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Host-specific *Wolbachia* strains in widespread populations of *Phlebotomus perniciosus* and *P. papatasi* (Diptera: Psychodidae), and prospects for driving genes into these vectors of *Leishmania*

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Abstract

A single strain of *Wolbachia* (α-proteobacteria, Rickettsiales) was found in widespread geographical populations of each of two *Phlebotomus* species, within which there was no indication of 'infectious speciation'. The two strains were identified by sequencing a fragment of *wsp* (a major surface protein gene), amplified by polymerase chain reaction from DNA extracted from the body parts of individual sandflies. Infection rates were high in the males and females of both sandflies, but they were lower for the B-group wPrn strain of *Wolbachia* in *Phlebotomus perniciosus* Newstead (60.3% overall) than for the A-group wPap strain in *P. papatasi* (Scopoli) (81.7%). Infections were frequent in the thorax, where *Leishmania* develops infective forms, as well as in the abdomen, where *Wolbachia* must infect the reproductive tissues to ensure its vertical transmission. These findings were related to knowledge of the population biology of *Wolbachia* in other insects, leading to the conclusion that this endosymbiont could be useful for driving transgenes through wild populations of both sandflies. This will require characterizing the cytoplasmic incompatibility phenotypes of *Wolbachia*–sandfly combinations, as well as estimating for them the incidence of paternal transmission and the fidelity of maternal transmission. Paternal transmission is one explanation for finding a single *Wolbachia* strain associated with all mitochondrial haplotypes and lineages of each sandfly species. However, this distribution pattern could also result from multiple horizontal transmissions or the failure of *wsp* to provide strain markers.

Introduction

Wolbachia is an intracellular maternally-inherited commensal (often a symbiont or parasite) belonging to the α-proteobacteria (Rickettsiales). It infects filarial worms (Sironi *et al*., 1995; Egyed *et al*., 2002), as well as a wide range of arthropods in which it can cause reproductive abnormalities such as parthenogenesis, feminization and cytoplasmic incompatibility (O'Neill *et al*., 1992; Weeks *et al*., 2002). The latter is the most common phenotype, and it results in embryonic mortality when *Wolbachia*-infected males mate either with uninfected females or females infected with a different *Wolbachia* strain (Weeks *et al*., 2002). This ability of *Wolbachia* (when living in germ-line cytoplasm) to modify the reproductive success of its hosts enables it to spread rapidly in a host population without the need of horizontal transmission. Such rapid vertical transmission favours genetically engineering populations of insect pests with genes carried by *Wolbachia* (Curtis & Sinkins, 1998; Turelli & Hoffmann, 1999).

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The present report investigates the distribution of *Wolbachia* in two species of phlebotomine sandflies (Diptera: Psychodidae) and considers the significance of the findings for any attempts to drive through their populations transgenes advantageous for intervening in the transmission of the parasitic protozoan *Leishmania* (Kinetoplastida: Trypanosomatidae). This is the causative agent of leishmaniasis, a cutaneous and visceral disease with a worldwide prevalence of about 15 million human cases (Ashford *et al*., 1992). Only two *Phlebotomus* species have been shown to harbour *Wolbachia* (Zhou *et al*., 1998; Cui *et al*., 1999; Ono *et al*., 2001): *Phlebotomus (Phlebotomus) papatasi* (Scopoli), the principal vector of *Leishmania major* Yakimoff & Schokhor (causative agent of rural zoonotic cutaneous leishmaniasis) in arid regions of north Africa and Asia; and *Phlebotomus (Larroussius) perniciosus* Newstead, the most widespread vector of *Leishmania infantum* Nicolle (causative agent of infantile and canine leishmaniasis) in south-west Europe and north-west Africa.

