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Broader prevalence of Wolbachia in insects including potential human disease vectors

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Abstract

Wolbachia are intracellular, maternally transmitted bacteria considered the most abundant endosymbionts found in arthropods. They reproductively manipulate their host in order to increase their chances of being transmitted to the offspring, and currently are being used as a tool to control vector-borne diseases. Studies on distribution of *Wolbachia* among its arthropod hosts are important both for better understanding why this bacterium is so common, as well as for its potential use as a biological control agent. Here, we studied the incidence of *Wolbachia* in a broad range of insect species, collected from different regions of Brazil, using three genetic markers (*16S rRNA*, *wsp* and *ftsZ*), which varied in terms of their sensitivity to detect this bacterium. The overall incidence of *Wolbachia* among species belonging to 58 families and 14 orders was 61.9%. The most common positive insect orders were Coleoptera, Diptera, Hemiptera and Hymenoptera, with Diptera and Hemiptera having the highest numbers of *Wolbachia*-positive families. They included potential human disease vectors whose infection status has never been reported before. Our study further shows the importance of using quantitative polymerase chain reaction for high-throughput and sensitive *Wolbachia* screening.

Keywords: Brazil, insects, *Wolbachia*

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Introduction

Wolbachia are gram-negative alphaproteobacteria of the order Rickettsiales and family Anaplasmataceae exhibiting symbiotic relationships with their hosts (O'Neill *et al.*, 1992; Dumler *et al.*, 2001; Werren *et al.*, 2008). They were first reported in the reproductive tissues of the mosquito *Culex pipiens* (Hertig & Wolbach, 1924) and, therefore, the species was named *Wolbachia pipientis* (Hertig, 1936). However, due to uncertainty about the actual taxonomic status of *W. pipientis*, researchers commonly refer to it simply as *Wolbachia* (Lo *et al.*, 2007). Currently, based on gene sequence information, at least 13 major clades of *Wolbachia* known as 'supergroups' (A–F and H–N) have been reported (reviewed in Augustinos *et al.*, 2011). All but three of these supergroups are found in arthropods, while the remaining three have so far only been found in nematodes (Casiraghi *et al.*, 2005; Lo *et al.*, 2007; Haegeman *et al.*, 2009; Augustinos *et al.*, 2011). However, the great majority of arthropod *Wolbachia* so far described come from only two supergroups (A and B).

Wolbachia strains are globally distributed (Werren & Windsor, 2000) and currently these bacteria are considered the most abundant endosymbionts found in invertebrates. *Wolbachia* are referred to as reproductive parasites, because they induce diverse reproductive phenotypes, mainly in arthropods (Werren, 1997; Werren *et al.*, 2008). Commonly, they are

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associated with parthenogenesis (Weeks & Breeuwer, 2001), phenotypic feminization of genetic males (Rousset *et al.*, 1992), cytoplasmic incompatibility (O'Neill *et al.*, 1992) and male killing (Hurst & Jiggins, 2000). *Wolbachia* are also thought to play important roles in speciation and local adaptation (Brucker & Bordenstein, 2012). The importance of *Wolbachia* in reproductive processes depends ultimately on its prevalence, and how it is transmitted between species (Stouthamer *et al.*, 1999). In Arthropoda, *Wolbachia* are believed to be primarily maternally transmitted within species (Skinner, 1982), but horizontal transmission also frequently occurs between species over longer evolutionary time-scales (Werren *et al.*, 1995*a*; Schilthuizen & Stouthamer, 1997).

About 40% of arthropod species are estimated to be infected with *Wolbachia* (Zug & Hammerstein, 2012). They are common and widespread in insects (Werren *et al.*, 1995*b*), which represent the greatest diversity of all known animal groups on Earth (Rafael *et al.*, 2012), equivalent to around 60% of all currently described organisms (Grimaldi & Engel, 2005). They are important for maintenance of ecosystems, as agricultural pests and vectors of human diseases, and useful in medicine and scientific research, besides representing a commercial value food in some cultures (Triplehorn & Johnson, 2005).

Due to the importance of *Wolbachia*, some researchers have investigated the presence of these bacteria in insects from different locations (Duron *et al.*, 2008; Russell, 2012; Russell *et al.*, 2012). Hilgenboecker *et al.* (2008) estimated that over 65% of insect species carry *Wolbachia*. However, other studies reported that up to 76% (Jeyaprakash & Hoy, 2000) or as few as 20% of insect species are infected with *Wolbachia* (Werren *et al.*, 1995*b*). In the first published survey of *Wolbachia* distribution, Werren *et al.* (1995*b*) found over 16% of sampled insect species from Panama were infected with *Wolbachia*, within several insect orders. In the UK, 22% of insects sampled were infected with *Wolbachia*, mainly in the Lepidoptera and Hymenoptera (West *et al.*, 1998). In North America, insect species from 13 different orders were screened for *Wolbachia*, of which 19.3% were positive. The bacteria have been found in species within several major insect orders: Coleoptera, Diptera, Hymenoptera, Lepidoptera and Orthoptera (Werren & Windsor, 2000).

Wolbachia detection in Arthropoda has been traditionally performed through standard polymerase chain reaction (PCR) assays targeting the *16S rRNA* gene, or protein-coding genes such as the *Wolbachia* surface protein (*wsp*) gene and the bacterial cell division gene *ftsZ* (reviewed in Simões *et al.* (2011). In contrast, real-time quantitative PCR (qPCR), which possesses high reproducibility, sensitivity and precision of results, has never been used as a tool for *Wolbachia* screening in Arthropoda.

