Insect Molecular Biology (2000) **9**(4), 393–405

Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species

A. Jeyaprakash and M. A. Hoy

Department of Entomology and Nematology, University of Florida, Gainesville, Florida, USA

Abstract

Bacteria belonging to the genus *Wolbachia* are associated with a variety of reproductive anomalies in arthropods. Allele-specific polymerase chain reaction (= Standard PCR) routinely has been used to amplify *Wolbachia* DNA from arthropods. While testing the two-spotted spider mite *Tetranychus urticae* and other arthropods known to be infected with *Wolbachia*, Standard PCR frequently produced false negatives, perhaps because the DNA from the arthropod host interfered with amplification by *Taq* DNA polymerase. Long PCR, which uses two enzymes (*Taq* and *Pwo*), consistently amplified *Wolbachia* DNA and a sensitivity analysis indicated that Long PCR was approximately six orders of magnitude more sensitive than Standard PCR in amplifying plasmid DNA spiked into insect genomic DNA. A survey indicated that 76% of sixty-two arthropod species and two subspecies in thirteen orders tested positive for the *Wolbachia wsp* sequence by Long PCR, which is considerably higher than the rate of 16.9% obtained previously for the *ftsZ* sequence using Standard PCR (Werren, J.H., Windsor, D. and Gao, L. (1995a) *Proc R Soc Lond B* 262: 197–204). A subsample of Long PCR products from fourteen arthropod species and two subspecies were sequenced, both directly and after cloning. Two A- and eleven B-*Wolbachia* strains were detected and their *wsp* sequences displayed a maximum of 23.7% sequence divergence at this locus. Two new groups (named Fus and Ten) were identified in addition to nineteen reported earlier (Zhou, W., Rousset, F. and O'Neill, S.L. (1998) *Proc R Soc Lond B* 265: 1–7; van Meer, M.M.M., Witteveldt, J. and Stouthamer, R. (1999) *Insect Mol Biol* 8: 399–408), because they displayed more than 2.5% sequence divergence from other *Wolbachia wsp* sequences. PCR products from seventeen of twenty-nine (59%) arthropod species analysed could not be sequenced directly due to apparent infection by multiple *Wolbachia* strains. The *wsp* sequences cloned from two such species (*Plutella xylostella* and *Trichoplusia ni*) indicated both A- and B-*Wolbachia* were present in a single individual. Hence, superinfection also may be more widespread than the 1.2% incidence previously estimated.

Keywords: long PCR, *Wolbachia*, *wsp*, arthropod, phylogeny, distribution.

Introduction

Wolbachia, rickettsia-like proteobacteria, are found in the reproductive tissues (ovaries and testes) of a wide range of arthropod species (O'Neill *et al.*, 1997; Werren, 1997). *Wolbachia* are obligate intracellular parasites and transmitted maternally from infected females to their progeny. *Wolbachia* infection is associated with a variety of reproductive anomalies in the host, including cytoplasmic incompatibility between different populations (Laven, 1951, 1967) or closely related species (Breeuwer & Werren, 1990; Breeuwer *et al.*, 1992), thelytoky or parthenogenesis induction in parasitoid wasps (Stouthamer *et al.*, 1990, 1993), male-killing in coccinellid beetles (Hurst *et al.*, 1999), and feminization of genetic males in isopods (Rousset *et al.*, 1992).

Wolbachia cannot be cultured in defined media and detection within infected gonadal cells may be time consuming. Therefore, detection of *Wolbachia* infection has been based largely on amplification of *Wolbachia* DNA using allele-specific polymerase chain reactions (= Standard PCR). Primers designed from the 16S rDNA sequence initially were used to amplify *Wolbachia* DNA from a diverse array of arthropods (O'Neill *et al.*, 1992; Stouthamer *et al.*, 1993). A phylogenetic analysis of the 16S rDNA sequences indicated a 2% sequence divergence separated the *Wolbachia* into two distinct groups, which were placed in two subdivisions (A and B). Other protein coding genes

Received 2 February 2000; accepted following revision 7 April 2000. Correspondence: Dr Ayyamperumal Jeyaprakash, Department of Entomology and Nematology, PO Box 110620, University of Florida, Gainesville, FL 32611, USA. Tel.: (352) 392 1901 ext. 170; fax: (352) 392 0190; e-mail: ajey@gnv.ifas.ufl.edu

from *Wolbachia* were sequenced subsequently, including the *ftsZ* which is involved in cell division (Holden *et al*., 1993), the *dnaA* gene which is essential for DNA replication initiation (Bourtzis *et al*., 1994), the *wsp* gene which encodes a major cell surface coat protein (Braig *et al*., 1998), and the *groE* operon which encodes two heat shock proteins (Masui *et al*., 1997). A phylogenetic analysis of *Wolbachia ftsZ* gene sequences from twenty-six different neotropical arthropod species detected a 13–16% sequence divergence between the A- and B-*Wolbachia* (Werren *et al*., 1995b). Recently, the *Wolbachia wsp* gene was amplified from twenty-six insect and one isopod species by the Standard PCR. The *wsp* sequences displayed up to 23% DNA sequence divergence (Zhou *et al*., 1998; van Meer *et al*., 1999). Zhou *et al*. (1998) proposed to subdivide the A- and B-*Wolbachia* into smaller groups based on a sequence divergence of 2.5% of the *wsp* sequences. The new group name was based on the first three letters of the first arthropod species from which the *Wolbachia* strain was identified. As a result, the A-*Wolbachia* were subdivided into Mel, AlbA, Mors, Kue, Uni, Riv, Dro, Haw, Pap and Aus groups, while the B-*Wolbachia* were subdivided into Con, Dei, Sib, Kay, Div, For, Ori, Pip and Vul groups, respectively (van Meer *et al*., 1999; Zhou *et al*., 1998). To date, *Wolbachia* have been detected from insects (O'Neill *et al*., 1992; Werren *et al*., 1995a,b), crustaceans (Bouchon *et al*., 1998), mites (Johanowicz & Hoy, 1996) and nematodes (Sironi *et al*., 1995) by the Standard PCR.

When we tried to use *ftsZ* and *wsp* primers to amplify *Wolbachia* DNA by the Standard PCR from a population of the two-spotted spider mite, *Tetranychus urticae*, which was known to be infected with *Wolbachia* (Johanowicz & Hoy, 1996), the assay failed frequently when using single individuals. In addition, while attempting to amplify the greening bacterium *Liberobacter asiaticum* DNA by Standard PCR from infected citrus leaves, similar false negative results were produced, prompting us to evaluate a different PCR-based procedure, called Long PCR. Long PCR amplified the *L. asiaticum* DNA consistently without false negatives (Hoy *et al*., 1999). The purpose of this paper was to evaluate Long PCR as a technique for amplifying *Wolbachia* DNA from a diverse array of arthropods and to compare the relative efficiency of the Long and Standard PCR protocols.

Results

Wolbachia *DNA amplification by Standard PCR*

Initially, genomic DNA preparations made from individual *Tetranychus urticae*, obtained from a population known to be infected with *Wolbachia* (Johanowicz & Hoy, 1996), were amplified by the Standard PCR using *ftsZ*- or *wsp*-specific primers. A total of 200 individual *T. urticae* were screened on ten different occasions. False negatives were obtained on all occasions, although a faint DNA band of the expected 0.6 kb size was obtained from three of the 200 individual mites for the *ftsZ* gene and from one of the 200 individual mites for the *wsp* gene. The 0.6 kb *ftsZ* PCR products obtained from *T. urticae* were reamplified, cloned into the pTZ19R *Sma*I site by a T/A cloning strategy (pAJ69 and pAJ70) (Mead *et al*., 1991). The 570-bp *ftsZ* sequences obtained shared 99% identities with the B-*Wolbachia* sequences from the lepidoptera *Ephestia cautella*, *Phyllonorycter quinnata* and *P. froelichiella* (Werren *et al*., 1995b). Similarly, the 0.6 kb *wsp* PCR product from one *T. urticae* was cloned (pAJ85 and pAJ86) (Table 1) and found to share 99.2% identity with the *Wolbachia w*CauB sequence from the lepidopteran *E. cautella* (Zhou *et al*., 1998).