Any consideration of *Wolbachia* as a transgene vector requires knowledge of its distribution in host tissues (Dobson *et al*., 1999), because transgene products may not reach their targets in the anterior gut of the sandfly, where most *Leishmania* develop, if a *Wolbachia* strain is restricted to the germ-line tissues. The vectorial potential of a *Wolbachia* strain also depends on its host prevalences and the population differentiation of its insect hosts, because a strain may not spread throughout, or between, host populations if it occurs in low densities in many individual hosts and there are barriers to host dispersal (Turelli & Hoffmann, 1999; Noda *et al*., 2001). Until now, *Wolbachia* has been reported from only a few small pools of *Phlebotomus* genomic DNA: one pool from Italian *P. perniciosus* (Ono *et al*., 2001), and seven pools from *P. papatasi* originating from the Jordan Valley (Zhou *et al*., 1998; Cui *et al*., 1999; Ono *et al*., 2001), Egypt and Saudi Arabia (Cui *et al*., 1999) and India (Ono *et al*., 2001). All pools were of flies from laboratory colonies, except for a single pool of two to three wild-caught *P. papatasi* from the Jordan Valley (Ono *et al*., 2001). Tissue distribution and density of *Wolbachia* were not mentioned in any of these reports.

The present results come from a survey of the distribution of *Wolbachia* strains in the body parts of individual sandflies sampled from widespread wild populations of *P. perniciosus* and *P. papatasi*, using a polymerase chain reaction that specifically amplifies a fragment of a variable surface protein gene (*wsp*) of *Wolbachia pipientis* Hertig (Rickettsiales: Rickettsiaceae) (Zhou *et al*., 1998). A polymerase chain reaction was also used to amplify from each sandfly a fragment of their mitochondrial cytochrome b gene (Cyt b), which is a useful marker for geographical races of these sandflies (Esseghir *et al*., 1997, 2000). Direct sequencing of both amplified DNA fragments was then employed to identify the unique-sequence haplotypes of each gene. Both *Wolbachia* and mitochondrial genes are usually inherited cytoplasmically and maternally, and so any deviation from strict co-variation can help to identify natural barriers to transgene spread: paternal transmission or imperfect maternal transmission of either genome will uncouple them, preventing the one with enhanced selective fitness (e.g. a *Wolbachia* strain that induces strong cytoplasmic incompatibility) from 'sweeping' the other through a host population (Turelli & Hoffmann, 1999).

Materials and methods

Geographical origins, collection and identification of sandflies

The analysed populations are listed in tables 1 and 2.

All adults of *P. perniciosus* were captured overnight in CDC miniature light traps, placed peri-domestically in gardens and often close to domestic animal shelters (Mahamdallie *et al*., 2003). The same method was used for adults of *P. papatasi*, which were also captured in Iran using sticky (castor oil) papers left overnight at the entrances to gerbil burrows (Parvizi *et al*., 2003). All sandflies were stored either in 80% analytical grade ethanol at –20°C or dry in liquid nitrogen and then in a –70°C freezer.

All sandflies were identified by their species-specific Cyt b sequences (see next section). Most were also identified based on morphological characters of the head and abdominal terminalia, which were slide-mounted in Berlese fluid (Lewis, 1982) following dissection with sterilized forceps and micro-needles (Testa *et al*., 2002). This was carried out in a room away from the molecular biology laboratory, to reduce the risk of polymerase chain reaction carryover, i.e. the 'contamination' of a genomic DNA sample with a product that had already been amplified. Voucher specimens were placed in the phlebotomine collection of the Department of Entomology, The Natural History Museum, London.

DNA extraction, amplification and sequencing

DNA was extracted (Ready *et al*., 1991) from either the thorax, the thorax and the attached anterior abdomen, the anterior abdomen or the whole abdomen of individual sandflies. Four hundred and eighty eight base-pairs (bp) of Cyt b were amplified by the polymerase chain reaction using the primers CB1-SE and CB3-R3A (Esseghir *et al*., 2000) and directly sequenced using the general methods given below for *wsp* and the specific protocols of Testa *et al*. (2002). DNA samples that failed to yield a Cyt b fragment were not included in the study.

