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MOLECULAR DIAGNOSTICS AND DNA TAXONOMY

Wolbachia detection: an assessment of standard PCR Protocols

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Abstract

***Wolbachia* is a large monophyletic genus of intracellular bacteria, traditionally detected using PCR assays. Its considerable phylogenetic diversity and impact on arthropods and nematodes make it urgent to assess the efficiency of these screening protocols. The sensitivity and range of commonly used PCR primers and of a new set of 16S primers were evaluated on a wide range of hosts and *Wolbachia* strains. We show that certain primer sets are significantly more efficient than others but that no single protocol can ensure the specific detection of all known *Wolbachia* infections.**

Keywords: 16S rRNA gene, MLST, PCR methods, specificity, *Wolbachia* screening

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The intracellular bacterium *Wolbachia* is currently considered the most abundant endosymbiont in arthropods and shows a global geographic distribution (Werren & Windsor 2000; Werren *et al.* 2008); in insects, it is estimated that over 65% of the species carry members of the *Wolbachia* clade (Hilgenboecker *et al.* 2008). *Wolbachia* is also present in parasitic filarial nematodes that are causative agents of river blindness and elephantiasis (Taylor & Hoerauf 1999; Fenn & Blaxter 2004).

In addition to its abundant presence in arthropods and nematodes, the increasing interest in *Wolbachia* is motivated by the diversity of its phenotypic effects. In arthropods, it can manipulate the host reproduction through male-killing (Jiggins *et al.* 2001), cytoplasmic incompatibility (Poinsot *et al.* 2003), parthenogenesis induction (Stouthamer *et al.* 1999) and feminization of genetic males (Hiroki *et al.* 2002; Vandekerckhove *et al.* 2003) with large impact on host ecology and evolution (Jiggins *et al.* 2000; Bordenstein *et al.* 2001; Charlat *et al.* 2003, 2007; Kremer *et al.* 2009). Additionally, *Wolbachia* is known both to play a protective role against some RNA viral infections in *Drosophila* (Browlie and Johnson, 2009) and to establish obligate associations, as seen in all infected nematodes (Fenn & Blaxter 2004; Fenn *et al.* 2006), and more rarely in arthropods (Dedeine *et al.* 2005; Hosokawa *et al.* 2010). This diversity of effects offers promising applications to the fields of pest and disease vector control (Kambris *et al.* 2009; Moreira *et al.* 2009).

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The *Wolbachia* genus encompasses a large phylogenetic diversity, with deeply diverging 'supergroups' and a root situated approximately 100 Ma (Bandi *et al.* 1998). These supergroups show an asymmetric distribution within the host landscape: supergroups A and B are commonly found in arthropods; supergroups C and D are restricted to filarial nematodes; supergroup E is exclusive to springtails; supergroup F is present both in arthropods and in nematodes; supergroup H is found in a single genus of termites; and supergroup K in one spider mite species. Other *Wolbachia* strains have been detected, and their clustering into supergroups is either controversial (supergroup G) or as yet unclear (Lo & Evans 2007; Ros *et al.* 2009).

Detection of *Wolbachia* has been traditionally performed using PCR assays, targeting the 16S rRNA gene or protein-coding genes such as *wsp* and *ftsZ*. The wide diversity and large impact of *Wolbachia* make it urgent to assess the efficiency of these detection protocols. Here, we use an extensive collection of insects of both known and unknown *Wolbachia* infection status to compare the sensitivity and range of several PCR primers: those commonly used as screening PCR primers, a new set of 16S primers and the primers integrated in the *Wolbachia* MLST (Multi Locus Sequencing Typing) system (Baldo *et al.* 2006).

The samples used include representatives from most of the known *Wolbachia* supergroups (A, B, C, D, F, H and K) as well as two new groups: M (Jack Werren, personal communication) and I (Haegeman *et al.* 2009). Additionally, we included arthropod specimens of unknown infection status, collected in the French Polynesia

between March 2005 and July 2007 (see Table S1 for further details on the samples used).