An attempt to amplify *Wolbachia* DNA from the Caribbean fruit fly *Anastrepha suspensa*, which is known to be infected with *Wolbachia* (Werren *et al*., 1995b), also yielded frequent false negatives and failed to produce the expected *ftsZ* or *wsp* PCR products (A. Jeyaprakash and M. A. Hoy, unpublished data). We hypothesized that the arthropod DNA present in the mixture might interfere with *Taq* DNA polymerase activity, creating false negatives.

Wolbachia *DNA amplification by Long PCR*

Genomic DNAs prepared from six different arthropod species, including the brown citrus aphid *Toxoptera citricida*, the citrus leafminer *Phyllocnistis citrella*, the Caribbean fruit fly *Anastrepha suspensa*, the psyllid *Diaphorina citri*, the eulophid parasitoid *Tamarixia radiata*, and the encyrtid parasitoid *Diaphorencyrtus aligarhensis*, were amplified by both Standard and Long PCR, using both the *ftsZ* and *wsp* primer pairs. A bright 0.6 kb DNA band was amplified consistently by Long PCR from *T. citricida*, *A. suspensa, P. citrella* and *D. aligarhensis* using both primer sets, but the Standard PCR failed in all reactions (Fig. 1A and B). The *T. radiata* DNA preparation was negative by both PCR protocols, which could mean either that the insect was free of *Wolbachia* or that the primers did not match the *wsp* sequence of any *Wolbachia* present. Contamination was not found in the no-DNA water controls and no distinct DNA bands were produced except for some primer-dimer artifacts.

Sensitivity analysis to compare the Standard and Long PCR procedures

In order to compare directly the efficiency of the Standard and Long PCR procedures, a sensitivity analysis was performed by mixing *T. radiata* DNA (10 ng) with a known amount of serially diluted plasmid pAJ85 DNA (100 ng to 1 fg) carrying the cloned 596 bp *T. urticae wsp* gene sequence. When the plasmid DNA alone was used as the DNA template, Standard PCR successfully amplified seven different plasmid dilutions, ranging from 100 ng to 100 fg, indicating that the primers and *Taq* DNA polymerase

Table 1. Arthropods tested for *Wolbachia* using Long PCR Assay with *wsp* primers

Taxonomic category*	Laboratory (L) or field (F)	Long PCR	DNA sequence or Southern blot analysis§	*Wolbachia* Super-Group	GenBank accession
Phylum Arthropoda					
Subphylum Chelicerata					
Class Arachnida					
Order Prostigmata					
Family Tetranychidae					
Tetranychus urticae (Koch)	L	+	DS, CS	B	AF217719
Order Mesostigmata					
Family Phytoseiidae					
Metaseiulus occidentalis (Nesbitt)	L	–			
Subphylum Mandibulata					
Class Insecta					
Order Thysanura					
Lepisma saccharina (Linnaeus)	F	+	DS	B	
Order Odonata					
Family Libellulidae					
Perithemis tenera (Say)	F	+	DS	B	AF217725
Order Blattodea					
Periplaneta fuliginosa (Serville)	F	–			
Pycnoscelus surinamensis (Linnaeus)	F	–			
Order Isoptera					
Family Rhinotermitidae					
Reticulitermis flavipes (Kollar)	F	+	DU, SB		
Order Orthoptera					
Family Acrididae					
Schistocerca americana (Drury)	L	+	DU, SB		
Family Gryllotalpidae					
Scapteriscus abreviatus (Scudder)	L	–			
Family Tettigoniidae					
Atlanticus gibbosus (Scudder)	F	–			
Order Dermaptera					
Euborellia annulipes (Lucas)	F	+	DU, SB		
Order Homoptera					
Family Aleyrodidae					
Parabemisia myricae (Kuwana)	L	+	DU, SB		
Bemisia tabaci (Gennadius)	L	+	DS	B	AF217717
Family Aphididae					
Toxoptera citricida (Kirkaldy)	L	+	DS	A	
Aphis craccivora (Koch)	L	+	DU, SB		
Family Pseudococcidae					
Planococcus citri (Risso)	L	–			
Ferrisia virgata (Cockerell)	L	+	DU, SB		
Family Psyllidae					
Diaphorina citri (Kuwayama)	L	+	DS	B	AF217721
Order Thysanoptera					
Frankliniella fusca (Hinds)	F	+	DU, SB		
Frankliniella occidentalis (Pergande)	F	–			
Order Neuroptera					
Myrmeleon spp.	F	–			
Order Coleoptera					
Family Curculionidae					
Diaprepes abbreviatus (Linnaeus)	L	+	DU, SB		
Family Coccinellidae					
Coleomegilla maculata lengi (de Geer)	L	+	DS	B	AF217720
Coleomegilla maculata fuscilabris	L	+	DS	B	AF217724
Cryptolaemus montrouzieri (Muslant)	L	–			
Family Curculionidae					
Pachnaeus litus (Germar)	F	+	DU, SB		
Anthonomus eugenii (Cano)	L	+	SB		
Family Lampyridae					
Photinus collustrans (LeConte)¶	F	+	DU, SB		
Photinus tanytoxus (Lloyd)¶	F	+	SB		
Order Siphonaptera					
Family Pulicidae					
Ctenocephalides felis (Bouche)	F	+	DU, SB		

Table 1. (*continued*)

Taxonomic category*	Laboratory (L) or field (F)	Long PCR	DNA sequence or Southern blot analysis§	*Wolbachia* Super-Group	GenBank accession
Order Diptera					
Family Tephritidae					
Anastrepha suspensa (Loew)	L	+	DS	A	AF217713
Family Drosophilidae					
Drosophila melanogaster (Meigen)	L	+	SB		
Family Culicidae					
Aedes aegypti (Linnaeus)	L	–			
Family Muscidae					
Haemotobia irritans (Linnaeus)	L	+	DS	A	AF217714
Musca domestica (Linnaeus)	L	–			
Family Ephyderidae					
Hydrellia pakistanae (Deonier)	L	+	DS	B	AF217718
Order Lepidoptera					
Family Gracillariidae					
Phyllocnistis citrella (Stainton)	L	+	DS	A	
Family Plutellidae					
Plutella xylostella (Linnaeus)	L	+	CS	A & B	AF217723
Family Noctuidae					
Trichoplusia ni (Hubner)	L	+	CS	A & B	AF217716
Family Pyralidae					
Galleria mellonella (Linnaeus)	L	+	DU, SB		
Plodia interpunctella (Hubner)	L	+	DU, SB		
Family Papilionidae					
Papilio glaucus australis (Linnaeus)	F	–			
Papilio troilus ilioneus (Linnaeus)	F	+	SB		
Order Hymenoptera					
Family Aphelinidae					
Eretmocerus spp.	L	+	SB		
Family Braconidae					
Diachasmimorpha longicaudata (Ashmead)	L	–			
Meteorus autographae (Muesebeck)	L	+	SB		
Family Diapriidae					
Trichopria columbiana (Ashmead)	L	+	SB		
Family Eulophidae					
Tamarixia radiata Waterston	L	–			
Quadrastichus haitiensis (Gahan)	L	+	SB		
Family Encyrtidae					
Diaphorencyrtus aligarhensis (Shafee, Alam & Agarwal)	L	+	DS	B	AF217715
Ageniaspis citricola (Logvinovskaya)	L	+	SB		
Family Formicidae					
Paratrechina longicornis (Latreille)	L	+	SB		
Pheidole dentata (Mayr)	L	+	SB		
Linepithema humilie (Mayr)	L	+	SB		
Camponotus floridanus (Buckley)	L	+	SB		
Tapinoma melanocephalum (Fabricius)	L	+	SB		
Monomorium floricola (Jerdon)	L	+	SB		
Monomorium pharaonis (Linnaeus)	L	+	SB		
Solenopsis invicta (Buren)	L	+	CS	B	AF217722
Technomyrmex albipes (Smith)	L	+	SB		
Wasmannia auropunctata (Roger)	L	+	SB		
Family Pteromalidae					
Catolaccus hunteri (Crawford)	F	+	SB		
Family Trichogrammidae					
Ceratogramma etiennei (Delvare	L	–			
Family Ichneumonidae					
Diadegma insulare (Cresson)	L	+	DU, SB		

*Based on classification used in Common Names of Insects & Related Organisms 1997, Entomological Society of America. §DS = Direct sequencing of Long PCR products was successful; DU = Direct sequencing of Long PCR products unsuccessful because overlapping banding patterns were produced, perhaps due to multiple infections; CS = Cloned DNA was used for sequencing; SB = Positive by Southern blot analysis of Long PCR products. ¶Five Individuals were screened by Long PCR. For all other species, only one individual was screened by Long PCR.