Samples were screened for the presence of *Wolbachia* using the general *wsp* primers 81F (5′-3′ TGGTCCAATAAGTGATGAAGAAAC) and 691R (5′-3′ AAAAATTAAACGCTACTCCA), shown to be able to amplify an homologous *wsp* gene fragment from the most diverse strains of *Wolbachia* (Braig *et al*., 1998; Zhou *et al*., 1998). A 20 µl polymerase chain reaction mixture consisted of 1× Promega buffer, 2.5 mM $MgCl_2$, 250 µM dNTP, 0.5 µM each of the two primers and one unit of Taq DNA polymerase (Promega, USA). To ensure a reliable amplification of *wsp* fragments from each body part, 2 µl of DNA were added to the polymerase chain reaction mixture (equivalent to 15 ng in the undiluted samples). The amplification was carried out under the following thermal profile using a GeneAmp® PCR System 9700 thermal cycler (PE Applied Biosystems, USA): 2 min of denaturation at 94°C; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min; and a final extension at 72°C for 10 min. The size and quantity of the amplified DNA fragments were determined by fractionating the polymerase chain reaction products on 1.5% agarose gels along with marker DNA fragments of known sizes and quantities (Promega PCR Markers G316A, or Bioline (UK) HyperLadder IV). The DNA in the excised gel fragments was purified with glassmilk (Geneclean II Kit, BIO 101 Inc, USA) and each strand directly sequenced using one

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of the *wsp* primers (3.2 pmoles) and the ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (version 2.0, PE Applied Biosystems). Purification of the extension products was carried out using the ethanol precipitation method. Sequences were resolved using the ABI 373A or 377 system, then edited and aligned with database sequences using Sequencher software (Gene Codes Corporation, USA), all according to ABI protocols (PE Applied Biosystems).

Positive controls for the amplification of the *wsp* fragment were performed using genomic DNA extracted from triple-infected *Asobara tabida* (Nees) (Hymenoptera: Braconidae) donated by Dr James Cook (Imperial College at Silwood Park). The sequences obtained were those previously reported (Vavre *et al*., 1999), and they were not found in any sandfly DNA sample.

Sensitivity and reproducibility of the polymerase chain reaction method of screening for wsp

The threshold of detection and reproducibility of the polymerase chain reaction were determined using serial dilutions of genomic DNA samples (undiluted (= 15 ng), 1/10, 1/100, 1/1,000, 1/10,000) from six individuals of *P. perniciosus* and seven individuals of *P. papatasi* (table 3; fig. 1).

Results

A single host-specific strain of W. pipientis *occurs in all mitochondrial lineages and geographical populations of each* Phlebotomus *species*

The polymerase chain reaction assay successfully amplified the targeted fragment of the *wsp* gene from different body parts of 38/63 (60.3%) male and female *P. perniciosus* originating from all three populations with the Typical mitochondrial Cyt b lineage and all three populations with the Iberian Cyt b lineage (table 1). All *wsp* fragments were directly sequenced and found to have the same haplotype (= unique sequence), regardless of geographical origin and Cyt b lineage. The 555-base pair (bp) haplotype (minus primers) was identical to that of the B-group strain of *W. pipientis* (wPrn) previously found only in a pool of genomic DNA extracted from 2–3 flies from a laboratory colony of Italian *P. perniciosus* (GenBank accession no. AF237884; Ono *et al*., 2001).

Similarly, the targeted *wsp* fragment was amplified from different body parts of 125/153 (81.7%) male and female *P. papatasi* originating from all five geographical populations sharing the single Cyt b lineage known from this species (table 2). Most of these *wsp* fragments were sequenced (85%) and, as for *P. perniciosus*, only a single haplotype was found. However, the 564-bp haplotype (minus primers) found in all *P. papatasi* was distinct from that of *P. perniciosus*. It was identical to that of the A-group strain of *W. pipientis* (wPap) previously found only in pools of genomic DNA extracted from 2–20 individuals of *P. papatasi* originating from the Jordan Valley (GenBank accession no. AF237883; Ono *et al*., 2001; Cui *et al*., 1999), India (GenBank accession no. AF237882; Ono *et al*., 2001), Egypt and Saudi Arabia (Cui *et al*., 1999). Only one of these pools came from 'field collected' sandflies: the sequence accessioned as AF237883 was amplified from a single pool of two to three *P. papatasi* captured in the Jordan Valley (Ono *et al*., 2001). The wPap sequence was originally reported as having an ambiguous base (C/T) at nucleotide position 102 (GenBank accession no. AF020082; Zhou *et al*., 1998).

Tissue tropism of Wolbachia

Most specimens had been used for other studies, and so not all their body parts could be screened for *Wolbachia*. In most cases, the posterior third of the abdomen (including some or all of the reproductive organs) had been slide-mounted for morphological identification, and then genomic DNA had been extracted from either the thorax with the anterior abdomen attached or from only one of these parts if proteins had been extracted from the other.

