding insects, rely on symbiotic microorganisms either for growth or survival (Douglas 2006). There is an increasing number of studies relating the presence of these microorganisms with the pathogen transmission (van den Heuvel et al. 1997, Hogenhout et al. 1998, Morin et al. 2000, Akad et al. 2004, O'Neill 2007, Gottlieb et al. 2010, Kliot et al. 2014). The genus *Wolbachia* Hertig (Rickettsiales: Rickettsiaceae) is a group of obligate intracellular bacteria of great interest because of the reproductive abnormalities they cause to their hosts (La Scola et al. 2005, Bourtzis 2008). These bacteria infect 40% of insect species (Zug and Hammerstein 2012), suggesting it may be the most ubiquitous endosymbiont on earth (Bourtzis 2008).

Wolbachia has been detected in planthopper species regarded as rice pests in various regions of the world, such as *Laodelphax striatellus* (Fallén) (maize rough dwarf virus vector in Europe), *Nilaparvata lugens* (Stål) (vector of rice ragged stunt virus and rice grassy stunt virus), *Sogatella furcifera* (Horváth) (southern rice black streak dwarf virus vector), and *Tagosodes orizicolus* (Muir) (vector of the rice hoja blanca virus in tropical America; Noda 1984a,b; Noda et al. 2001; Hernández et al. 2004; Tang et al. 2010; Zhang et al. 2010; Pu et al. 2012). However, no similar studies have been conducted on the infection status of *Wolbachia* in delphacid vectors of virus of maize and wheat, to date. Therefore, the aim of this study was to detect *Wolbachia* in different planthopper vectors of viruses that affect economically important cereal crops in Argentina.

Materials and Methods

Insect Populations. Delphacid vectors of cereal crop viruses were collected using an entomological sweep net from seven localities in the province of Córdoba (Argentina), the endemic area of the Río Cuarto disease. Two extreme points, north and southeast of the production area, were also included (Fig. 1; Table 1). Insects were collected from the borders of maize and wheat fields between 2004 and 2010. Specimen collections were conducted during spring and summer, when planthopper populations increase (Remes Lenicov et al. 1991). Collected specimens were identified down to species level with a taxonomic key at the laboratory (Remes Lenicov and Virla 1999). Then, insects were individually kept in vials at −20°C with ethanol 100% until molecular analysis. Additionally, insect lines reared in our laboratory since 1997 were included.

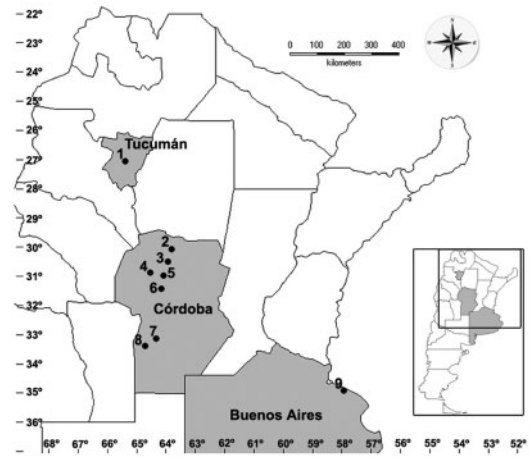

Fig. 1. Geographic location of planthopper sampling sites in three provinces of Argentina.

Total DNA Extraction. Total genomic DNA was individually extracted from adult planthoppers to screen for *Wolbachia* infection following the protocol of Sunnucks and Hales (1996), with an incubation temperature of 55°C during 1–4 h. A total of 344 specimens belonging to six species were collected (Table 1). For those populations in which >10 insects of the same species were collected, the extraction was carried out in bulk with 10 to 20 insects. The pellet obtained was resuspended in 50 µl DEPC water and the supernatant was used as template in subsequent PCRs.

Bacteria Detection by PCR. Universal primers were used to detect microorganisms belonging to *Eubacteria* group, and specific primers were used to amplify *Wolbachia* genes (Table 2). All bacterial fragments were amplified in a final volume of 50 µl containing 100 ng genomic DNA, 1X buffer (Invitrogen, Carlsbad, CA), 3.0 mM $MgCl_2$ (Invitrogen), 0.1 mM of each dNTP (Promega, Madison, WI), 0.5 µM of each primer (Invitrogen), 1 unit of Taq Polymerase 5 U/µl (Invitrogen), and sterile, double-distilled H_2O until the final volume was reached. As this is the first *Wolbachia* detection in planthoppers from Argentina, no positive or negative controls were available. Therefore, total genomic DNA from *Naupactus ambiguus* Boheman naturally infected with *Wolbachia* and *Naupactus versatilis* Hustache were used as positive and negative reaction controls, respectively. The primers used to assess the quality of DNA extraction were those