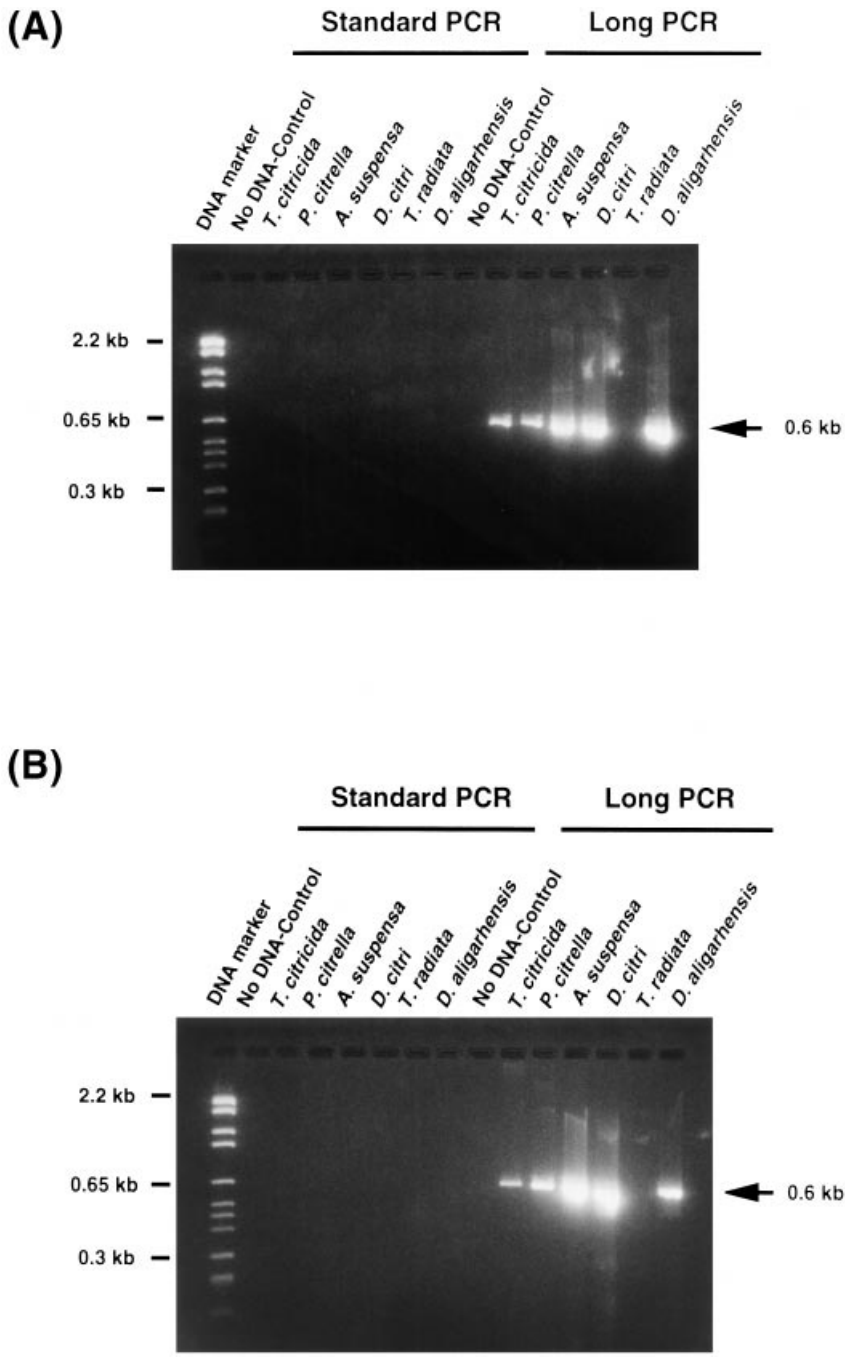

Figure 1. Long PCR successfully amplified (A) *ftsZ* and (B) *wsp* sequences from five of six arthropod species; the same DNA extracts were all negative for *Wolbachia* by Standard allele-specific PCR.

were functioning as expected (Fig. 2A). By contrast, in the presence of *T. radiata* genomic DNA (10 ng), only three plasmid DNA dilutions (from 100 ng to 1 ng) could be amplified by the Standard PCR, indicating that insect genomic DNA reduced the sensitivity of the assay by four orders of magnitude (Fig. 2B). When identical insect + plasmid template mixes were amplified by Long PCR, all plasmid dilutions (from 100 ng to as low as 1 fg) produced a bright 0.6 kb DNA band, indicating that the Long PCR assay was at least six orders of magnitude more efficient in amplifying the target *wsp* sequence (Fig. 2C).

A survey of arthropods by Long PCR for Wolbachia *infection*

The apparent increase in sensitivity obtained when using the Long PCR protocol suggested it would be interesting to screen additional arthropod species in order to estimate the proportion infected with *Wolbachia*. Genomic DNA