In Brazil, there are some reports regarding the detection of *Wolbachia* in limited, specific arthropod groups, but no general surveys of *Wolbachia* distribution among arthropods have so far been conducted. For example, infection of *Wolbachia* has been detected in two species of *Balloniscus* (Crustacea, Oniscidea) (Almerão *et al.*, 2012) and in some species of Diptera in Culicidae (de Albuquerque *et al.*, 2011; de Almeida *et al.*, 2011; Morais *et al.*, 2012; Baton *et al.*, 2013) and in Hymenoptera (Formicidae) (Martins *et al.*, 2012). Here, we show the incidence of *Wolbachia* in different insect orders from the northern and southeastern regions of Brazil using three different markers (*16S rRNA*, *wsp* and *ftsZ*), and the observed incidence corroborates the previously reported widespread nature of this bacterium. We also emphasize the importance of using qPCR for *Wolbachia* high-throughput screening.

Materials and methods

Insect collection sites

Insects were collected from various field sites spanning the northern and southeastern regions of Brazil, from 2009 to 2012. Samples were obtained from urban, non-urban, forest and forest fragments from Manaus, Careiro da Várzea, Coari and Lábrea in the state of Amazonas; from Belo Horizonte, Belo Vale, Campo Belo and São João da Missões in the state of Minas Gerais; and from Niterói and Rio de Janeiro city in the state of Rio de Janeiro (table 1).

Insect collection and identification

Insects were manually collected using forceps, nets or traps: HP trap with light attraction (HP Biomédica, Sabará, Minas Gerais, Brazil; Pugedo *et al.*, 2005), CDC trap+CO_2 (John W. Hock Company, Gainesville, Florida, USA) and BG-Sentinel traps (Biogents AG, Regensburg, Germany). Whole insects were individually preserved (to prevent potential cross-contamination) in 96% ethanol and stored at 4°C until identification and DNA extraction. Specimens were identified based on morphology to family level according to Rafael *et al.* (2012) and Triplehorn & Johnson (2005). Sand flies were identified to species level through genital morphology according to Galati (2003) and mosquitoes to species according to Consoli & de Oliveira (1994), Faran & Linthicum (1981) and Linthicum (1988). Photos were taken for voucher samples with a stereomicroscope (Zeiss Stemi DV4) and digital camera (Canon SX30 IS). Insects that had bristles and spots on the wings, which were important for identification, were not preserved in ethanol but kept in silica.

DNA extraction

Small insects had their bodies homogenized, whereas larger insects were dissected in 1X PBS, to remove ovaries, fat body, thorax and/or abdomen. In the latter case, individual organs were used for DNA extraction.

Crude DNA samples were prepared from individual insects by homogenization in 80 µl 'squash buffer' (0.4 mM EDTA, 4 mM Tris, 20 mM NaCl) using a Mini-Beadbeater-16 (BioSpec Products, Inc., Bartlesville, Oklahoma, USA) (modified from Fu *et al.*, 2010). All samples were measured using a NanoDrop (Thermo Scientific Waltham, MA, USA) and diluted to a final concentration of 20 to 50 ng genomic DNA μl^{-1}.

Template and PCR reaction

Insects were screened for the presence of *Wolbachia* using PCR. Standard PCR was used for the ribosomal *16S rRNA* gene with the primers 16S-2 (originally called Wspec; Werren & Windsor, 2000; Simões *et al.*, 2011). Real-time qPCR was performed for the *wsp* and *ftsZ* genes using the *wsp* primers (Moreira *et al.*, 2009) and newly designed primers to the *ftsZ* gene, as follows; *ftsZ*qPCR Forward: 5′-GCATTGCAGAGCTTGGACTT-3′ and *ftsZ*qPCR Reverse: 5′-TCTTCTCCTTCTGCCTCTCC-3′. The *ftsZ*qPCR primers were designed using Primer3 (Rozen & Skaletsky, 2000;

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Table 1. Insect collection sites. Insects were collected from different settings: urban, non-urban, forest and forest fragments in northern (Amazonas state) and southeastern (Belo Horizonte and Rio de Janeiro), Brazil (2009–2012).