All tissue samples were conserved in 96% ethanol at −20 °C. DNA extraction was performed with the Nucleospin® Tissue kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany), according to the manufacturer's instructions, with elution in 150 μL of water. For small specimens (<5 mm long), extraction was performed on the entire body while abdominal tissue was used for larger specimens. Samples received from colleagues as dehydrated DNA were re-suspended at room temperature for 2–3 h in 90 μL of water. All samples were stored at −20 °C. The quality of the samples that failed to amplify *Wolbachia* DNA was assessed using the metazoan CO1 primers LCO-HCO (Folmer *et al.* 1994); samples that failed to amplify the CO1 locus were further tested using eukaryotic 18S and arthropod 18S primers (Medlin *et al.* 1988; Halanych *et al.* 2001; Duron *et al.* 2008).

Thirteen different *Wolbachia* PCR primers, targeting six loci, were evaluated in this study, hereafter denominated using the following short names: 16S-1 to 16S-6 (O'Neill *et al.* 1992; Werren & Windsor 2000; Casiraghi *et al.* 2001; Sakamoto *et al.* 2006; this study); wsp (Zhou *et al.* 1998), GatB, CoxA, HcpA, Fbp, FtsZ-1 (Baldo *et al.* 2006) and FtsZ-2 (Casiraghi *et al.* 2001). Primer sequences and expected amplicon size are provided in Table S2. PCR protocols (Table S3 and S4) followed recommendations from the original publications, except for the 16S-6 pair, designed in this study (see Data S1 for details on primer design). All reactions used EuroTaq® DNA polymerase (EUROBIO, Les Ulis, France) and took place in a Tetrad®Thermocycler (Bio-Rad, Hercules, CA, USA) with 1 μL of template. Fragments were separated by 1% agarose gel electrophoresis, stained with Ethidium Bromide and visualized under UV light.

We first performed a preliminary assessment of 12 existing pairs of primers using a set of 12 DNA extracts, including arthropods and nematodes and at least one representative of the majority of the known supergroups: A (2 samples), B (2 samples), C (1 sample), D (1 sample), F (3 samples), I (1 sample), H (1 sample) and M (1 sample) (Table S1). Our results show that the different primers can produce very distinct patterns (Table 1 and Fig. S1). The primers 16S-2, 16S-5, wsp, FtsZ-2 and the 5 MLST primers detected 10 or more infections of 12 in total. Discrimination among these pairs is mainly based on two samples, which harbour infections from supergroups F (*Mansonella pertans*) and I (*Rhadopholus similes*). Overall, only two pairs of primers (16S-2 and CoxA) produced identical results and only the FbpA primers detected the 12 infections, although one sample (F infection from *M. pertans*) produced a very faint band.

Next, we assessed the specificity (detection of *Wolbachia* infections only) of all the primer pairs that detected at least 10 of the 12 *Wolbachia* infections (Table 1 and Fig. S2). To this purpose, we used DNA templates from six close relatives of *Wolbachia*: *Rickettsia conovii*, *Rickettsia*

Table 1 Preliminary assessment of PCR protocols: primers are denominated using short names (see text)