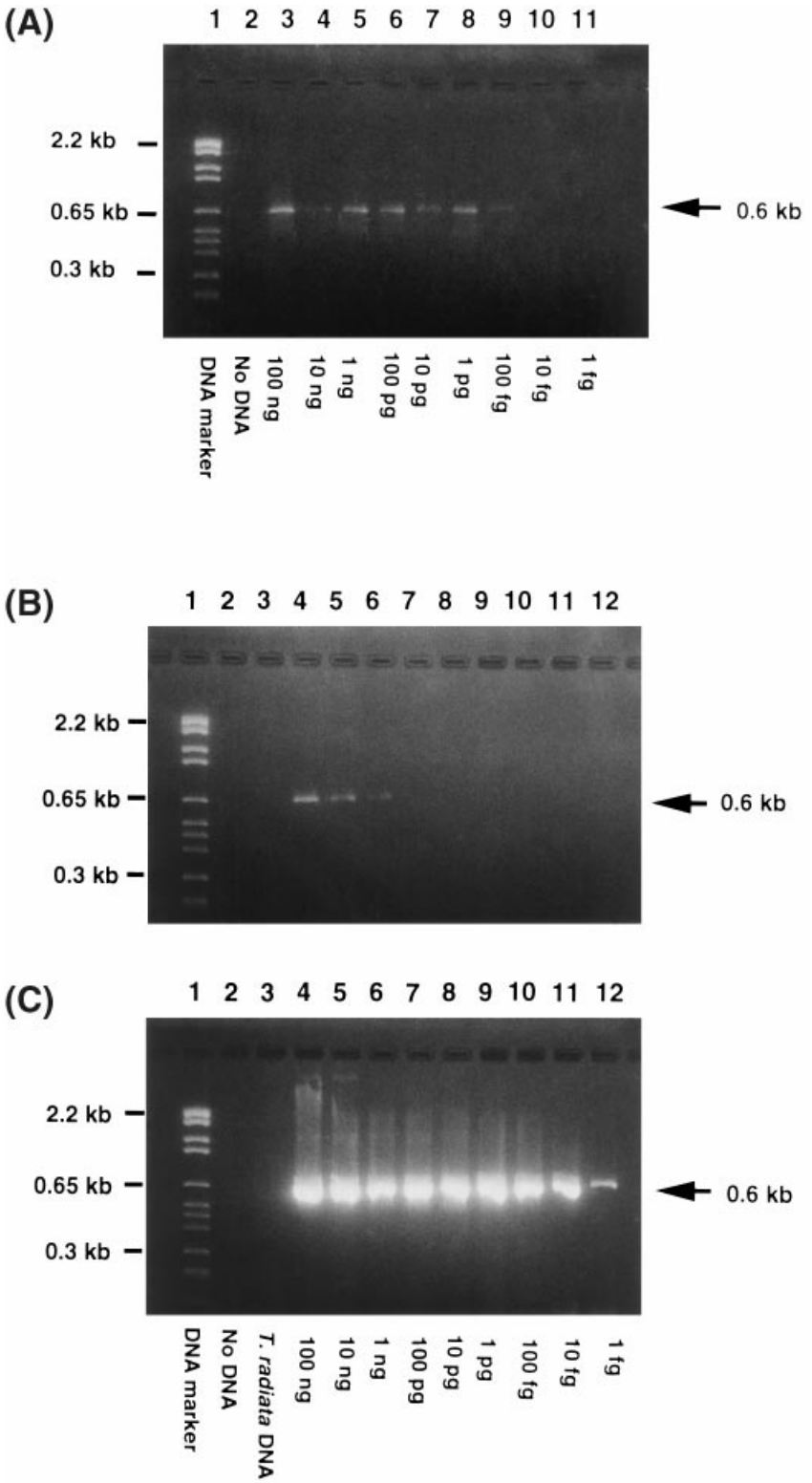

Figure 2. Sensitivity analysis of Standard and Long PCR protocols using serially diluted plasmid pAJ85 DNA and *wsp* primers. (A) standard PCR + plasmid alone; lane (1) DNA marker VI, (2) no DNA-control, and (3–11) plasmid DNA amount used are shown below the lanes. (B) standard PCR + plasmid + *T. radiata* DNA. (C) Long PCR + plasmid + *T. radiata* DNA; lane (1) DNA marker VI, (2) no DNA-control, (3) *T. radiata* DNA-control, and (4–12) plasmid DNA amount added to *T. radiata* DNA are shown below the lanes.

preparations made from single individuals of both field-collected and laboratory-maintained arthropod species were amplified by the Long PCR using *Wolbachia wsp* primers because this locus is variable and useful for typing *Wolbachia* strains (Zhou *et al*., 1998). Of the sixty-two species and two subspecies belonging to thirteen Orders of arthropods in two Subphyla (Chelicerata and Mandibulata) tested by Long PCR, forty-seven species and two subspecies

(76%) were positive (Table 1). Of the forty-six laboratory colonies tested, thirty-seven were positive (80%), while eleven of the seventeen field-collected species were positive (65%). The high levels of *Wolbachia* infection among both laboratory and field populations of these diverse arthropods suggest that *Wolbachia* is substantially more common than previously estimated (Werren *et al*., 1995a). A single individual had been used from each species to obtain a positive result, with the exception of the two firefly species (*Photinus collustrans* and *P. tanytoxus*) where only one of five individuals screened were positive.

To confirm that the PCR products obtained were not due to contamination, an attempt was made to sequence directly the Long PCR products from twenty-eight different arthropod species and two subspecies (Table 1). Long PCR products from only eleven of the twenty-eight species and two subspecies could be sequenced directly, because seventeen produced overlapping banding patterns, indicating they might be infected with two or more *Wolbachia* strains. To determine if this hypothesis could be confirmed, Long PCR products from three of the seventeen species (*Trichoplusia ni*, *Plutella xylostella* and *Solenopsis invicta*) were cloned into plasmid pCR2.1-TOPO and several independent clones were sequenced. The sequences indicated both A- and B-*Wolbachia* were present in *T. ni* and *P. xylostella* (Table 1). Because a single *T. ni* and *P. xylostella* individual had been used for Long PCR, the double infections observed should be considered real. In *S. invicta*, only a single (B) strain could be cloned, because the majority of the 100 clones analysed contained a *wsp* DNA fragment of < 0.1 kb.

Phylogenetic analysis of the Wolbachia wsp *sequences*

The eighteen *Wolbachia wsp* sequences obtained from fourteen different arthropod species and two subspecies were compared and thirteen unique sequences were found (Table 1). A Blast search of GenBank was performed using the thirteen unique *Wolbachia* sequences. Sequences belonging to twenty-eight other *Wolbachia* strains were extracted from GenBank, including ten A- and nine B-*Wolbachia* reference strains (a strain used to identify the group), plus one A- and eight B-closely related *Wolbachia* strains. Phylogenetic analyses were conducted to establish the relationships between the strains and place them in groups by following the established convention of deleting a third hypervariable region (41 bp) from the *wsp* gene and placing sequences in different groups if sequences diverged more than 2.5% (Zhou *et al*., 1998). Separate DNA and protein alignments were performed for all forty-one *Wolbachia wsp* sequences by CLUSTAL W (Thompson *et al*., 1994). The DNA alignment was manually adjusted using the position of amino acids to generate a data set consisting of 544 aligned characters (data not shown). The data were transported into PAUP 4.0b2 to generate a midpoint rooted Neighbour-joining tree with the optimality criterion set for Distance and Kimura 2-parameter (Fig. 3). Bootstrap analysis detected values above 50% for a majority of the *Wolbachia* lineages, providing strong support for this Neighbour-joining tree (Fig. 3). An overall sequence divergence of 25.6% was found in the *wsp* locus between the thirteen A- and twenty-eight B-*Wolbachia* strains included in this analysis. Sequence divergence was 17.5% for A strains and 23.3% for B strains.

Five A-*Wolbachia* sequences, amplified from *Anastrepha suspensa*, *Toxoptera citricida*, *Phyllocnistis citrella*, *Plutella xylostella* and *Trichoplusia ni*, shared 100% sequence identities (*w*Sus-A1) and exhibited 0.6% sequence divergence from the A-*Wolbachia* reference strain of *D. melanogaster* (Zhou *et al*., 1998), which allowed them to be placed in the Mel group. One *Wolbachia* sequence (*w*Irr-A1), obtained from *Haematobia irritans*, displayed 2% sequence divergence from the A-*Wolbachia* reference strain of *Glossina morsitans morsitans* (Zhou *et al*., 1998) and another *Wolbachia* strain from *G. brevipalpis* (accession number AF164685) belonging to the Mors group; it was therefore placed in the Mors group (Fig. 3).

Two B-*Wolbachia wsp* sequences amplified from the ladybird beetle *Coleomegilla maculata fuscilabris* and the silverfish *Lepisma saccharina* shared 100% sequence identity (*w*Fus-B1), but displayed 15.1% sequence divergence from all other B-*Wolbachia* reference strains (Zhou *et al*., 1998; van Meer *et al*., 1999) and were placed in a new Fus group (Fig. 3). *Wolbachia wsp* sequences obtained from the encyrtid parasitoid *Diaphorencyrtus aligarhensis*, the cabbage looper *Trichoplusia ni*, the whitefly *Bemisia tabaci*, and the dipteran *Hydrellia pakistanae* were all unique and found to be most closely related to the Pip group because they displayed less than 1.5% sequence divergence from the B-*Wolbachia* reference strain of *Culex pipiens* (Zhou *et al*., 1998). The new strains were named *w*Ali-B1, *w*Ni-B1, *w*Tab-B1 and *w*Pak-B1, respectively. One *Wolbachia wsp* sequence from the two-spotted spider mite (*T. urticae*) was unique (*w*Urt-B1) and displayed 1.7% sequence divergence from the B-*Wolbachia* reference strain of *Tagosedes orizicolus* (van Meer *et al*., 1999), and was placed in the Ori group (Fig. 3). Four other *Wolbachia wsp* sequences, from the ladybird beetle *C. maculata lengi*, the psyllid *Diaphorina citri*, the fire ant *Solenopsis invicta* and the diamondback moth *Plutella xylostella*, also were unique and named *w*Len-B1, *w*Cit-B1, *w*Inv-B1 and *w*Xyl-B1, respectively (Fig. 3). These were placed in the Con group because they all displayed less than 2.5% sequence divergence from the Con group B-*Wolbachia* reference strain of *Tribolium confusum*. Finally, a *wsp* sequence from the dragonfly *Pherithemis tenera* was unique (*w*Ten-B1) displaying 10.9% sequence divergence from all other B-*Wolbachia* reference strains. It could be placed in a new Ten group (Fig. 3).