City	Site	State	Environment	GPS coordinates	Collection date
Manaus	Centro	Amazonas	Urban	S3°6.4315′, W60°1.5676′	September/2011
Manaus	Petrópolis	Amazonas	Urban forest fragments	S3°09.5018′, W59°98.8075′	
Careiro da Várzea	Br319 – Km 106	Amazonas	Forest	S3°17.6238′ e W59°51.8484′	August/2009 and October/2010
Coari	Gasoduto	Amazonas	Forest	S4°10.1303′ e W63°14.0305′	May/2010
Lábrea	Terra Indígena Caititu, Aldeia Castanheira	Amazonas	Forest	S07°27′28.7′, W64°43′42.2¢¢	May/2012
Niteroi	Jurujuba	Rio de Janeiro	Urban	S22°93.3332′, W43°11.6669′	October and November/2012
Rio de Janeiro	Tubiacanga	Rio de Janeiro	Urban	S22°78.5780′, W43°22.6513′	
Rio de Janeiro	Vila Valqueire	Rio de Janeiro	Urban	S22°88.3333, W43°36.6665′	
Rio de Janeiro	Urca	Rio de Janeiro	Urban	S22°95.4769′, W43°16.6557′	
Belo Horizonte	Barro Preto	Minas Gerais	Urban	S19°55.1703′, W43°57.973′	
Belo Horizonte	Sion	Minas Gerais	Urban	S19°57.3132′, W43°56.2222′	
Belo Horizonte	Luxemburgo	Minas Gerais	Urban	S19°94.8444′, W43°95.6791′	April/2011 and September/2012
Belo Horizonte	São Pedro	Minas Gerais	Urban	S19°94.2450′, W43°93.6733′	
Belo Horizonte	Magabeiras	Minas Gerais	Urban forest fragments	S19°57.2520′, W43°54.3821′	
Belo Horizonte	UFMG	Minas Gerais	Colony	S19°51.4953′, W 43°57.60002′	August/2013
Belo Horizonte	CPqRR/Fiocruz	Minas Gerais	Colony	S19°55.4390′ W43°56.3806′	May/2011
Belo Vale	private property	Minas Gerais	Non-urban forest fragments	S20°24.4796′ W44°1.0909′	April/2012
Campo Belo	private property	Minas Gerais	Non-urban forest fragments	S20°51.9503′ W45°16.3921′	
São João da Missões	Xacriabá	Minas Gerais	Forest	S14°88.2146′ W44°21.8105′	August/2012

Untergasser *et al*., 2012) to amplify a 271 bp fragment of the *ftsZ* gene from as broad a spectrum as possible of known sequences from Supergroups A and B, but not C and D, *Wolbachia*. The specificity of the *ftsZ*qPCR primers to *Wolbachia* was checked using NCBI Primer-BLAST against the non-redundant database. Control DNA samples were prepared using adult females of the mosquito *Aedes aegypti* artificially infected with either the *w*Mel (Walker *et al*., 2011) or *w*MelPop strains of *Wolbachia* (McMeniman *et al*., 2009).

Standard PCR had the following components: a final concentration of 0.5X Buffer A and 0.5X Buffer B, 0.13 mM dNTP, 1 µM of each 16S-2 F/R primer, together with 0.3 µl of Elongase (Applied Biosystems®,Grand Island, New York, USA) and a total of 20–50 ng μl^{-1} of sample DNA, made up with water to a total volume of 25 µl. Amplifications were performed in an automatic thermocycler (Veriti™ Dx Thermal Cycler, Applied Biosystems®,Grand Island, New York, USA) using 35 cycles (30 s 94°C, 30 s 52°C, 1.5 min 68°C) preceded by 5 min at 94°C and followed by a final extension step of 10 min at 68°C. PCR products were visualized on 2% agarose gels stained with Gel Red (diluted 1000×, Biotium, Inc. Hayward, California, USA). qPCR had a final concentration of 1× SYBR® Green PCR Master Mix (Applied Biosystems) and 0.5 µM of each primer (*wsp* F/R or *ftsZ*qPCR F/R), with a total of 20–50 ng of sample DNA and water to a total volume of 20 µl. The DNA was amplified through 40 cycles (15 s at 95°C and 30 s at 60°C) for the *wsp* R/F primers, and for 40 cycles (15 s at 95°C, 60 s at 60°C) for the *ftsZ*qPCR F/R primers. All qPCR reactions were carried out in a 96-well microtitre plate (Model 7500, Applied Biosystems). Results were analyzed with the 7500 software v2.0.5, through individual analysis of each amplification curve (compared to the pattern of a positive control) and also their melting curves to check the specificity of the amplification.

In order to confirm the PCR results and therefore, *Wolbachia* infection status, we sequenced a subset of 61 samples (table 2), that exhibited positive results for only one set of primers. For that, DNA was amplified through conventional PCR under the same conditions as the qPCR (see above). After conventional PCR, the samples were then purified (PCR Purification Kit, Qiagen; Venlo, Limburg, Netherlands), lyophilized and sent for sequencing (Macrogen; Seoul, Korea). As a control, we also sequenced the DNA of *A. aegypti* artificially infected with the *w*MelPop (McMeninam *et al*., 2008), using the *16S rRNA, ftsZ* and *wsp* primers. The raw sequencing reads were trimmed and analyzed using the nucleotide-nucleotide BLAST (BLASTN) tool from NCBI and results are shown on table 2.

Results

A total of $n = 396$ insect specimens from 194 species were screened for *Wolbachia* in 14 orders and 58 families. The largest group belonged to Diptera ($n = 191$; 48% of all specimens examined) followed by Hemiptera ($n = 56$; 14%), Hymenoptera ($n = 56$; 14%) and Coleoptera ($n = 34$; 9%). The highest number of species belonged to Diptera ($n = 65$; 34% of all species examined), followed by Hymenoptera ($n = 35$; 18%), Hemiptera ($n = 33$; 17%) and Coleoptera ($n = 25$; 13%) (fig. 1a, b and table 3).

We used three sets of primers to increase the chance of detecting different strains of *Wolbachia* in our insect samples: *16S rRNA, wsp* and *ftsZ*. We found 28.3% specimens positive for the *16S rRNA* marker, 46.2% for *wsp* and 39.7% for the *ftsZ* primer (table 3). As expected, the *wsp* and *ftsZ* primers were more sensitive in detecting *Wolbachia* infections than the *16S rRNA* primers, which were used for qPCR.