However, one result was clear: *Wolbachia* was usually detectable in the thorax of both species. Indeed, infection rates for the thorax were usually higher than those for the anterior abdomen of males and females of *P. perniciosus* (table 1) and only a little less than infection rates for the whole abdomen of both species (tables 1, 2).

Maternal transmission of Wolbachia, *and infection rates in male and female sandflies*

The Italian *P. perniciosus* came from three laboratory-reared F1 broods (table 1). *Wolbachia* was detected in all six siblings (four males, two females) from one brood, but in neither of the two females from each of the other two broods. This is consistent with the successful maternal transmission of *Wolbachia* by only one out of three of the wild mothers, probably because only this mother was infected and had a high enough density of bacteria to permit transmission (Turelli & Hoffmann, 1999). Alternatively, *Wolbachia* may have been transmitted from the other two mothers to their eggs, but the densities of the bacteria may have been too low to permit detection by the polymerase chain reaction.

Wolbachia was detected consistently less often in wild-caught males of *P. perniciosus* (table 1) and *P. papatasi* (table 2) than in the females of the same species, except when genomic DNA from complete abdomens was screened. This is likely to result from true differences in the densities of *Wolbachia* in various tissues of each sex, rather than from any difference in the quantities of genomic DNA extracted, as explained in the following section.

This is the first report to identify *Wolbachia* in both sexes of these two species. Previously, *wsp* was isolated from unspecified material (Zhou *et al*., 1998), from mixed pools of ten males and ten females (Cui *et al*., 1999) or from two to three flies of unspecified sex (Ono *et al*., 2001).

Wolbachia *density in host populations*

The method used to screen for the *wsp* gene fragment was not a quantitative polymerase chain reaction (Sinkins *et al*., 1995a; Noda *et al*., 2001), i.e. the quantity of the *wsp* fragment amplified could not be expected to be strictly proportional to the density of *Wolbachia* in each tissue sample. However, there was strong empirical evidence that the absence/presence of an amplified fragment and its quantity were both determined by the density of *Wolbachia* in each tissue sample, rather than by the quantity of genomic DNA extracted from it. This conclusion was based on two observations. First, the sensitivity of the polymerase chain

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Table 1. Prevalences and densities of *Wolbachia* (B-group strain wPrn) in *Phlebotomus perniciosus* from different geographical regions, estimated by detecting the *wsp* gene fragment.

Population (settlement, province / department, country)	Cyt b lineage*	% sandflies wsp+ve (no. screened) Part of body used for DNA extraction										
		Thorax		Thorax + Anterior abdomen		Anterior abdomen		Abdomen		Any body part		
		Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	All
Parabitá, Apulia , Italy Laboratory-reared, F1	Typical	ND	ND	100.0 (4) {++/+++}	33.3 (6) {+++}	ND	ND	ND	ND	100.0 (4)	33.3 (6)	60.0 (10)
Goubellat, Medjez El-Bab, Tunisia Field collected	Typical	ND	ND	40.0 (10)	ND	ND	ND	ND	ND	40.0 (10)	ND	40.0 (10)
Beaulieu-sur-mer, Alpes Maritimes, France Field collected	Typical	50.0 (4) {+/++}	100.0 (2) {+/++}	ND	ND	50.0 (6) {+++}	100.0 (1) {+++}	ND	ND	50.0 (10)	100.0 (3)	61.5 (13)
St Just, Barcelona , Spain ** Field collected	Iberian	80.0 (5) {+/++}	100.0 (5) {+/++}	ND	ND	ND	ND	100.0 (5) {+/++}	100.0 (5) {++}	100.0 (5)	100.0 (5)	100.0 (10)
Torvizcón, Granada, Spain Field collected	Iberian	ND	60.0(5) {+/++}	ND	ND	ND	60.0 (5) {+/++}	ND	ND	ND	60.0 (10)	60.0 (10)
Trevélez, Granada, Spain Field collected	Iberian	ND	40.0 (5) {+/++}	ND	ND	0.0 (2)	66.6 (3) {+/++}	ND	ND	0.0 (2)	50.0 (8)	40.0 (10)
Total % *wsp* +ve (no.) (All samples)		66.7 (9)	70.6 (17)	57.1 (14)	33.3 (6)	37.5 (8)	66.7 (9)	100.0 (5)	100.0 (5)	58.1 (31)	62.5 (32)	60.3 (63)
Total % *wsp* +ve (no.) (Samples with both sexes)		66.7 (9)	100.0 (7)	–	–	37.5 (8)	75.0 (4)	100.0 (5)	100.0 (5)	66.7 (21)	63.6 (22)	65.1 (43)

{Density: + to +++ = increasing quantity of polymerase chain reaction product} *Esseghir *et al*. (2000). **The thorax and abdomen of each of five males and five females. ND, not done.