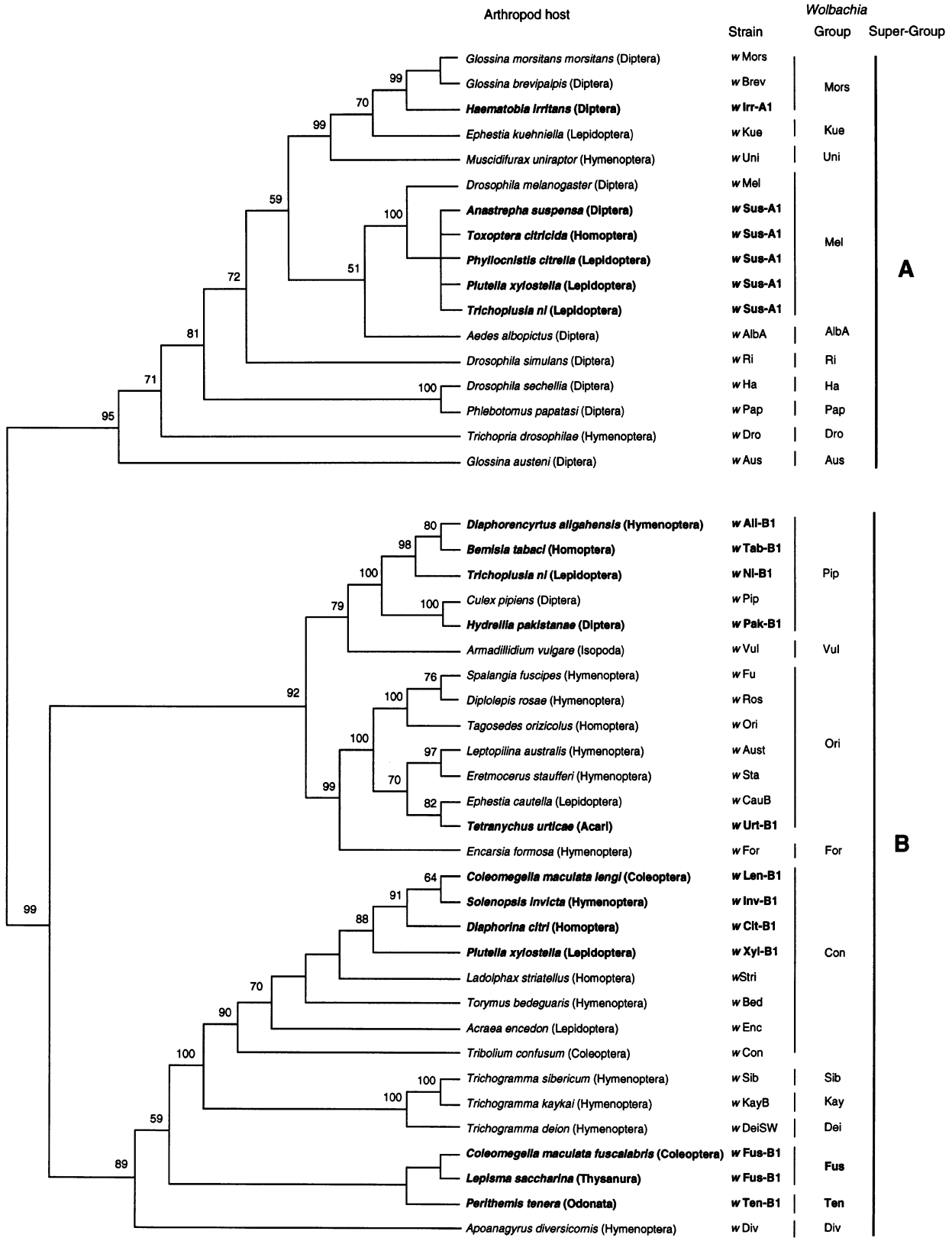

Figure 3. A midpoint rooted Neighbour-joining tree was generated for all forty-one different *Wolbachia* strains using a CLUSTAL W alignment after excluding the 41-bp third hypervariable region of *wsp* gene with optimality criterion set for Distance and Kimura 2-parameter in PAUP 4.0b2. Bootstrap values detected above 50% for 100 replicates are given before the branch. New *Wolbachia* strains and groups are shown in bold.

Additional phylogenetic analysis also was performed to determine whether including the 41 bp third hypervariable region of the *wsp* gene could improve the tree topology. A CLUSTAL W alignment was made after including the third hypervariable region in the data set, adjusted using the protein alignment and a total of 623 aligned characters were transported into PAUP 4.0b2. A midpoint rooted Maximum likelihood tree was generated using the Substitution model assuming the number of substitution types as 2, all sites evolving at the same rate and transition/transversion ratio set for 2 (Fig. 4). The branching pattern of Maximum likelihood tree (Fig. 4) was similar to the Neighbour-joining tree (Fig. 3), but the *w*Fus, *w*Ten and *w*Div strains clustered together with a high bootstrap value support of 61% for the *w*Fus-*w*Ten/*w*Div branch and 74% for *w*Ten-*w*Div branch. Thus, it appears that including the 41 bp third hypervarible region for phylogenetic analysis did improve the tree topology and could be used in the future to establish correct relationships between the *Wolbachia* strains when additional unique *wsp* sequences become available from other arthropod species.

Detection of *Wolbachia wsp* sequences with 100% sequence identities on two occasions from divergent arthropod species by Long PCR (Fig. 3) could be due to horizontal transfer, but also could be attributed to aerosol contamination by *wsp* PCR products. In order to resolve this issue, the Long PCR was used to amplify *ftsZ* sequences from *T. citricida, P. citrella*, *A. suspensa*, *D. citri* and *D. aligarhensis* (Fig. 1A) and these *ftsZ* products were sequenced directly and compared. The *ftsZ* sequences obtained from *T. citricida, P. citrella* and *A. suspensa* (510 bp) were also 100% identical to each other and shared 99.8% identity with A-*Wolbachia* sequences from *Aphytis lingnanensis*, *A. chrysomphali*, *Nasonia giraulti*, *A. suspensa*, *Drosophila recens*, *D. simulans*, *D. sechellia* and *Trichogramma bourarachae* in GenBank (accession number Y13281, Y13666, U228174, U28179, U28182, U28184, U28185 and AF062593). The *ftsZ* sequences amplified simultaneously from *D. citri* and *D. aligarhensis* by Long PCR (Fig. 1A) were different, and the *D. aligarhensis ftsZ* sequence (510 bp) shared 100% identity with a male-killing B-*Wolbachia* from *Acraea encedon* in GenBank (accession number AJ130892). No amplification occurred in the no DNA-water control (Fig. 1A), thus there seems to be no evidence for aerosol contamination by PCR products. Identical *ftsZ* and *wsp* sequences obtained from three divergent arthropod species (*T. citricida*, *P. citrella* and *A. suspensa*) thus indicates possible horizontal transfer of *Wolbachia.*

Finally, no attempt was made to clone or sequence Long PCR products from several arthropod species (Table 1). However, high stringency Southern blot analyses conducted have confirmed that these Long PCR products were actually *Wolbachia wsp* sequences (Blot not shown here) and not a PCR-generated artifact or false positives.