Table 1. *Wolbachia* detection by specific gene amplification and percentage of infection in planthopper vectors of cereal crop viruses

Species	Origin	Lat.	Long.	Collection year	*n*	Percentage of infection (%)	Genes amplified by specific primers		
							16S	wsp	ftsZ
D. kuscheli	Breeding colony, Río Cuarto (Córdoba)[7]	33° 08′ S	64° 21′ W	1997	55	93	+	+	+
	Capilla del Monte (Córdoba)[4]	30° 52′ S	64° 33′ W	2006	9	89	+	+	+
	Jesús María (Córdoba)[5]	30° 59′ S	64° 06′ W	2005	8	0	−	n/a	n/a
	La Plata (Buenos Aires)[9]	34° 55′ S	57° 57′ W	2007	7	0	−	−	n/a
	Río Cuarto (Córdoba)[7]	33° 08′ S	64° 21′ W	2006	10	40	n/a	+	+
	Río Cuarto (Córdoba)[7]	33° 08′ S	64° 21′ W	2008	6	0	−	−	n/a
T. orizicolus	Breeding colony, Capilla del Monte (Córdoba)[4]	30° 52′ S	64° 33′ W	2005	48	0	−	−	n/a
	El Rodeo (Córdoba)[2]	30° 05′ S	63° 50′ W	2008	2	50	+	n/a	+
	IPAVE (Córdoba)[6]	31° 25′ S	64° 11′ W	2006	2	0	−	−	n/a
	Sampacho (Córdoba)[8]	33° 23′ S	64° 44′ W	2007	1	0	−	−	n/a
Ch. haywardi	Capilla del Monte (Córdoba)[4]	30° 52′ S	64° 33′ W	2007	12	0	n/a	−	n/a
C. teapae	Famaillá (Tucumán)[1]	27° 04′ S	65° 25′ W	2004	16	[a]	+	+	−
P. tigrinus	Breeding colony, Jesús María (Córdoba)[5]	30° 59′ S	64° 06′ W	2004	40	0	−	n/a	n/a
	El Simbolar (Córdoba)[3]	30° 29′ S	63° 58′ W	2008	68	6	n/a	+	+
	Jesús María (Córdoba)[5]	30° 59′ S	64° 06′ W	2004	3	0	n/a	−	n/a
	IPAVE (Córdoba)[6]	31° 25′ S	64° 11′ W	2005	5	0	n/a	−	n/a
T. propinqua	Capilla del Monte (Córdoba)[4]	30° 52′ S	64° 33′ W	2006	20	[a]	+	+	−
	IPAVE (Córdoba)[6]	31° 25′ S	64° 11′ W	2005	35	[a]	+	+	+

+: positive; −: negative; n/a: not analyzed; Lat.: latitude; Long.: longitude; [1-9]: references on map (Fig. 1).
[a] Not calculated, coming from bulk of insect.

Table 2. Oligonucleotides and thermal profiles used in PCR amplifications of *Eubacteria* and *Wolbachia*

Gene	Primer	Target group	Thermal profile	Fragment size (bp)	Reference
16S rDNA	16S F 5′-GCTTAACACATGCAAG-3′ 16S R 5′-CCATTGTAGCACGTGT-3′	Eubacteria	95°C 2 min 92°C 30 s 50°C 30 s 72°C 30 s } x 40 72°C 5 min	≈1,180	O'Neil et al. 1992
16 S rDNA	99F 5′-TTGTAGCCTGCTATGGTATAACT-3′ 994R 5′-GAATAGGTATGATTTTCATGT-3′	*Wolbachia*	95°C 2 min 95°C 1 min 55°C 1 min 72°C 1 min } x 35 72°C 20 min	≈900	O'Neil et al. 1992
wsp	81F 5′-TGGTCCAATAAGTGATGAAGAAAC-3′ 691R 5′-AAAAATTAAACGCTACTCCA-3′	*Wolbachia*	94°C 1 min 94°C 30 s 50°C 1 min 72°C 1 min } x 35 72°C 10 min	≈600	Braig et al. 1998
ftsZ	FtsZfl 5′-GTTGTCGCAAATACCGATGC-3′ FtsZrl 5′-CTTAAGTAAGCTGGTATATC-3′	*Wolbachia*	94°C 2 min 94°C 1 min 55°C 1 min 72°C 1.5 min } x 40 72°C 10 min	≈1,055	Werren et al. 1995

designed by Normark (1994) for mitochondrial *cytochrome oxidase subunit I* (*COI*) gene. To confirm *Wolbachia* presence in the tested insects, amplified products for *wsp* gene were purified using the commercial kit Illustra MicroSpin S-300 HR Columns (GE Healthcare, Little Chalfont, United Kingdom) and sequenced in both directions by Macrogen Inc., South Korea.