Table 2. Prevalences and densities of *Wolbachia* (A-group strain wPap) in wild-caught *Phlebotomus papatasi*, estimated by detecting the *wsp* gene fragment.

Population (settlement, province, country)	Cyt b lineage*	% sandflies *wsp*+ve (no. screened) Part of the body used for DNA extraction								
		Thorax**		Thorax + Anterior abdomen		Abdomen**		Any body part		
		Males	Females	Males	Females	Males	Females	Males	Females	All
Granada , Spain	Typical	ND	ND	50.0 (6) {+/++}	ND	ND	ND	50.0 (6)	ND	50.0 (6)
Habib Abad, Isfahan, Iran	Typical	100.0 (5) {+}	90.0 (10) {++/+++}	69.7 (43) {+/++/+++}	92.0 (25) {+/++/+++}	100.0 (5) {+/++}	100.0 (10) {++/+++}	75.5 (48)	94.3 (35)	83.1 (83)
Khorzoogh, Isfahan, Iran	Typical	ND	ND	73.1 (26) {+/++/+++}	91.6 (24) {+/++/+++}	ND	ND	73.1 (26)	91.7 (24)	82.0 (50)
Hamadan, Hamadan , Iran	Typical	ND	ND	83.3 (12) {+/++/+++}	ND	ND	ND	83.3 (12)	ND	83.3 (12)
Karaj, Tehran , Iran	Typical	ND	ND	100.0 (2) {+/+++}	ND	ND	ND	100.0 (2)	ND	100.0 (2)
Total % *wsp* +ve (no.) (All samples)		100.0 (5)	90 .0(10)	73.0 (89)	91.8 (49)	100.0 (5)	100.0 (10)	74.5 (94)	93.2 (59)	81.7 (153)
Total % *wsp* +ve (no.) (Samples with both sexes)		100.0 (5)	90.0 (10)	71.0 (69)	91.8 (49)	100.0 (5)	100.0 (10)	74.3 (74)	93.2 (59)	82.7 (133)

{Density: + to +++ = increasing quantity of polymerase chain reaction product.} *Esseghir *et al.* (1997). **The thorax and abdomen of each of five males and ten females. ND, not done.

reaction (fig. 1) was not affected by a ten-fold reduction in the quantity of target genomic DNA of either *Phlebotomus* species (table 3). Secondly, the quantities of tissue used for DNA extraction varied two-fold to five-fold in volume, and not ten-fold, between different body parts.

Based on this reasoning, it is concluded that the densities of *Wolbachia* were higher in *P. papatasi* (compared with *P. perniciosus*; fig. 1), higher in the abdomen of both species (compared with the thorax), and higher in the female of both species. The largest observed differences between the sexes were for the anterior abdomen of *P. perniciosus* (table 1) and the thorax-with-anterior abdomen of *P. papatasi* (table 2). These differences are consistent with the highest densities of *Wolbachia* being in the posterior abdomen (including the gonads), more of which can be lost by dissection in the males than in the females.

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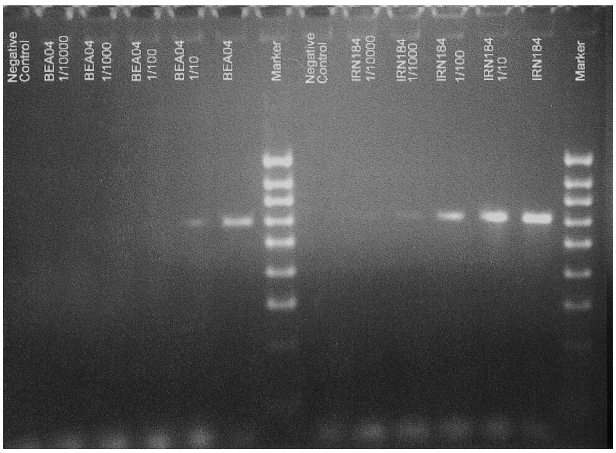