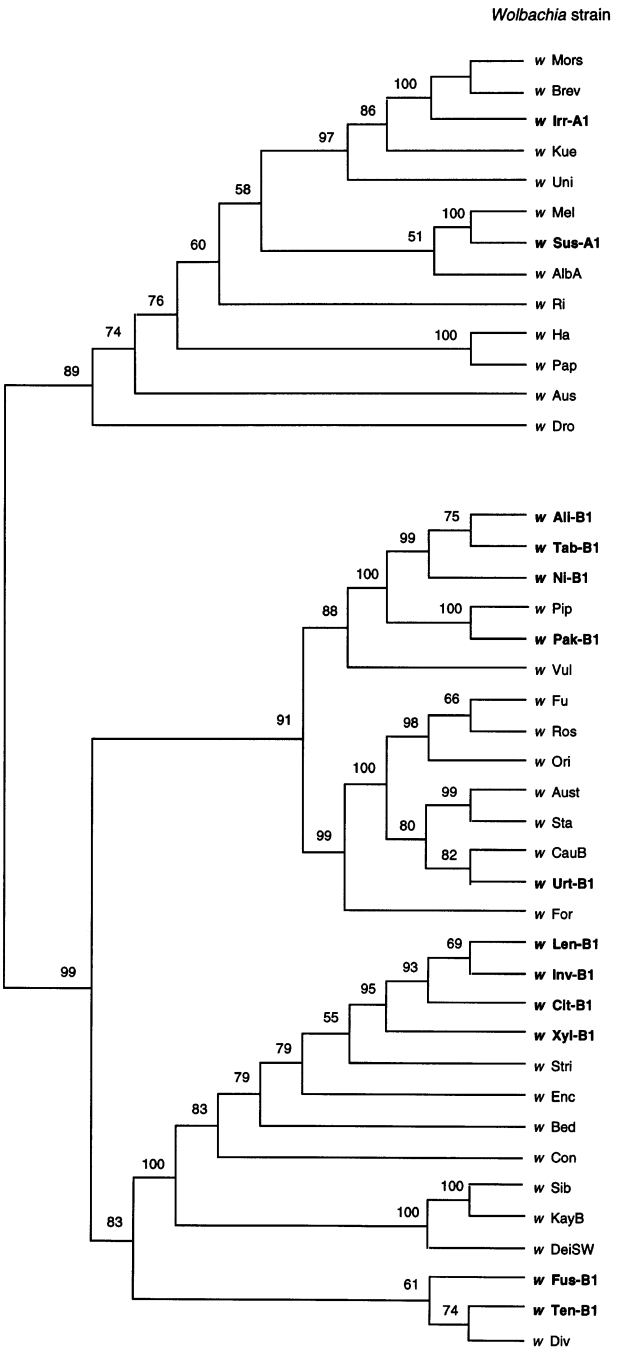

Figure 4. A midpoint rooted Maximum likelihood tree was generated for all forty-one different *Wolbachia* strains using a CLUSTAL W alignment after including the 41-bp third hypervariable region of *wsp* gene with optimality criterion set for a Substitution model in PAUP 4.0b2. Bootstrap values detected above 50% for 100 replicates are given before the branch. New *Wolbachia* strains are shown in bold. Alignment is deposited in the EMBL Nucleotide Sequence Database (accession number DS43430).

Discussion

Although Standard allele-specific PCR has been accepted widely as an efficient method for amplifying *Wolbachia* DNA, little information is available regarding the frequency of false

negative and false positive results. Turelli & Hoffman (1995) compared Standard PCR and cross-breeding tests with *Wolbachia*-infected and -uninfected *D. simulans* lines, and were always able to detect Standard PCR positive individuals using 16S rDNA primers from infected lines identified by the cross-breeding test. When attempts were made to amplify *Wolbachia* DNA by Standard PCR from single individuals of *T. urticae*, *M. occidentalis* and *A. suspensa* known to be infected with *Wolbachia* (Johanowicz & Hoy, 1996), few produced PCR products, suggesting that false negatives were obtained frequently. Some of the failures could have been due to a failure of females to transmit the *Wolbachia* to all progeny.

By contrast, the Long PCR procedure consistently amplified *Wolbachia* DNA when mixed with insect DNA even when there were as few as 100 copies of the plasmid DNA (1 fg of pAJ85) present (Figs 1 and 2). Reasons for the increased sensitivity could be due to differences in the two PCR protocols. Long PCR uses long primers (30-mers recommended); template denaturation for only 10 s; a buffer with higher pH (9.2), a slightly higher Mg^{2+} ion concentration (1.75 mM), higher concentrations of dNTPs (350 μM each), a mixture of two different DNA polymerases at a higher concentration (5 units of *Taq* and 1 of *Pwo*) in a 50-μl reaction volume, lower annealing (65 °C) and extension (68 °C) temperatures, and a linked PCR profile for thirty-five cycles with each of the last twenty-five cycles having an additional 20 s added to the extension segment (Barnes, 1994).

The failure of Standard PCR to amplify *Wolbachia* DNA consistently could be due to one, or more, of the following factors. It is likely that relatively small amounts of *Wolbachia* DNA are mixed with large amounts of arthropod genomic DNA, which could result in a nonoptimal primer : DNA template ratio. Prolonged denaturation could cause breakage of the DNA template and depurination of bases, which could stop the extension by *Taq* (Chu *et al*., 1986). Low buffer pH (8) also can enhance depurination (Lindahl, 1993). *Taq* is more error-prone, compared to other DNA polymerases such as T4, introducing one base-pair substitution for every 9000 bases amplified. By contrast, T4 DNA polymerase introduces one substitution for every 160 000 bases (Kunkel & Eckert, 1989). *Taq* has terminal deoxynucleotidyltransferase activity and adds the nucleotide A to the 3′ end of the amplified DNA (Clark, 1988). If incompletely synthesized DNA strands containing mismatches or with added As at the 3′ end accumulate during early PCR cycles, these incomplete products (also known as 'megaprimers') could interfere with the annealing of the primers to the DNA template during subsequent PCR cycles. Additionally, *Taq* completely lacks any DNA editing ability and cannot correct mismatches or remove the added A from megaprimers (Tindall & Kunkel, 1988). The megaprimers also could sequester and deplete the Mg^{2+} ions from the reaction buffer.

The Long PCR procedure utilizes both *Taq* and a thermostable DNA polymerase (*Pfu*, Vent or Deep Vent), which exhibits a 3′- to 5′-exonuclease activity (Barnes, 1994). The *Pwo* DNA polymerase from *Pyrococcus woesei* shares 100% DNA sequence identity with *Pfu* from *P. furiosus* and exhibits similar exonuclease activity (Dabrowski & Kur, 1998). The successful amplification of *Wolbachia* DNA mixed with arthropod genomic DNA by the Long PCR may be due to the exonuclease activity of the *Pwo* polymerase. Evidence for this was provided by Barnes (1994) when he showed successful amplification of the 1500 bp CryV insecticidal protein gene sequence from *Bacillus thuringiensis* by Long PCR using a 384 bp megaprimer with an added A on the 3′ end and a 43-mer primer. The sequence obtained indicated that the added A was indeed removed from the megaprimer by *Pfu*.

Others have successfully amplified *Wolbachia* DNA from arthropods by Standard PCR (Werren *et al*., 1995a,b; Johanowicz & Hoy, 1996; Zhou *et al*., 1998; van Meer *et al*., 1999), perhaps because the titre of the bacteria was high in their specimens or they used multiple pooled specimens. Our sensitivity analysis indicated that the Standard PCR protocol could produce false negatives and suggests that individuals with low titres of *Wolbachia* could have been misdiagnosed. Previously, 16.9% (24/154) of neotropical arthropod species screened tested positive by the Standard PCR (Werren *et al*., 1995a), and 27.4% of parasitoids of temperate Lepidoptera (45/164) were reported positive in a survey (Cook & Butcher, 1999). Infection estimates as high as 50% (25/50) were reported when Indo-Australian ants were tested for *Wolbachia* (Wenseleers *et al*., 1998). An estimate of 30% (22/85) infection was made for isopod species (Bouchon *et al*., 1998). Unfortunately, no clear indications were given in these surveys of the number of individuals tested that gave false negative or false positive results. In our tests, almost 76% (48/63) of arthropods screened by the Long PCR protocol tested positive on the first and only attempt using a single individual. Assuming the arthropods we tested are representative, *Wolbachia* infection may be more widespread and genetically diverse than previously estimated. *Wolbachia* infection rates also may have been underestimated in this study due to the limited number of individuals tested.