Overall, *Wolbachia* was found in 10 of the 14 insect orders surveyed, with 232 (58.6%) specimens and 120 (61.9%) species positive. We found 100% species infected with *Wolbachia* in Orthoptera/Blattodea/Neuroptera/Siphonaptera, 73% in Hemiptera, 69% in Hymenoptera, 62% in Diptera, 50% in Isoptera, 48% in Coleoptera and 40% in Lepidoptera

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Table 2. Sequenced insect samples. Insects samples were sequenced for *Wolbachia* using wsp, *16S rRNA* and *fstZ* primers.

Order	Family/order/species	Primer	Sequenced specimens	Positive for Wolbachia	Max score	Query cover (%)	*E* value	Ident (%)	Accession
Hymenoptera	Formicidae	*16S rRNA*	2	2	708	92	0.0	100	JQ726771.1
Hymenoptera	Vespidae	*16S rRNA*	1	1	675	56	0.0	99	AB746405.1
Diptera	Psychodidae and Phlebotominae	*16S rRNA*	5	2	682	55	0.0	99	AB772263.1
		wsp			148	27	5.00×10^{-32}	89	AY916133.1
Diptera	Psicodidae Phebotominae *Sciopemyia sordellii*	*FstZ*	1	1	350	48	1.00×10^{-92}	98	AY916134.1
Diptera	Psicodidae Phebotominae *Psychodopygus llanosmartinsi*	*FstZ*	1	1	392	53	2.00×10^{-105}	98	KJ659910.1
Diptera	Drosophilidae	16S rRNA	7	7	682	94	0.0	97	KF250093.1
		FstZ			412	49	2.00×10^{-111}	99	AY095164.1
Diptera	Culicidae *Culex quinquefasciatus*	16S rRNA	6	5	665	89	0.0	99	HG428761.1
		FstZ			379	46	2.00×10^{-101}	98	KJ659910.1
Diptera	Culicidae/*Culex* sp.	16S rRNA	3	3	462	94	2.00×10^{-126}	88	HG428761.1
		FstZ			139	21	3.00×10^{-29}	95	JX296508.1
Diptera	Culicidae/*Mansonia titilans*	*FstZ*	2	2	409	88	1.00×10^{-110}	100	GU573908.1
Diptera	Tachinidae	16S rRNA	1	1	460	92	5.00×10^{-126}	89	KF250093.1
Diptera	Tipulidae	*FstZ*	1	1	333	41	1.00×10^{-87}	99	HG970644.1
Diptera	Tabanidae	*FstZ*	1	1	195	24	1.00×10^{-45}	88	AY157007.1
Diptera	Dolichopodidae	*wsp*	1	1	159	64	1.00×10^{-35}	89	U83105.1
Coleoptera	Anobiidae	16S rRNA	3	3	728	97	0.0	99	CP003883.1
Isoptera	Rhinotermitidae	16S rRNA	9	8	616	92	9.00×10^{-179}	96	AB632591.1
		wsp			259	51	1.00×10^{-65}	97	AJ833931.1
Hemiptera	Pirrhocoridae	16S rRNA	1	1	555	95	2.00×10^{-154}	92	KF250093.1
Hemiptera Heteroptera	Rhopalidae	16S rRNA	2	2	339	92	3.00×10^{-89}	83	EU914940.1
Hemiptera Achenorrhyncha	Cicadellidae	*Wsp*	1	1	265	36	5.00×10^{-67}	98	KC137230.1
Hemiptera Auchenorrhyncha	*Coreidae*	*wsp*	1	1	241	31	8.00×10^{-60}	99	KJ648498.1
Hemiptera	Reduviidae *Triatoma infestans*	*FstZ*	2	0					
		Wsp							
Hemiptera	Reduviidae *Rhodnius prolixus*	*FstZ*	1	0					
		wsp							
Hemiptera	Reduviidae *Triatoma brasiliensis*	*FstZ*	2	0					
		wsp							
Hemiptera	Reduviidae *Panstrongylus* megistus	*FstZ*	4	0					
		Wsp							
Hemiptera	Berytidae	*wsp*	1	1	248	17	1.00×10^{-61}	97	KC161952.1
Hemiptera		*wsp*	1	1	189	36	3.00×10^{-44}	90	KF036313.1
Neuroptera	Chrisopidae	*FstZ*	1	0					
Total			61 samples	46 positive for *Wolbachia*					