Fig. 1. Threshold of detection of the *wsp* gene fragment by the polymerase chain reaction, tested using 1/10 serial dilutions of total genomic DNA extracted from a female *Phlebotomus perniciosus* (BEA04) and a female *P. papatasi* (IRN184). Two microlitres of DNA were added to each polymerase chain reaction, equivalent to 15 ng in the undiluted samples. Marker: Bioline HyperLadder IV has nine DNA fragments (top to bottom) of 1000, 800, 700, 600, 500, 400, 300, 200 (weak), 100 (weak) base pairs (bp). *wsp* fragments have migrated about as far as the 600 bp band (60 ng of DNA), consistent with their sequence lengths of 599 bp (*P. perniciosus*) and 608 bp (*P. papatasi*).

Discussion

Apparent absence in Phlebotomus *of strict co-variation of* Wolbachia *and mitochondrial DNA: no 'infectious speciation'*

Phylogenetic concordance between *Wolbachia* strains and mitochondrial DNA (mtDNA) lineages has been noted for arthropod sibling species, e.g. Crustacea (Marcadé *et al*., 1999), Hymenoptera (Shoemaker *et al*., 2000) and Diptera (Behura *et al*., 2001). However, naturally occurring *Wolbachia* infections do not always cause cytoplasmic incompatibility (Hoffmann *et al*., 1996), and this phenotype will not always lead to reproductive isolation and the creation of new species by 'infectious speciation' (Wade, 2001). The preliminary evidence suggests there is no infectious speciation within *P. perniciosus* and *P. papatasi*.

Phlebotomus papatasi has a single mtDNA lineage throughout its wide geographical range in the arid belt from Spain and Morocco to India. The Cyt b haplotypes differ little (by 1–6/441 bp in pairwise comparisons), and their geographical distribution is consistent with a genetic bottleneck followed by a radial dispersal from the eastern Mediterranean subregion within the last 135,000 years (Esseghir *et al*., 1997). More intensive sampling in Iran has shown that there can be many local Cyt b haplotypes (> 30) derived by one to two mutations from the predominant regional haplotype (Parvizi *et al*., 2003), but all are a part of the single network (or minimum-spanning phylogenetic tree) that was first recognized by Esseghir *et al*. (1997). Despite this large amount of small-scale mtDNA variation, only one *wsp* strain of *Wolbachia* (wPap) has been discovered in *P. papatasi* originating from India, Saudi Arabia, the Jordan Valley and Egypt (Cui *et al*., 1999; Ono *et al*., 2001), and now from Iran and Spain. Based on the findings of Turelli & Hoffmann (1995, 1999), there are three likely explanations for this apparent absence of *Wolbachia*-sandfly co-variation. Firstly, the wPap strain may have recently invaded *P. papatasi* by multiple horizontal transmissions, and the ectoparasitic mites found on this sandfly (Lewis, 1982) could be vectors. Secondly, wPap may have invaded by a single horizontal transmission and then become associated with different mtDNA haplotypes throughout the sandfly's range because of paternal transmission. In this case, the failure of wPap to sweep along the mtDNA haplotype of the sandfly it first invaded would be explained by one or more of the following: a weak cytoplasmic incompatibility phenotype in *P. papatasi*, curing of *Wolbachia* infections (by natural antibiotics), and imperfect maternal inheritance of one or both of the cytoplasmic genomes. Thirdly, there may be different strains of *Wolbachia* associated with some or all of the regional Cyt b haplotypes of *P. papatasi*, but the *wsp* gene may evolve too slowly to provide markers. The latter would indicate that *wsp* evolves far more slowly than its discoverers (Braig *et al*.,

Table 3. Sensitivity of polymerase chain reaction detection of *Wolbachia*, tested using serial dilutions of sandfly DNA.