	Host	Unknown arthropod						*Bm*	*Ov*	*Cp.*	*Mp.*	*Rm*	Zsp							
Primers	Supergroup	A	F	A	B	B	M	D	C	F	F	I	H	Number of positives	*Rc*	*Ap*	*Eca*	*Ech*	*Rt*	*Rb*
16S-1		2	0	0	3*	2	0	0	0	0	*	0	0	3	na	na	na	na	na	na
16S-2		3	3	3	3	3	3	3	1	3	0	0	3	9 + (1)	0	*	0	0	0	0
16S-3		0	*	0	1	3*	0	1	0	*	0	0	*	1 + (2)	na	na	na	na	na	na
16S-4		3*	2	3	3	2	3	3	*	2*	*	0	3*	9	na	na	na	na	na	na
16S-5		3	3	3	3	3	3	3	2	3*	*	2	3	11	*	2*	2*	*	*	*
16S-6		3	3	3	3	3	3	3	3	3	0	2*	3	11	0	0	0	0	0	0
FbpA		3	3	3	3	3	3	3	3	3	1*	3*	3	11 + (1)	1*	*	*	*	1*	1*
FtsZ-1		3	3	3	3*	3	3	3	3	3*	0	*	2*	10	1*	*	*	*	*	0
CoxA		3	3	3	3	3	3	2	3	3	0	0	3	10	*	*	*	0	1*	0
GatB		3	3	2*	3	3*	2	2	3	1*	*	2*	2	10 + (1)	3*	3*	3*	3*	3*	1*
HcpA		3	3	3	3	3	3	3*	3*	2*	1*	*	3	10 + (1)	*	*	2*	*	2*	*
wsp		3*	3*	3	3*	3*	2	2	3	1*	*	*	3*	9 + (1)	2*	1*	*	2*	*	0
FtsZ-2		3	3*	3	3	3*	3*	3*	3	3*	1*	*	3*	10 + (1)	1*	*	*	3*	*	*

Results are coded as follows: '0' = no amplification; '1' = very weak band of correct size; '2' = faint band of correct size; '3' = strong band of correct size.

*Amplification(s) of incorrect size (false bands and 'na' = not applicable. Abbreviations: *Bm*, *Brugia malayi*; *Ov*, *Onchocercus volvulus*; *Cp*, *Chorthippus paralelus*; *Mp*, *Mansonella pertans*; *Rs*, *Rhadopholus similes*; *Zsp*, *Zootermopsis* spp; Rc, *Rickettsia conovii*; *Ap*, *Anaplasma phagocytophylum*; Eca, *Ehrlichia cannis*; Ech, *Ehrlichia chaffensis*; Rt, R*ickettsia typhi* and Rb, *Rickettsia bellii*.

Table 2 Assessment of detection protocols on a large sample of known *Wolbachia* results is coded as in Table 1. All samples are from unknown arthropods unless otherwise stated. Abbreviations are as in Table 1 except Pch (ld): *Pityogenes chalcographus*

	Supergroup							
Protocol	A	B	B	F	F	A	A	A**
16S-2	3	3	3	3	0	3	3	3
16S-6	3	3	3	3	0	3	3	3
FbpA	3	3	3	3	1	3	3	3
	A	F**	A	B	F	B	A	B
16S-2	3	3	3	3	3	3	2	3
16S-6	3	3	3	3	3	3	3	3
FbpA	3	3	3	3	3	3	3	3
	B	A	A**	B**	A	F	A	B
16S-2	3	3	3	3	3	3	3	3
16S-6	3	3	3	3	3	3	3	3
FbpA	3	3	3	3	3	3	3	3
	B	B	B	F	B	B**	B	B
16S-2	3	3	3	3	3	3	3	3
16S-6	3	3	3	3	3	3	3	2
FbpA	3	3	3	2	3	3	3	3
	A	A	B	F	A	M**	B	F
16S-2	3	3	3	3	3	3	3	3
16S-6	3	3	3	3	3	3	3	3
FbpA	3	3	3	3	3	3	3	3
	D** (Bm)	D (Bm)	D (Bm)	D (Bm)	C** (Oc)	C (Oc)	C (Oc)	C (Oc)
16S-2	2	3	1	na	3	3	3	3
16S-6	3	3	3	na	3	0	0	0
FbpA	3	3	2	na	3	3	3	3
	F** (Cp)	F (Cp)	F (Cp)	F** (Mp)	A (Pch (ld))	A (Pch (ld))	A (Pch (ld))	A (Pch (ld))
16S-2	3	3	3	0	0	0	0	0
16S-6	3	3	*	0	0	0	0	0
FbpA	3	3	2	1*	*	0	2	0
	I** (Rs)	H** (Zsp)	H (Zsp)	K (Bsp)				
16S-2	0	3	3	3				
16S-6	1	3	2	3				
FbpA	3*	3	3	3				

typhi, *Anaplasma phagocytophilum*, *Ehrlichia canis*, *Ehrlichia chaffensis* and *Rickettsia bellii*. Only the 16S-2 pair exhibited complete specificity to *Wolbachia* infections.