Results and Discussion

The 16 S eubacterial ribosomal gene is widely used in the detection of a great variety of bacteria. The amplification of this gene has indicated the presence of prokaryotes in *C. teapae*, *Ch. haywardi*, *D. kuscheli*, *P. tigrinus*, *T. orizicolus*, and *T. propinqua*, which are all MRCV or BYSMV vectors (Remes Lenicov et al. 1985; Velazquez et al. 2003, 2006; Virla et al. 2004; Mattio et al. 2008; Velazquez 2010; Dumón et al. 2011). The primers used for this gene amplified fragments of the expected ∼1150 bp size in all the tested species. These results are in agreement with those reported by Douglas (2006), who stated that sap-feeding insects host endosymbiotic microorganisms. *C. teapae*, *D. kuscheli*, *P. tigrinus*, *T. orizicolus*, and *T. propinqua* all tested positive for *Wolbachia* after the amplification of at least one specific gene (Table 1). The sizes of the amplified fragments were similar to those expected for the *ftsZ*, 16 S rDNA, and *wsp Wolbachia* genes (O'Neill et al. 1992, Werren et al. 1995, Braig et al. 1998).

PCR products of the *wsp* gene of *Wolbachia* in *C. teapae*, *D. kuscheli*, and *T. propinqua* (MRCV vectors in the field) were sequenced. The sequences obtained in *C. teapae* and *D. kuscheli* were identical (GenBank KM386824 and KM386825, respectively), but different from that of *T. propinqua* (GenBank KM386826). BLAST homology searches indicated that the sequence of both species shared 97% nucleotide identity with the *Wolbachia* outer surface protein (WSP) gene from different groups of insects (e.g., GenBank AY566423.1 E-value = 0.0). This analysis also showed that the *wsp* sequence from *T. propinqua* shared 100% nucleotide identity with the *Wolbachia wsp* gene from several mosquito species belonging to the *Culex* genus (e.g., GenBank KJ140129.1 E-value = 0.0) and Lepidoptera (e.g., GenBank AB094390.1 E-value = 0.0). These differences in the nucleotide sequences indicate the presence of different *Wolbachia* strains in the planthoppers studied. The *wsp* gene has a high evolutionary rate, which allows the discrimination of different strains (Zhou et al. 1998). However, its high rate of recombination does not allow its use for inferring phylogenetic relationships (Baldo et al. 2005). Therefore, further research will include a thorough typing of these bacteria using other genes to define the supergroup to which they belong.

Furthermore, data showed that not all individuals or populations of the same species were positive for *Wolbachia*. For example, while *D. kuscheli* populations from Capilla del Monte (2006), Rio Cuarto (2006), and the breeding colony, amplified the *Wolbachia*-specific genes, populations of La Plata (2007) and Jesús María (2005) were negative. This geographic variability in *Wolbachia* infection status was already observed in *T. orizicolus* in Costa Rica and in the genus *Perkinsiella* in Australia (Hernández et al. 2004, Hughes et al. 2011). The collection of a greater number of specimens in future studies would help to get a clearer picture of the variability of infection among regions.

The lack of detection of *Wolbachia* could also be a consequence of an infection density below the threshold detectable by the PCR, as noted in Australian planthopper populations (Hughes et al. 2011). This aspect could not be evaluated in the present work, but to rule out this factor as an explanation for the negative results, another amplifying strategy as nested PCR would be engaged in future research.

As demonstrated in studies on the *Wolbachia* genome, this bacterium is unable to synthesize a variety of amino acids (Brownlie and O'Neill 2006), essential for the proper development of these phloem-feeding insects. Therefore, there must be another endosymbiont aside *Wolbachia*, supplying the amino acids lacking in the diet of the insects.

The amplification of eubacterial ribosomal gene in planthoppers tested negative for *Wolbachia* indicated indeed, the presence of other procarionts. Accordingly, in a recent study by Tang et al. (2010), the presence of a variety of bacteria belonging to the phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* in *Nilaparvata lugens*, a rice pest in Asia, has been evidenced. Therefore, our future works will incorporate the bacterial diversity harbored by planthopper vectors that are mentioned as plant pathogenic viruses in Argentina.

In conclusion, this is the first report on the presence of *Wolbachia* in delphacid species of agronomic importance in Argentina. Considering the potential of *Wolbachia* as a biological control agent of pests, these results provide an initial basis for its application as an alternative to chemical control, in a context of sustainable crop management. Moreover, *Wolbachia* can influence the capacity of insects to transmit pathogens causing substantial economic losses in maize and wheat in Argentina, so further studies are needed to address this point.

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