In some cases identical *wsp* sequences have been amplified from divergent arthropods; in the Kue group, identical *wsp* sequences were detected from *Ephestia kuehniella* (Lepidoptera) and *Trichogramma kaykai* (Hymenoptera) (Zhou *et al*., 1998; van Meer *et al*., 1999). In this study for example *A. suspensa* (Diptera), *T. citricida* (Homoptera), *P. citrella*, *P. xylostella* and *T. ni* (Lepidoptera) had identical sequences. These findings may indicate horizontal transfer of *Wolbachia* between unrelated arthropod species and suggest improved amplification by Long PCR might help to detect more such cases.

The presence of both an A- and B-*Wolbachia* in *T. ni* and *P. xylostella* individuals were confirmed by sequencing (Table 1) (Fig. 3). The Long PCR products from 59% (17/29) of the *Wolbachia*-positive arthropods could not be sequenced, probably because they were infected with multiple *Wolbachia* (Table 1). Previous surveys have suggested that only about 1.2% of arthropod species are infected with both A- and B-*Wolbachia* (Werren *et al*., 1995a). It is thus likely that multiple *Wolbachia* infections may be more widespread than previously estimated (Werren *et al*., 1995a), suggesting that the Long PCR is more versatile.

These results indicate that Long PCR could detect *Wolbachia* infections within individuals of arthropod populations and could evaluate *Wolbachia* transfer efficiency in progenies from crosses involving infected and uninfected individuals. Long PCR might be used to confirm that antibiotic- or heat-treated individuals are actually free of *Wolbachia*. Detection of *Wolbachia* from so many arthropod species by Long PCR also suggests that some infections with *Wolbachia* might be ancient, involving mutualistic relationships with the host and not causing cytoplasmic incompatibility, parthenogenesis, feminization or male-killing in the hosts they infect.

The Long PCR procedure successfully amplified the *nus*G-*rpl*K segment of a plant pathogenic greening bacterium of citrus (*Liberobacter asiaticum*) when standard PCR failed to amplify the same DNA segment from known infected leaves (Hoy *et al*., 1999). Sensitivity analysis also indicated that the Long PCR protocol could consistently detect as few as 100 copies of plasmid DNA when mixed with 10 ng of plant or arthropod DNA. However, at concentrations of 0.1 fg (approximately ten copies), only about half of the known positives were detected, indicating a lower limit to the sensitivity of the protocol (M. A. Hoy *et al*., unpublished data).

The level of sensitivity obtained in two different experiments (*Wolbachia* DNA plus arthropod DNA and *Liberobacter* DNA plus plant or insect DNA) suggests that the Long PCR procedure could be used to amplify other microbial DNA from both plants or animals. The Long PCR also might be useful for amplifying DNA from very tiny individuals such as single nematodes. Standard PCR of the rDNA ITS1 region from the small plant parasitic nematode *Belonolaimus longicaudatus* (2 mm long and 40 μm diameter) failed frequently in our laboratory. Yet, Long PCR consistently amplified a bright DNA band from the *B. longicaudatus* ITS1 region even though the DNA concentration in the reaction was low (< 1 ng) and shorter primers (18-mer and 23-mer) with low annealing temperature (51 °C) were used (H.-R. Han *et al*., unpublished data). It would be interesting to determine whether the Long PCR procedure can be used to amplify DNA from museum or fossil specimens, from which the amount of DNA template available for amplification may be low.

Experimental procedures

DNA extractions

A total of sixty-three insect and mite species were obtained from laboratory colonies ($n = 46$) in Gainesville, Florida or collected directly from the field ($n = 17$) in Florida. Genomic DNA was extracted by the PUREGENE method using the manufacturer's suggested procedure (Gentra Systems, Minneapolis, MN). If the insect or mite was < 1 mm in length, a whole individual was used to extract DNA, which was then resuspended in 10 μl of sterile water. If the insect was larger (1–5 mm), the abdomen from a single individual was used to extract DNA and the genomic DNA was resuspended in 50 μl of sterile water. If the adult insect was > 5 mm, the ovary or testis was dissected from the abdomen and the genomic DNA was resuspended in 100 μl of sterile water.

Wolbachia-*PCR*

Wolbachia ftsZ gene sequences from several arthropods available in GenBank (accession numbers U28203-U28210, U28199, U97531) were aligned and two highly conserved regions identified in order to design primers (30-mers, FtsZ-F, 5′-TACTGACTGTTGGAGT TGTAACTAAGCCGT-3′ and FtsZ-R, 5′-TGCCAGT TGCAAGAACAGAAACTCTAACTC-3′) to amplify a 0.6-kb DNA fragment. Primers (30-mers, Wsp-F, 5′-TGGTCCAATAAGTGATGAAGAAACTAGCTA-3′ and Wsp-R, 5′-AAAAAT TAAACGCTACTCCAGCT TCTGCAC-3′) were designed from the *Wolbachia wsp* gene sequence of *Drosophila simulans* (accession number AF020070) to amplify a variable region of about 0.6 kb (Braig *et al*., 1998). Both standard and Long PCR assays were performed using approximately 10 ng of arthropod genomic DNA extracted from a single individual of each of the sixty-two arthropod species and two subspecies.

Standard PCR was performed by a hot start method in a 25 μl volume containing 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM dATP, dGTP, dCTP, dTTP, 400 pM of primers (FtsZ-F and FtsZ-R or Wsp-F and Wsp-R) and 0.8 unit *Taq* DNA Polymerase (Roche Molecular Biochemicals, Indianapolis, IN) (Saiki, 1989). The DNA preparations were amplified for thirty-five cycles, with each cycle consisting of denaturation at 94 °C for 30 s and extension at 72 °C for 1 min.

Long PCR was performed in a 50 μl volume containing 50 mM Tris (pH 9.2), 16 mM ammonium sulphate, 1.75 mM $MgCl_2$, 350 μM dATP, dGTP, dCTP, dTTP, 800 pM of primers (FtsZ-F and FtsZ-R or Wsp-F and Wsp-R), 1 unit of *Pwo* and 5 units of *Taq* DNA polymerases (Barnes, 1994). The Long PCR was carried out using three linked profiles: (i) one cycle consisting of denaturation at 94 °C for 2 min, (ii) ten cycles each consisting of denaturation at 94 °C for 10 s, annealing at 65 °C for 30 s and extension at 68 °C for 1 min, and (iii) twenty-five cycles each consisting of denaturation at 94 °C for 10 s, annealing at 65 °C for 30 s and extension at 68 °C for 1 min, plus an additional 20 s added for every consecutive cycle.

Clean laboratory practices, sealed pipette tips and fresh reagents were used to avoid contamination. A PERKIN-ELMER DNA Thermal Cycler 480 was used for all PCR assays.

Recombinant DNA techniques

Recombinant DNA techniques, including restriction digests, ligation, bacterial transformation and plasmid DNA preparation were performed by standard methods (Sambrook *et al*., 1989). PCR

products were purified using QIAquick PCR purification column (QIAGEN Inc., Valencia, CA). Standard PCR products were cloned into the *E. coli* plasmid pTZ19R *Sma*I site by a T/A cloning strategy (Mead *et al*., 1991). Subsequently, Long PCR products were cloned into the plasmid pCR2.1-TOPO using the procedure suggested by the manufacturer (Invitrogen Corporation, Carlsbad, CA). DNA sequencing was performed at the University of Florida ICBR Core Facility using a PERKIN-ELMER Applied Biosystems ABI PRISM Automated DNA Sequencer. MacDNASIS software program (Hitachi Software Engineering America Ltd, San Bruno, California) was used to assemble and handle the sequences. DNA sequences were aligned by using CLUSTAL W and transported into MacClade and PAUP*4.0b2 to generate a phylogenetic tree.