Following the sensitivity and specificity tests, we decided to analyse more exhaustively the most satisfactory sets of primers only. Our selection was based on the

following criteria: (i) sensitivity (detection of the maximum diversity of *Wolbachia* strains) and (ii) specificity and, preferably, absence of false (wrong size) amplifications. Only the 16S-2 primers thus qualified for a more extensive test. However, this pair of primers does not detect all *Wolbachia* infections. We therefore designed new 16S primers (16S-6) in an attempt to improve the detection of *Wolbachia* without compromising specificity and tested them on the above-mentioned 12 *Wolbachia* and 6 non-*Wolbachia* templates (see Data S1 for details, Fig. S3, Table S5). This resulted in the amplification of 11 *Wolbachia* infections of 12 and no amplification of closely related groups (Table 1 and Fig. S4). Therefore, we tested the most promising pairs of primers, namely 16S-2 and 16S-6, on a more extensive set of samples (59 DNA extracts) of known infection status including a sample from supergroup K, more samples from the A, B, C, D, F and I supergroups (including isolates from different hosts) and four low-density infections (A supergroup). Notably, the large set of A and B infections include most of the known diversity within these groups based on a MLST characterization (Jack Werren, personal communication). The 16S-2 primers produced 15% (7 of 59) false negatives while the 16S-6 pair produced 17% (10 of 59) false negatives (contingency test, $P = 0.6$) (Tables 2 and S6). Both pairs seem equally sensitive to most supergroups, in particular the widespread A and B infections. The 16S-2 pair seems more efficient for the C group while only the 16S-6 pair amplifies the I group (albeit very weakly). The two pairs detected the K and H infections, but the 16S-2 primers produced stronger signals for the H group (Fig. S5).

This extensive test also included the FbpA primers, despite their lack of specificity, since they were the only ones detecting the 12 *Wolbachia* infections in the preliminary assessment (although one signal was very weak). They were the only pair to detect both an F infection (from an unknown Arthropod host) and a low-density A infection, producing 9% of false negatives. Overall, the false negative rate did not vary among the three pairs of primers tested (contingency test, $P = 0.12$) and none of the three pairs (16S-2, 16S-6 or FbpA) was able to detect all the infections in this extended test. As one cannot rule out the detection of non-*Wolbachia* strains using the FbpA primers, in particular the detection of *R. bellii*, the assessment of this primer pair was ended at this stage.

A final comparison between the 16S-2 and 16S-6 pairs was conducted with a set of 90 arthropods of unknown infection status. As expected from the previous tests, the results differed slightly among primer pairs (Table S7 and Fig. S6). Quantitatively, the 16S-6 pair detected 26 infections, three of which were exclusive to these primers; the 16S-2 pair detected 24 infections, of which a single amplification was exclusive to this pair. Overall, the two pairs of primers agreed in 23 of 27 (85%) positive samples. The 16S-2 pair produced, in general, stronger signals, and the detection rate did not differ significantly between the two pairs (contingency test, $P = 0.87$).

Standard PCR can be implemented on large samples and remains at present the most effective method to screen *Wolbachia* infections. Our results highlight one constraint associated with these methods, that is, the trade-off between sensitivity and specificity. We observed that two sets of 16S primers (including the ones designed in this study) amplify only *Wolbachia* infections. However, these showed some level of variation in terms of sensitivity. The FbpA primers appeared slightly more sensitive but lacked specificity. In an attempt to satisfy the sensitivity and specificity criteria in a single reaction, we evaluated the feasibility of a multiplex PCR (see Data S1 for details). Annealing temperature constraints suggested that the 16S-6 and FbpA pairs could be combined. However, we observed a reduced efficiency of each pair when used in combination.

