# Intense Transpositional Activity of Insertion Sequences in an Ancient Obligate Endosymbiont

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The streamlined genomes of ancient obligate endosymbionts generally lack transposable elements, such as insertion sequences (IS). Yet, the genome of *Wolbachia*, one of the most abundant bacterial endosymbionts on Earth, is littered with IS. Such a paradox raises the question as to why there are so many ISs in the genome of this ancient endosymbiont. To address this question, we investigated IS transpositional activity in the unculturable *Wolbachia* by tracking the evolutionary dynamics and history of ISWpi1 elements. We show that 1) ISWpi1 is widespread in *Wolbachia*, being present in at least 55% of the 40 sampled strains, 2) ISWpi1 copies exhibit virtually identical nucleotide sequences both within and among *Wolbachia* genomes and possess an intact transposase gene, 3) individual ISWpi1 copies are differentially inserted among *Wolbachia* genomes, and 4) ISWpi1 occurs at variable copy numbers among *Wolbachia* genomes. Collectively, our results provide compelling evidence for intense ISWpi1 transpositional activity and frequent ISWpi1 horizontal transmission among strains during recent *Wolbachia* evolution. Thus, the genomes of ancient obligate endosymbionts can carry high loads of functional and transpositionally active transposable elements. Our results also indicate that *Wolbachia* genomes have experienced multiple and temporally distinct ISWpi1 invasions during their evolutionary history. Such recurrent exposure to new IS invasions may explain, at least partly, the unusually high density of transposable elements found in the genomes of *Wolbachia* endosymbionts.

## Introduction

Insertion sequences (ISs) are prokaryotic autonomous transposable elements that encode a transposase gene mediating their transposition (i.e., their ability to move to another locus in a genome) (Chandler and Mahillon 2002). ISs are widespread among prokaryotic genomes (e.g., present in >75% of 262 representative genomes surveyed; Touchon and Rocha 2007), in which they can represent substantial proportions (Chandler and Mahillon 2002; Siguier et al. 2006; Filee et al. 2007). However, when host lifestyle is considered, it is notable that ISs are largely missing from the genomes of obligate endosymbionts, that is, intracellular bacteria that replicate exclusively in the cells of other organisms and typically have no extracellular state (Moran and Plague 2004; Bordenstein and Reznikoff 2005; Touchon and Rocha 2007). This is generally ascribed to the confined and isolated intracellular environment in which these bacteria reside, which reduces opportunities for acquisition of genetic material. This view is supported by the strikingly stable genomes of various obligate endosymbionts of insects such as *Buchnera*, which lack IS and have experienced no genomic rearrangement and gene acquisition for the past 50–70 Myr (Tamas et al. 2002). Yet, comparative genomic analyses of various Rickettsiales, a diverse group of intracellular alpha-Proteobacteria, have demonstrated striking exceptions to this pattern in that these genomes exhibit extensive variability in their mobile element content, including IS (Darby et al. 2007). However, the within-species IS dynamics has not been studied for this group of bacteria, making difficult the analysis of the microevolutionary events responsible for this variability.

Within Rickettsiales, *Wolbachia* bacteria are ancient obligate endosymbionts that have been associated with arthropod and nematode hosts for >100 Myr (Rousset et al. 1992; O'Neill et al. 1997; Bandi et al. 1998; Bourtzis and Miller 2003) and possibly represent one of the most abundant bacterial endosymbionts on Earth (Werren, Windsor, and Guo 1995). These maternally inherited bacteria are often referred to as reproductive parasites because they are able to manipulate the reproduction of their arthropod hosts to increase their own transmission (O'Neill et al. 1997; Bourtzis and Miller 2003; Cordaux et al. 2004). In addition to vertical transmission, *Wolbachia* from arthropods are occasionally transmitted horizontally (Werren, Zhang, and Guo 1995; Vavre et al. 1999; Cordaux et al. 2001). Contrary to expectations, genome sequencing of the *Wolbachia* strain harbored by the fruit fly *Drosophila melanogaster* (*w*Mel) revealed an unusually high proportion of repetitive and mobile DNA, including IS (Moran and Plague 2004; Wu et al. 2004; Bordenstein and Reznikoff 2005). This result is particularly significant given that *w*Mel otherwise exhibits many typical features of a long-term symbiotic lifestyle, such as reduced genome size and A + T nucleotide composition richness (Wernegreen 2002; Wu et al. 2004). Such a paradox raises the question as to why there are so many ISs in the genome of this endosymbiont.