Acknowledgements

We acknowledge Denise Johanowicz (USDA, Gainesville) for critically reading the manuscript and thank Juan Alvarez, Debbie Boyd, Jerry Butler, John Capinera, Pete Coon, Sheila Gomez, Jim Lloyd, Oscar Perez, Lucy Skelley, Lois Wood and Tom Walker of the University of Florida, Gainesville and Ru Nguyen (Florida Division of Plant Industry, Gainesville) for providing insect specimens. This work was supported in part by the Davies, Fischer and Eckes Endowment in Biological Control at the University of Florida. This is Florida Agricultural Experiment Station journal publication R-07346.

References

Barnes, W. (1994) PCR amplification of up to 35-kb DNA with high fidelity and high yield from λ bacteriophage templates. *Proc Natl Acad Sci USA* **91**: 2216–2220.

Bouchon, D., Rigaud, T. and Juchault, P. (1998) Evidence for widespread *Wolbachia* infection in isopod crustaceans: molecular identification and host feminization. *Proc R Soc Lond B* **265**: 1081–1090.

Bourtzis, K., Nirgianaki, A., Onyango, P. and Savakis, C. (1994) A prokaryotic *dnaA* sequence in *Drosophila melanogaster*: *Wolbachia* infection. *Insect Mol Biol* **3**: 131–142.

Braig, H.R., Zhou, W., Dobson, S.L. and O'Neill, S.L. (1998) Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. *J Bacteriol* **180**: 2373–2378.

Breeuwer, J.A.J. and Werren, J.H. (1990) Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature* **346**: 558–560.

Breeuwer, J.A.J., Stouthamer, R., Burns, D.A., Pelletier, D.A., Weisburg, W.G. and Werren, J.H. (1992) Phylogeny of cytoplasmic incompatibility microorganisms in the parasitoid wasp genus *Nasonia* (Hymenoptera: Pteromalidae) based on 16S ribosomal DNA sequences. *Insect Mol Biol* **1**: 25–36.

Chu, G., Vollrath, D. and Davis, R.W. (1986) Separation of large DNA-molecules by contour-clamped homogeneous electric-fields. *Science* **234**: 1582–1585.

Clark, J.M. (1988) Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. *Nucl Acids Res* **16**: 9677–9686.

Cook, J.M. and Butcher, R.D.J. (1999) The transmission and effects of *Wolbachia* bacteria in parasitoids. *Res Popul Ecol* **41**: 15–28.

Dabrowski, S. and Kur, J. (1998) Cloning and expression in *Escherichia coli* of the recombinant His-tagged DNA polymerases from *Pyrococcus furiosus* and *Pyrococcus woesei*. *Prot Expres Purific* **14**: 131–138.

Holden, P.R., Brookfield, J.F.Y. and Jones, P. (1993) Cloning and characterization of an *ftsZ* homologue from a bacterial symbiont of *Drosophila melanogaster*. *Mol Gen Genet* **240**: 213–220.

Hoy, M.A., Nguyen, R. and Jeyaprakash, A. (1999) Classical biological control of Asian citrus psylla. Citrus Industry **80** (9): 20–22.

Hurst, G.D.D., Graf von der Schulenburg, J.H., Majerus, T.M.O., Bertrand, D., Zakharov, I.A., Baungaard, J., Volkl, W., Stouthamer, R. and Majerus, M.E.N. (1999) Invasion of one insect species, *Adalia bipunctata* by two different male-killing bacteria. *Insect Mol Biol* **8**: 133–139.

Johanowicz, D.L. and Hoy, M.A. (1996) *Wolbachia* in a predator-prey system: 16S ribosomal DNA analysis of two phytoseiids (Acari: Phytoseiidae) and their prey (Acari: Tetranychidae). *Annal Entomol Soc Am* **89**: 435–441.

Kunkel, T.A. and Eckert, K.A. (1989) Fidelity of DNA polymerases used in polymerase chain reactions. *Polymerase Chain Reaction* (Erlich, H.A., Gibbs, R. and Kazazian, H.H., eds), pp. 5–10. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Laven, H. (1951) Crossing experiments with *Culex* strains. *Evolution* **5**: 370–375.

Laven, H. (1967) Eradication of *Culex pipiens fatigans* through cytoplasmic incompatibility. *Nature* **216**: 383–384.

Lindahl, T. (1993) Instability and decay of the primary structure of DNA. *Nature* **362**: 709–715.

Masui, S., Sasaki, T. and Ishikawa, H. (1997) *groE*-Homologous operon of *Wolbachia*, an intracellular symbiont of arthropods: a new approach for their phylogeny. *Zool Sci* **14**: 701–706.

Mead, D.A., Pey, N.K., Herrnstadt, C., Marcil, R.A. and Smith, L.M. (1991) A universal method for the direct cloning of PCR amplified nucleic acid. *Biotechnology* **9**: 657–663.

O'Neill, S.L., Giordano, R., Colbert, A.M.E., Karr, T.L. and Robertson, H.M. (1992) 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc Natl Acad Sci USA* **89**: 2699–2702.

O'Neill, S.L., Hoffmann, A.A. and Werren, J.H. (1997) *Influential Passengers*. Oxford University Press, Oxford.

Rousset, F., Bouchon, D., Pintureau, B., Juchault, P. and Solignac, M. (1992) *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. *Proc R Soc Lond B* **250**: 91–98.

Saiki, R.K. (1989) The design and optimization of the PCR. *PCR Technology* (Erlich, H.A., ed.), pp. 7–16. Stockton Press, New York.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, Vols I–III. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sironi, M., Bandi, C., Sacchi, L., Di Sacco, B., Damiani, G. and Genchi, C. (1995) Molecular evidence for a close relative of the arthropod endosymbiont *Wolbachia* in a filarial worm. *Mol Biochem Parasitol* **74**: 223–227.

Stouthamer, R., Luck, R.F. and Hamilton, W.D. (1990) Antibiotics cause parthenogenetic *Trichogramma* (Hymenoptera/

Trichogrammatidae) to revert to sex. *Proc Natl Acad Sci USA* **87**: 2424–2427.

Stouthamer, R., Breeuwer, J.A.J., Luck, R.F. and Werren, J.H. (1993) Molecular identification of microorganisms associated with parthenogenesis. *Nature* **361**: 66–68.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nuc Acid Res* **22**: 4673–4680.

Tindall, K.R. and Kunkel, T.A. (1988) The fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Biochemistry* **27**: 6008–6013.

Turelli, M. and Hoffmann, A.A. (1995) Cytoplasmic incompatibility in *Drosophila simulans*: Dynamics and parameter estimates from natural populations. *Genetics* **140**: 1319–1338.

van Meer, M.M.M., Witteveldt, J. and Stouthamer, R. (1999) Phylogeny of the arthropod endosymbiont *Wolbachia* based on the *wsp* gene. *Insect Mol Biol* **8**: 399–408.

Wenseleers, T., Ito, F., Borm, S.V., Huybrechts, R., Volckaert, F. and Billen, J. (1998) Widespread occurrence of the micro-organism *Wolbachia* in ants. *Proc R Soc Lond B* **265**: 1447–1452.

Werren, J.H., Windsor, D. and Gao, L. (1995a) Distribution of *Wolbachia* among neotropical arthropods. *Proc R Soc Lond B* **262**: 197–204.

Werren, J.H., Zhang, W. and Guo, L.R. (1995b) Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. *Proc R Soc Lond B* **261**: 55–71.

Werren, J.H. (1997) Biology of *Wolbachia*. *Annu Rev Entomol* **42**: 587–609.

Zhou, W., Rousset, F. and O'Neill, S.L. (1998) Phylogeny and PCR based classification of *Wolbachia* strains using *wsp* gene sequences. *Proc R Soc Lond B* **265**: 1–7.