Species	Population	Individual sandflies screened	Sex	Body part	Dilutions of sandfly DNA				
					Undiluted	1/10	1/100	1/1000	1/10000
P. perniciosus	Parabitá , Italy	Ita121	Female	Thor.+ ant.ab.	+++	++	–	–	–
	Parabitá , Italy	Ita122	Female	Thor.+ ant.ab.	+++	++	–	–	–
	Beaulieu-sur-mer, France	BEA04	Female	Ant. ab.	++	+	–	–	–
	Beaulieu-sur-mer, France	BEA06	Male	Ant. ab.	+++	++	+	–	–
	Beaulieu-sur-mer, France	BEA16	Male	Thorax	+	+	–	ND	ND
	Beaulieu-sur-mer, France	BEA18	Female	Thorax	+	+	–	ND	ND
P. papatasi	Habib Abad, Iran	IRN40 (1)	Male	Thor.+ ant.ab.	+++	++	+	+	–
	Habib Abad, Iran	IRN76 (1)	Male	Thor.+ ant.ab.	+++	+++	–	–	–
	Habib Abad, Iran	IRN184	Female	Thor.+ ant.ab.	+++	+++	++	+	+
	Khorzoogh, Iran	IRN66	Male	Thor.+ ant.ab.	+++	++	+	–	ND
	Habib Abad, Iran	IRN40 (2)	Male	Thor.+ ant.ab.	+++	++	+	+	ND
	Habib Abad, Iran	IRN76 (2)	Male	Thor.+ ant.ab.	+++	+++	+	ND	ND
	Habib Abad, Iran	IRN80	Male	Thor.+ ant.ab.	+	+	–	ND	ND
	Shapour Abad, Iran	IRN104	Male	Thor.+ ant.ab.	+	+	–	ND	ND
	Zardjane J, Iran	IRN176	Male	Thor.+ ant.ab.	+	+	–	ND	ND

ND, not done; -, no polymerase chain reaction product; + to +++ = increasing quantity of polymerase chain reaction product, comparable for both species; Thor., thorax; Ant.ab., anterior abdomen; 1, first test; and 2, second test of the same sample.

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1998) could ever have expected of a surface protein gene that is the most variable of *Wolbachia* markers (Zhou et *al.*, 1998). All the samples contained amplifiable mtDNA and most samples contained amplifiable *Wolbachia* DNA, and so it is most unlikely that alternative *wsp* alleles were not amplified because of the absence of available target DNA. However, it is possible that only one *wsp* allele was amplified because it preferentially hybridized to one or both of the 'general' *wsp* primers used, even though their sequences are highly conserved across taxa (Braig *et al.*, 1998; Zhou et *al.*, 1998). Where allele-specific hybridization of primers has been suspected, the amplification of multiple alleles of other *Wolbachia* genes has occasionally permitted the identification of double infections (Mitsuhashi *et al.*, 2002).

The apparent absence of *Wolbachia*-mtDNA co-variation is even more remarkable for *P. perniciosus*, because this species has two geographically distinct lineages of Cyt b – the Iberian, and the Typical in Tunisia, Malta, Italy and France (Esseghir *et al.*, 1997, 2000; Mahamdallie *et al.*, 2003) – and within the Iberian lineage there is strong evidence for little or no gene flow between north-east and southern Spain, based on population genetics analyses of polymorphic microsatellite DNA and isoenzyme loci (Aransay *et al.*, 2001, 2003; Mahamdallie *et al.*, 2003). The timeframe of dispersal is different from *P. papatasi*. The Iberian and Typical lineages of *P. perniciosus* are believed to have been isolated during the last Pleistocene Ice Age, at the end of which (*c.* 12,000 years ago) they dispersed northwards (Esseghir *et al.*, 2000; Aransay *et al.*, 2003). A single *wsp* strain of *Wolbachia* (wPrn) was found in *P. perniciosus*, and the explanations proposed for *P. papatasi* also apply.