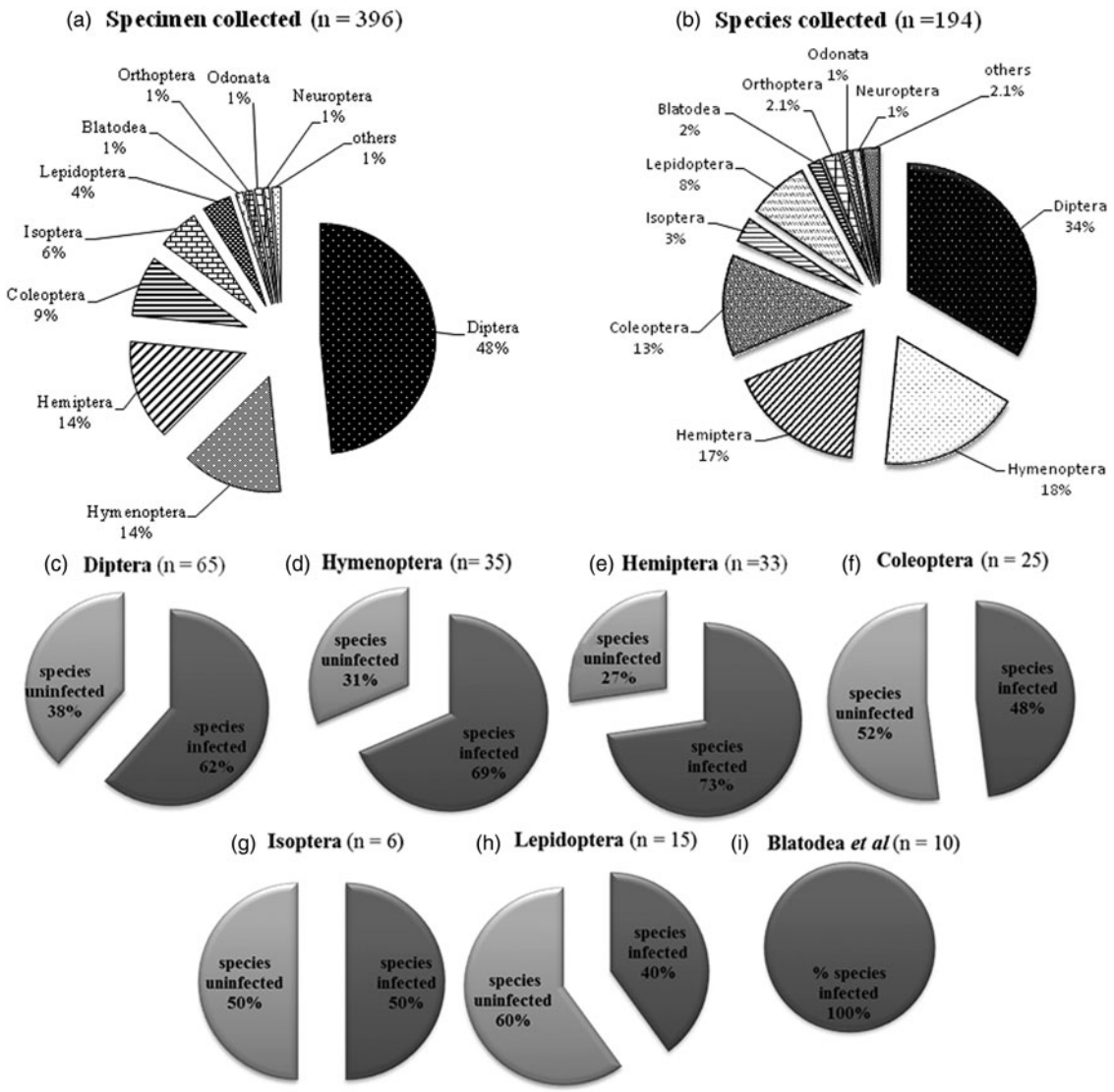

Fig. 1. Diversity and abundance of insects collected, and the proportion of species infected with *Wolbachia* in each order. In figures C–I: infected (dark grey) and uninfected (light grey) with *Wolbachia*.

(fig. 1c–i). *Wolbachia* was not detected in four insect orders: Odonata, Psocoptera, Diplura or Thysanura. This probably reflects the small sample sizes for these insect groups, rather than the absence of *Wolbachia*, as previous studies have found *Wolbachia* in the Odonata and Psocoptera (Thipaksorn *et al*., 2003; Dong *et al*., 2006). *Wolbachia* were present in 46 families from the 10 PCR-positive orders screened. Orders with the largest number of families infected with *Wolbachia* were Hemiptera ($n = 12$; 20.7%), Diptera (12; 21%) and Coleoptera (7; 12.1%) (table 3).

Within Diptera (families Culicidae and Psychodidae) and Hemiptera (Reduviidae), which include several human disease vectors species, we screened 41 species and 19 were positive for *Wolbachia* (table 3). In Culicidae, we found *Wolbachia* in four species and two genera. Positive results for *Culex quinquefasciatus* Say, 1823 and *Aedes albopictus* (Skuse, 1894) and *Culex* sp. were expected as their infectious status is widely reported. However, for *Mansonia titillans* (Walker, 1848), *Psorophora cingulata* (Fabricius, 1805) and *Limatus* sp. this is first report of *Wolbachia*. In Psychodidae, we report here for the first time the presence of *Wolbachia* in four phlebotomine species: *Psychodopygus llanosmartinsi* (Fraiha & Ward, 1980), *Sciopemyia sordellii* (Shannon & Del Ponte, 1927), *Psychodopygus davisi* (Root, 1934), *Trichophoromyia flochi* (Abonnenc & Chassignet, 1948), and two genera whose species have not been identified: *Evandromyia* sp. and *Psychodopygus* sp. In Reduviidae we did not find *Wolbachia* in any of screened species of triatominae: *Triatoma infestans* (Klug, 1934), *Panstrongylus megistus* (Klug, 1934), *Triatoma brasiliensis* Neiva, 1911 and *Rhodnius prolixus* Stål, 1859. These species are exclusively hematophagous, and have been reported with their association with Chagas disease transmission (table 4).

Sequencing a subset of samples allowed us to confirm the majority of samples that showed positivity with the PCR

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Table 3. Number of insects collected and infected with *Wolbachia*. Insects were screened for Wolbachia using *wsp*, *16S rRNA* and *fstZ* primers.