Projects involving PCR followed by sequencing may give priority to sensitivity since non-*Wolbachia* DNAs will be identified at the sequencing stage. In addition, a protein-coding locus would be more informative than an rRNA locus if the sequences are to be used for typing or phylogenetic inference. The FbpA primers would then represent the best candidates. The additional cost incurred will depend on the actual incidence of close relatives to *Wolbachia*, in particular *R. bellii*. In contrast, PCR-based projects aimed at filtering *Wolbachia* infections only should give priority to specificity. The 16S-2 and 16-6 primers would then be most appropriate. Independent reactions involving these different primer pairs would ensure the lowest false positive and false negative rates.

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References

Baldo L, Hotopp Dunning JC, Jolley KA *et al.* (2006) Multilocus sequence typing system for the endosymbiont *Wolbachia* pipientis. *Applied and Environmental Microbiology*, **72**, 7098–7110.

Bandi C, Anderson JC, Genchi C, Blazter ML (1998) Phylogeny of Wolbachia in filarial nematodes. *Proceedings of the Royal Society of London Series B*, **265**, 2401–2413.

Bordenstein SR, O'Hara FP, Werren JH (2001) *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in Nasonia. *Nature*, **409**, 707–710.

Brownlie J, Johnson K (2009) Symbiont-mediated protection in insect hosts. *Trends in Microbiology*, **17**, 348–354.

Casiraghi M, Anderson TJ, Bandi C, Bazzocchi C, Genchi C (2001) A phylogenetic analysis of filarial nematodes: comparison with the phylogeny of Wolbachia endosymbionts. *Parasitology*, **122**, 93–103.

Charlat S, Hurst GDD, Mercot H (2003) Evolutionary consequences of *Wolbachia* infections. *Trends in Genetics*, **19**, 217–223.

Charlat S, Reuter M, Dyson EA *et al.* (2007) Male-killing bacteria trigger a cycle of increasing male fatigue and female promiscuity. *Current Biology*, **17**, 273–277.

Dedeine F, Bouletreau M, Vavre F (2005) *Wolbachia* requirement for oogenesis: occurrence within the genus Asobara (Hymenoptera, Braconidae) and evidence for intraspecific variation in A. tabida. *Heredity*, **95**, 394–400.

Duron O, Bouchon D, Boutin S *et al.* (2008) The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. *BMC Biology*, **6**, 27.

Fenn K, Blaxter M (2004) Are filarial nematode *Wolbachia* obligate mutualist symbionts? *Trends in Ecology & Evolution*, **19**, 163–166.

Fenn K, Conlon C, Jones M *et al.* (2006) Phylogenetic relationships of the *Wolbachia* of nematodes and arthropods. *PLoS Pathogens*, **2**, e94.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, **3**, 294–299.

Haegeman A, Vanholme B, Jacob J *et al.* (2009) An endosymbiotic bacterium in a plant-parasitic nematode: member of a new *Wolbachia* supergroup. *International Journal for Parasitology*, **39**, 1045–1054.

Halanych K, Feldman R, Vrijenhoek R (2001) Molecular evidence that *Sclerolinum brattstromi* is closely related to Vestimentiferans, not to Frenulate Pogonophorans (Siboglinidae, Annelida). *The Biological Bulletin*, **201**, 65–75.

Hilgenboecker K, Hammerstein P, Telschow A, Werren JH (2008) How many species are infected with *Wolbachia*? A statistical analysis of current data. *FEMS Microbiology Letters*, **281**, 215–220.

Hiroki M, Kato Y, Kamito T, Miura K (2002) Feminization of genetic males by a symbiotic bacterium in a butterfly, Eurema hecabe (Lepidoptera: Pieridae). *Naturwissenschaften*, **89**, 167–170.

Hosokawa T, Koga R, Kikuchi Y, Meng X-Y, Fukatsu T (2010) *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 769–774.

Jiggins FM, Hurst GDD, Majerus ME (2000) Sex-ratio-distorting *Wolbachia* causes sex-role reversal in its butterfly host'. *Proceedings of the Royal Society B Biological Sciences*, **267**, 69–73.

Jiggins FM, von Der Schulenburg JH, Hurst GDD, Majerus ME (2001) Recombination confounds interpretations of *Wolbachia* evolution. *Proceedings of Biological Sciences*, **268**, 1423–1427.