To address this question, we investigated IS transpositional activity in the unculturable *Wolbachia* by tracking the evolutionary dynamics and history of ISWpi1, a group of IS related to the IS5 family, the distribution of which is so far exclusively restricted to *Wolbachia* bacteria (Cordaux 2008). Previous results suggest that ISWpi1 transposase may potentially be functional because 1) the 2 overlapping open reading frames constituting ISWpi1 transposase are intact in many copies (Cordaux 2008) and 2) several ISWpi1 copies are differentially inserted in various *Wolbachia* strains (Duron et al. 2005; Iturbe-Ormaetxe et al. 2005; Riegler et al. 2005). Here, we show that *Wolbachia* endosymbionts have recently experienced, and probably continue to experience, high levels of ISWpi1 transpositional activity

**Table 1**
**Distribution of ISWpi1 in 40 *Wolbachia* Strains**

| Host Species (Strain) | Taxonomic Group | Geographic Origin | *Wolbachia* Supergroup | ISWpi1 Presence |
|---|---|---|---|---|
| *Aleochara bilineata* | Insecta, Coleoptera | Canada | A | Yes |
| *Delia radicum* | Insecta, Diptera | Brittany, France | A | Yes |
| *Drosophila ananassae* | Insecta, Diptera | Rio de Janeiro, Brazil | A | Yes |
| *Drosophila auraria* | Insecta, Diptera | Tokyo, Japan | A | Yes |
| *Drosophila melanogaster* (*w*Mel)[a] | Insecta, Diptera | Antibes, France | A | Yes |
| *Drosophila simulans* (*w*Au) | Insecta, Diptera | Yaounde, Cameroon | A | Yes |
| *D. simulans* (*w*Ri) | Insecta, Diptera | Antibes, France | A | Yes |
| *Drosophila suzukii* | Insecta, Diptera | Tokyo, Japan | A | Yes |
| *Drosophila triauraria* | Insecta, Diptera | Tokyo, Japan | A | Yes |
| *Drosophila yakuba* | Insecta, Diptera | Ogoue River, Gabon | A | Yes |
| *Zaprionus sepsoides* | Insecta, Diptera | Sao Tomé | A | Yes |
| *Asobara tabida* (*w*Atab3) | Insecta, Hymenoptera | Antibes, France | A | Yes |
| *Asobara japonica* | Insecta, Hymenoptera | Sapporo, Japan | A | Yes |
| *Leptopilina heterotoma* (*w*Lhet1) | Insecta, Hymenoptera | Antibes, France | A | Yes |
| *Pachycrepoideus dubius* | Insecta, Hymenoptera | France | A | Yes |
| *Amaurobius ferox* | Arachnida, Araneae | Poitiers, France | B | Yes |
| *Segestria florentina* | Arachnida, Araneae | Chizé, France | B | No |
| *Talitrus saltator* | Crustacea, Amphipoda | La Rochelle, France | B | Yes |
| *Lepas anatifera* | Crustacea, Cirripedia | La Rochelle, France | B | No |
| *Armadillidium vulgare* (*w*VulC) | Crustacea, Isopoda | Saint Cyr, France | B | Relic only |
| *A. vulgare* (*w*VulM) | Crustacea, Isopoda | Méry sur Cher, France | B | Relic only |
| *Cylisticus convexus* | Crustacea, Isopoda | Avanton, France | B | Relic only |
| *Helleria brevicornis* | Crustacea, Isopoda | Bastia, France | B | No |
| *Oniscus asellus* | Crustacea, Isopoda | Golbey, France | B | Relic only |
| *Philoscia muscorum* | Crustacea, Isopoda | Poitiers, France | B | No |
| *Platyarthrus hoffmannseggi* | Crustacea, Isopoda | Liniers, France | B | Yes |
| *Porcellio dilatatus petiti* | Crustacea, Isopoda | Saint Honorat, France | B | No |
| *Porcellionides pruinosus* (*w*PruIII) | Crustacea, Isopoda | Nevers, France | B | Relic only |
| *Sphaeroma hookerii* | Crustacea, Isopoda | Graye sur Mer, France | B | No |
| *Sphaeroma rugicauda* | Crustacea, Isopoda | Alresford Creek, United Kingdom | B | No |
| *Drosophila sechellia* (*w*Sn) | Insecta, Diptera | Seychelles Archipelago | B | Yes |
| *Reticulitermes santonensis* | Insecta, Isoptera | Charente, France | B | Yes |
| *Charanyca trigrammica* | Insecta, Lepidoptera | Pinail, France | B | No |
| *Lomaspilis marginata* | Insecta, Lepidoptera | Poitiers, France | B | Yes |
| *Maniola jurtina* | Insecta, Lepidoptera | Poitiers, France | B | No |
| *Peribatodes rhomboidaria* | Insecta, Lepidoptera | Poitiers, France | B | Yes |
| *Spilosoma lubricipeda* | Insecta, Lepidoptera | Poitiers, France | B | No |
| *Dysdera crocata* | Arachnida, Araneae | Chizé, France | G | No |
| *Dysdera erythrina* | Arachnida, Araneae | Saint Benoit, France | G | No |
| *Musca domestica* | Insecta, Diptera | Poitiers, France | G | No |
| Water control | | | — | No |