Order	Family	Number of specimens collected	Number of infected specimens	Number of species collected	Number of infected species	*16S rRNA*	*wsp*	*fstZ*
Diptera	Drosophilidae	20	18	4	3	13	17	18
	Chironomidae	9	4	7	4	1	3	1
	Cecidomyiidae	5	1	1	1	0	1	0
	Tachinidae	2	1	2	1	1	1	1
	Calliphoridae	6	5	4	4	2	4	2
	Culicidae	70	65	13	6	52	55	61
	Tabanidae	3	2	2	2	1	1	1
	Psychodidae	66	26	24	13	5	19	13
	Anthomyiidae	1	0	1	0	0	0	0
	Muscidae	1	1	1	1	0	1	1
	Tipulidae	2	1	2	1	0	0	1
	Dolichopodidae	1	1	1	1	0	1	0
	Sarcophagidae	1	1	1	1	0	1	1
	Unidentified	4	4	2	2	0	3	3
	Total	191	130	65	40	75	107	103
Hymenoptera	Apidae	20	5	8	4	2	3	0
	Formicidae	21	12	13	9	5	9	8
	Vespidae	9	7	9	7	2	5	2
	Braconidae	1	1	1	1	0	1	0
	Pompilidae	1	1	1	1	0	1	1
	Unidentified	4	2	3	2	0	2	2
	Total	56	28	35	24	9	21	13
Hemiptera/ Stemorrhyncha	Psyllidae	4	4	2	2	1	4	2
	Aphididae	1	1	1	1	0	1	1
	Gerridae	4	4	4	4	1	4	1
	Corixidae	1	1	1	1	0	1	1
	Reduviidae	20	0	4	0	1	14	3
	Cydnidae	1	1	1	1	0	1	1
Hemiptera/Heteroptera	Berytidae	2	2	2	2	1	2	1
	Pyrrhocoridae	4	4	4	4	2	3	3
	Rhopalidae	5	4	2	2	2	4	4
	Pentatomidae	1	1	1	1	0	1	0
	Coreidae	1	1	1	1	0	1	0
	Cicadellidae	7	4	6	3	1	3	2
Hemiptera/ Auchenorrhyncha	Cixiidae	2	2	1	1	1	2	2
	Cicadidae	1	0	1	0	0	0	0
Hemiptera	Unidentified	2	1	2	1	0	1	0
	Total	56	30	33	24	11	23	17
Coleoptera	Cantharidae	3	1	1	1	0	1	0
	Chrysomelidae	9	3	9	3	2	0	2
	Curculionidae	6	5	3	2	0	5	4
	Tenebrionidae	1	0	1	0	0	0	0
	Scarabaeidae	1	0	1	0	0	0	0
	Passalidae	1	0	1	0	0	0	0
	Haliplidae	1	1	1	1	0	1	1
	Nitidulidae	1	0	1	0	0	0	0
	Cerambycidae	1	1	1	1	0	1	0
	Anobiidae	5	4	1	1	3	4	4
	Brentidae	1	1	1	1	0	1	0
	Unidentified	4	3	4	2	1	3	1
	Total	34	19	25	12	6	16	12
Odonata	Libellulidae	2	0	1	0	0	0	0
	Coenagrionidae	2	0	1	0	0	0	0
	Total	4	0	2	0	0	0	0
Orthoptera	Acrididae	1	1	1	1	1	0	0
	Anostostomatidae	1	1	1	1	1	1	1
	Tettigoniidae	1	1	1	1	0	1	1
	Unidentified	1	1	1	1	0	1	1
	Total	4	4	4	4	2	3	3
Lepidoptera	Sphingidae	1	0	1	0	0	0	0
	Nymphalidae	2	1	2	1	0	1	0
	Unidentified	13	5	12	5	1	4	3
	Total	16	6	15	6	1	5	3

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Table 3. (*Cont.*)

Order	Family	Number of specimens collected	Number of infected specimens	Number of species collected	Number of infected species	*16S rRNA*	*wsp*	*fstZ*
Blatodea	Blaberidae	1	1	1	1	1	1	0
	Blattidae	3	2	2	2	1	2	1
	Total	4	3	3	3	2	3	2
Diplura	Parajapygidae	1	0	1	0	0	0	0
Siphonaptera	Pulicidae	2	1	1	1	0	1	1
Thysanura	Lepismatidae	1	0	1	0	0	0	0
Neuroptera	Chrysopidae	3	1	2	1	0	1	1
Psocoptera	Psocidae	1	0	1	0	0	0	0
Isoptera	Rhinotermitidae	23	10	6	3	6	3	2
Total		396	232	194	120	112	183	156
%			58.6		61.9	28.3	46.2	39.39

Table 4. Species and genus of hemipterans, Culicidae and phlebotomines collected and screened for Wolbachia. Hemipterans from colony, Culicidae from several localities, and phlebotomines from colony and Amazon.