Prospects for using Wolbachia *to drive genes into* Phlebotomus *populations*

Three observations favour the use of *Wolbachia* for the genetic manipulation of *P. perniciosus* and *P. papatasi.* Firstly, widespread populations of these sandflies are naturally infected. The absence of natural infections of *Wolbachia* in anopheline mosquitoes (Diptera: Culicidae), the vectors of malaria and dengue, was discouraging for Kittayapong *et al.* (2000). *Wolbachia* might be rarer in neotropical sandflies (Psychodidae), because only one strain was detected (in both *Lutzomyia shannoni* (Dyar) and *L. whitmani* (Antunes & Coutinho)) among the ten species screened (Ono *et al.*, 2001). Few insect species have been sampled extensively for *Wolbachia*, some exceptions being *Drosophila simulans* Sturtevant (Diptera: Drosophilidae) (Hoffmann & Turelli, 1988; Turelli & Hoffmann, 1991; 1995; Turelli *et al.*, 1992; Hoffmann *et al.*, 1996), tsetse flies (Diptera: Glossinidae) (Cheng *et al.*, 2000) and mosquitoes (Kittayapong *et al.*, 2000). Most surveys have involved very small numbers of each species, often taken from colonies, but even so it is clear that *Wolbachia* infects 16–76% of insect species in a range of natural communities (Jeyaprakash & Hoy, 2000).

The second favourable observation was that both sandfly species usually had *Wolbachia* infections in the thorax, where *Leishmania* establishes infections and produces infective forms. *Wolbachia* was at first thought to be restricted to the reproductive tissues of insects (Weeks *et al.*, 2002), but it is now known to be widespread in the somatic tissues of some species, including fruit flies and mosquitoes (Dobson *et al.*, 1999) and tsetse-fly vectors of *Trypanosoma* (Cheng *et al.*, 2000).

The third favourable observation was that some populations of sandflies had high infection rates and densities of *Wolbachia*, which are necessary for the successful genetic manipulation of field populations (Sinkins *et al.*, 1995a; Turelli & Hoffmann, 1999). Iranian populations of *P. papatasi* certainly have these characteristics (table 2; Parvizi *et al.*, 2003), but this was not the case for all populations of *P. perniciosus* (table 1).

The failure to detect associations between sandfly mtDNA haplotypes and *Wolbachia* strains need not indicate an absence of cytoplasmic incompatibility, as the following example illustrates. The mosquito *Culex pipiens* Linnaeus (Diptera: Culicidae) has a number of incompatibility crossing types, but these are not obviously correlated with different mtDNA haplotypes or distinctive *Wolbachia* strains (Guillemaud *et al.*, 1997). The presence of uninfected *Phlebotomus*, too, need not indicate an absence of cytoplasmic incompatibility. For example, imperfect maternal transmission of *Wolbachia*, curing of its infections (by natural antibiotics) and immigration of flies from uninfected populations were sufficient to explain the presence of uninfected *D. simulans* in wild Californian populations (Turelli & Hoffmann, 1995, 1999), even at a time when a strain of *Wolbachia* with a cytoplasmic incompatibility phenotype was spreading through these populations and sweeping along with it a single mtDNA haplotype (Turelli & Hoffmann, 1991; Turelli *et al.*, 1992). Furthermore, the introduction of transgenes into wild populations would not necessarily require *Wolbachia* strains of sandfly origin (Braig *et al.*, 1994). Bi-directional cytoplasmic incompatibility (Sinkins *et al.*, 1995b; Curtis & Sinkins, 1998) would have to be used for the genetic manipulation of sandflies if their naturally occurring *Wolbachia* have a cytoplasmic incompatibility phenotype.

To drive a transgene through a wild insect population, it is first necessary to introduce the engineered gene into some individuals and to couple it to the maternal transmission of *Wolbachia*. Theoretically, this would be best achieved by directly integrating the transgene into the *Wolbachia* genome (Turelli & Hoffmann, 1999), which is feasible if there are insect cell cultures susceptible to *Wolbachia*, as there are for the mosquito *Aedes albopictus* Skuse (Diptera: Culicidae) (O'Neill *et al.*, 1997). Cell cultures are available for *P. papatasi* (Saraiva *et al.*, 2000).

Based on all these considerations, it would be worthwhile to test experimentally for cytoplasmic incompatibility and geographical variability of incompatibility types (Hoffmann & Turelli, 1988; Turelli & Hoffmann, 1995) in *P. perniciosus* and *P. papatasi*. Even then, the rational use of cytoplasmic incompatibility to drive transgenes through sandfly populations would be possible only if more was known about *Wolbachia* population biology. Predictive models of *Wolbachia* spread (Turelli & Hoffmann, 1999) are particularly sensitive to variations in the incidence of paternal transmission (Hoffmann & Turelli, 1988), maternal transmission fidelity (including natural curing), biotic environment (including aging and diapause) and abiotic environment (including temperature). All of these should be assessed for *P. perniciosus* and *P. papatasi*.

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