Kambris Z, Cook PE, Phuc HK, Sinkins SP (2009) Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. *Science*, **326**, 134–136.

Kremer N, Charif D, Henri H *et al.* (2009) A new case of *Wolbachia* dependence in the genus Asobara: evidence for parthenogenesis induction in Asobara japonica. *Heredity*, **103**, 248–256.

Lo N, Evans TA (2007) Phylogenetic diversity of the intracellular symbiont *Wolbachia* in termites. *Molecular Phylogenetics and Evolution*, **44**, 461–466.

Medlin L, Elwood H, Stickel S, Sogin M (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. *Gene*, **71**, 491–499.

Moreira LA, Iturbe-Ormaetxe I, Jeffery JA *et al.* (2009) A *Wolbachia* symbiont in Aedes aegypti limits infection with dengue, Chikungunya, and Plasmodium. *Cell*, **139**, 1268–1278.

O'Neill SL, Giordano R, Colbert AM, Karr TL, Robertson HM (1992) 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proceedings of the National Academy of Sciences*, **89**, 2699–2702.

Poinsot D, Charlat S, Merçot H (2003) On the mechanism of *Wolbachia*-induced cytoplasmic incompatibility: confronting the models with the facts. *Bioessays*, **25**, 259–265.

Ros VID, Fleming VM, Feil EJ, Breeuwer JAJ (2009) How diverse is the genus *Wolbachia*? Multiple-gene sequencing reveals a putatively new Wolbachia supergroup recovered from spider mites (Acari: Tetranychidae). *Applied and Environmental Microbiology*, **75**, 1036–1043.

Sakamoto JM, Feinstein J, Rasgon JL (2006) Wolbachia infections in the Cimicidae: museum specimens as an untapped resource for endosymbiont surveys. *Applied and Environmental Microbiology*, **72**, 3161–3167.

Stouthamer R, Breeuwer JA, Hurst GDD (1999) *Wolbachia* pipientis: microbial manipulator of arthropod reproduction. *Annual Review of Microbiology*, **53**, 71–102.

Taylor MJ, Hoerauf A (1999) *Wolbachia* bacteria of filarial nematodes. *Parasitology Today*, **15**, 437–442.

Vandekerckhove T, Watteyne S, Bonne W (2003) Evolutionary trends in feminization and intersexuality in woodlice (Crustacea, Isopoda) infected with Wolbachia pipientis (alpha-Proteobacteria). *Belgian Journal of Zoology*, **133**, 61–69.

Werren JH, Windsor DM (2000) *Wolbachia* infection frequencies in insects: evidence of a global equilibrium?. *Proceedings of Biological Sciences*, **267**, 1277–1285.

Werren JH, Baldo L, Clark ME (2008) *Wolbachia*: master manipulators of invertebrate biology. *Nature Reviews Microbiology*, **6**, 741–751.

Zhou W, Rousset F, O'Neil S (1998) Phylogeny and PCR-based classification of Wolbachia strains using wsp gene sequences. *Proceedings Biological Sciences*, **265**, 509–515.

Supporting Information

Additional supporting information may be found in the online version of this article.

Data S1 Design of new primers.

Fig. S1 Preliminary assessment of existing *Wolbachia* detection protocols.

Fig. S2 Assessment of the specificity of existing protocols.

Fig. S3 The figure shows the 16S primers aligned with the following sequences.

Fig. S4 Preliminary assessment of a new *Wolbachia* detection protocol (16S-6 primers).

Fig. S5 Assessment of detection protocols on a large sample of *Wolbachia*-infected hosts.

Fig. S6 Blind test on a random sample of arthropods.

Table S1 Tissue and DNA material used.

Table S2 Primers assessed in the present study.

Table S3 PCR mix concentrations.

Table S4 PCR programs.

Table S5 The 11 *Wolbachia* sequences chosen as representatives of the known supergroups.

Table S6 Comparison among 16S-6, 16S-2 and FbpA.

Table S7 DNA material used in the blind test on a random sample of arthropods.

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