Species	Family	Number collected	Number infected with *Wolbachia*	*wsp*	*16S rRNA*	*fstZ*
Triatoma infestans[1,2]	Triatominae	5	0	0	0	0
Triatoma brasiliensis[1,2]	Triatominae	5	0	0	0	0
Rhodniusprolixus[1,2]	Triatominae	5	0	0	0	0
Panstrongylus megistus[1,2]	Triatominae	5	0	0	0	0
Anopheles darlingi[2]	Culicidae	1	0	0	0	0
Anopheles sp.	Culicidae	1	0	0	0	0
Urotaenia sp.	Culicidae	1	0	0	0	0
Culex quinquefasciatus[2]	Culicidae	8	7	4	5	6
Culex spp.	Culicidae	31	31	29	27	30
Mansonia titilans	Culicidae	4	4	4	3	4
Limatus sp.	Culicidae	1	1	1	0	0
Psorophora cingulata	Culicidae	2	0	0	0	2
Aedes albopictus[2]	Culicidae	19	17	16	16	17
Trichophoromyia ubiquitalis	Psychodidae	3	0	0	0	0
Trichophoromyia flochi	Psychodidae	1	1	0	1	0
Psychodopygus claustrei[2]	Psychodidae	3	0	0	0	0
Psychodopygus davisi[2]	Psychodidae	2	1	1	0	1
Psychodopygus serie chagasi[2]	Psychodidae	1	0	0	0	0
Psychodopygus llanosmartinsi[2]	Psychodidae	1	1	1	0	1
Psychodopygus sp.	Psychodidae	2	2	2	0	2
Evandromyia begonae	Psychodidae	1	0	0	0	0
Evandromyia sp.	Psychodidae	2	2	1	1	1
Nyssomyia richardwardi	Psychodidae	2	0	0	0	0
Nyssomyia antunesi	Psychodidae	1	0	0	0	0
Nyssomyia sp.	Psychodidae	4	0	0	0	0
Psathyromyia aragaoi	Psychodidae	1	0	0	0	0
Sciopemyia sordellii	Psychodidae	3	1	1	0	1
Lutzomyia longipalpis[2,3]	Psychodidae	5	0	0	0	0
Deanemyia maruaga	Psychodidae	1	0	0	0	0

[1]Specimens from CPqRR/Fiocruz colony.
[2]Species vectors of disease.
[3]Specimens from UFMG (Minas Gerais) colony.

analysis. From a total of 61 DNA samples, 46 returned sequences belonging to *Wolbachia* (table 2).

Discussion

We studied the incidence of *Wolbachia* in insects collected from northern and southern parts of Brazil. Most of the insects collected belong to Coleoptera, Diptera, Hemiptera and Hymenoptera. Although we used light and CO_2 traps, as well as manual sampling to collect insects near or within urban areas, targeting a great diversity of insect groups, most of the insects sampled were Diptera, Heteroptera, Hymenoptera and Coleoptera. This is because these orders are large and well-diversified, making it easier to collect representatives in different habitats. The higher prevalence of *Wolbachia* in Diptera was expected, since many species in this order have previously been reported to be infected with the endosymbiont, and we collected more specimens and species from this order, so that we would be more likely to detect rare infections (Duron *et al*., 2008; Hilgenboecker *et al*., 2008;

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Zug & Hammerstein, 2012). In dipteran insects, especially mosquitoes (Hertig & Wolbach, 1924) and drosophilids, *Wolbachia* is commonly found (Boyle *et al.*, 1993; Braig *et al.*, 1994). Furthermore, many other insect groups are known to carry *Wolbachia*: e.g., leafhoppers, thrips and whiteflies (Nirgianaki *et al.*, 2003), termites (Bandi *et al.*, 1997; Lo *et al.*, 2002; Bordenstein & Rosengaus, 2005), beetles (Werren & Windsor, 2000; Nirgianaki *et al.*, 2003), odonates (dragonflies and damselflies) (Thipaksorn *et al.*, 2003) and crickets (Kamoda *et al.*, 2000). Although in our collections, Hemiptera and Hymenoptera had fewer species and specimens collected compared to Diptera, *Wolbachia* had a higher incidence.

Heteroptera, known as true bugs, is one of the most diverse groups of insects with incomplete metamorphosis. *Wolbachia* infection was previously reported in this group (Kikuchi & Fukatsu, 2003) and here we observed a 28.6% frequency of infection distributed in eight different families (Gerridae, Corixidae, Cydnidae, Berytidae, Pyrrhocoridae, Rhopalidae, Pentatomidae and Coreidae), six of them previously reported by Kikuchi & Fukatsu (2003). In many groups of Heteroptera, the removal of the endosymbionts can result in stunted growth and/or mortality of the nymphs, suggesting a major role for *Wolbachia* in this host association (Fukatsu & Hosokawa, 2002).

Wolbachia also influence reproductive patterns in social Hymenoptera. Studies on ants in Indonesia showed that *Wolbachia* was common, with 50% of the species infected (Wenseleers *et al.*, 1998). In our study, from 13 species of ants screened, nine were infected with *Wolbachia*, representing an incidence of infection greater than 69%. *Wolbachia* infection has been reported to cause parthenogenesis in some families of Coleoptera (Werren *et al.*, 1995*a*; Rodriguero *et al.*, 2010). Furthermore, evidence of horizontal transfer of *Wolbachia* was also found in Curculionidae, Chrysomelidae and Tenebrionidae (Rodriguero *et al.*, 2010). We collected 19 species of beetles from these and others families. *Wolbachia* was present in 12 species: Cantharidae ($n = 1$), Chrysomelidae ($n = 3$), Curculionidae ($n = 2$), Haliplidae ($n = 1$), Cerambicidae ($n = 1$), Anobiidae ($n = 1$), Brentidae ($n = 1$) and two other species. Based on *16S rRNA* and *wsp* sequence detection, *Wolbachia* had already been reported in siphonapteran hosts (Jeyaprakash & Hoy, 2000; Gorham *et al.*, 2003; Dittmar & Whiting, 2004) and in this study we collected a flea [*Ctenocephalides canis* Curtis (Siphonaptera, Pulicidae)] from a domestic dog that was also positive for *Wolbachia*. According to Dittmar & Whiting (2004), the discovery of symbiotic bacteria in wild populations of Siphonaptera suggests a potentially widespread association with fleas. Although we collected only two specimens of the same species, one specimen was positive.

In the present study, the overall incidence of *Wolbachia* among species was similar to that reported by Hilgenboecker *et al.* (2008) who estimated that the percentage of infected *Wolbachia* species is approximately 66%, when rarely infected species are included. Most of the species that we screened were based on one or only a few individuals. Within each species from the same population, we found that 40 to 100% specimens were infected with *Wolbachia* (i.e., the intra-specific prevalence of *Wolbachia* varied from low to high frequency). This could be because the levels of infection within a host population may depend on the age of the endosymbiont–host association (i.e., whether there has been sufficient time for *Wolbachia* to invade the host population) and how *Wolbachia* manipulates the reproduction of their hosts (Hurst & Jiggins, 2000).

Wolbachia is naturally present in many genera of mosquitoes, including *Aedes*, *Culex*, *Mansonia* and *Coquillettidia* (Kittayapong *et al.*, 2000; Ricci *et al.*, 2002; Dean & Dobson, 2004) and recently it has been reported in *Anopheles gambiae* (Baldini *et al.*, 2014). Our survey also revealed the presence of *Wolbachia* in a number of other potential vectors of human pathogens. *Wolbachia* has previously been found in the gonads and salivary glands of *Rhodnius pallescens* Barber, 1932, which is considered the most important vector of *Trypanosoma cruzi* and *Trypanosoma rangeli* in the Neotropics (Espino *et al.*, 2009), but the role of this endosymbiont in the relationship between the insect and parasite is not yet known. In Brazil, there are several kissing bug species, which are important vectors of Chagas disease, such as *T. infestans*, *T. brasiliensis*, *R. prolixus* and *P. megistus* (Costa & Lorenzo, 2009), but there are no reports about the presence of *Wolbachia* in these insects. Although the *wsp* marker detected *Wolbachia* in five specimens of *P. megistus* and *T. brasiliensis*, while the *ftsZ* primers detected the bacterium in two specimens of *R. prolixus* and one *P. megistus*, the infection was not confirmed by sequencing (table 2), as the blasted sequences had no hits to *Wolbachia*. It is important to emphasize that these particular samples were derived from the laboratory. Broader screening of field specimens should be envisaged, increasing the chance of *Wolbachia* detection.

Wolbachia has also been reported in the Phlebotominae (Diptera: Psychodidae) both in New (Ono *et al.*, 2001; Azpurua *et al.*, 2010) and Old World species (Zhou *et al.*, 1998). Phlebotomines are vectors of several viral, bacterial and protozoal diseases of humans and other animals, but there are few studies on the presence of *Wolbachia* in sand flies (Cui *et al.*, 1999; Ono *et al.*, 2001; Benlarbi & Ready, 2003; Matsumoto *et al.*, 2008; Azpurua *et al.*, 2010; de Sousa *et al.*, 2013) and about the biological relationship of the endosymbiont with the host (Kassem *et al.*, 2003; Kassem & Osman 2007). In Iran, a new strain of *Wolbachia* was recently found in *Phlebotomus perfiliewi transcaucasicus* Perfil'ev, 1937 (Parvizi *et al.*, 2013), increasing the list of phlebotomines known to be infected with this endosymbiont. Further studies should explore the potential for *Wolbachia* to be used as a biological control agent for *Leishmania* vectors. Here, we collected 21 sand fly species (20 wild species from Amazonas and one from a colony), and *Wolbachia* was found only in wild species. In six wild species, the bacterium was found using both *wsp* and *ftsZ* primers. Only in a single wild species of the genus *Evandromyia* was *Wolbachia* detected by all three markers.

Conclusions

Due to the high diversity amongst different *Wolbachia* strains, it is difficult to detect a wide range of strains using one set of universal primers. Currently, new strains of *Wolbachia* in different host species have been found, mainly due to the use of a combination of primers to improve detection of this bacterium (Lo *et al.*, 2002). Here, we used three different primer sets and two PCR methods to enhance the detection of *Wolbachia* in an extensive collection of insects. According to Simões *et al.* (2011), the *16S rRNA* primers are sensitive to detect a broad-spectrum of *Wolbachia*. However, these primers do not detect all *Wolbachia* strains. It was clear in our results that the primers used for real-time qPCR (*wsp* and *ftsZ*) showed a higher number of positive samples than conventional PCR (using the *16S rRNA* primer set), which can be explained by the higher sensitivity provided by qPCR.

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In summary, one should take into account the difficulty of designing primers covering all existing groups of *Wolbachia*, but on the other hand be cautious of using a single marker, such as *wsp* or *ftsZ*, as this could potentially underestimate *Wolbachia* prevalence in a given sample. Finally, we recommend the use of real-time qPCR because it is the most sensitive and fastest method to detect *Wolbachia* in a wide variety of arthropod samples.

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