[a] Used as a positive control because ISWpi1 presence is confirmed by in silico analyses (Wu et al. 2004; Cordaux 2008).

within genomes and horizontal transfers among genomes. Our results thus provide compelling evidence that ancient obligate endosymbionts can carry high loads of functional and transpositionally active transposable elements. This may explain, at least partly, why the genomes of *Wolbachia* endosymbionts are littered with IS.

## Materials and Methods
### *Wolbachia* Strains

Forty *Wolbachia* strains identified from 23 insect (5 different orders), 13 crustacean (3 different orders), and 4 arachnid individual hosts were used (table 1). Some animals originated from laboratory strains, whereas others were caught in the wild. Total DNA was extracted as previously described (Bouchon et al. 1998). To confirm the presence of *Wolbachia* DNA of suitable quality in the samples, 2–3 loci from *Wolbachia* chromosomal DNA (*wsp*, *16S rRNA*, and *GroE*) were amplified by polymerase chain reaction (PCR), as previously described (Bouchon et al. 1998; Cordaux et al. 2001; Verne et al. 2007). Purified

*wsp* PCR products were directly sequenced as previously described (Cordaux et al. 2001). Each of the 40 samples was infected by a single *Wolbachia* strain, as indicated by the lack of ambiguity in the electrophoregrams. Sequences generated in this study were deposited in GenBank under accession numbers EU288004–EU288015.

### ISWpi1 Detection Assay

To investigate the distribution of ISWpi1 among the 40 *Wolbachia* strains, we designed an intra-ISWpi1 PCR assay, using primers internal to the ISWpi1 consensus sequence. A 681-bp long region internal to ISWpi1 was amplified using specific oligonucleotide primers ISWpi1-F (5′-GATCTAAGCGAAAGGGAATGG) and ISWpi1-R (5′-CAACCCATCTTCTTGGCTGT). PCR amplification was performed using a standard protocol, with an annealing temperature of 60 °C (Cordaux et al. 2006). Resulting PCR products were separated on 1.5% agarose gels, stained with ethidium bromide, and visualized using UV fluorescence. To confirm the results, PCR amplifications were performed

at least twice for each sample, and purified PCR products were directly sequenced as above. ISWpi1 sequences were deposited in GenBank under accession numbers EU288016–EU288038 and EU684314–EU684317. To further confirm the results, *Wolbachia* strains inferred to lack ISWpi1 based on the above PCR assay were subjected to a second PCR assay amplifying 197 bp of ISWpi1 internal sequence, using specific oligonucleotide primers ISWpi1-F1 (5′-CGAAAGGGAATGGTCAAGAA) and ISWpi1-R1 (5′-GCTTCTTCCATTGCCTGAAC) and an annealing temperature of 54 °C.

## ISWpi1 Locus Genotyping

To evaluate the timing of ISWpi1 transpositional activity during *Wolbachia* evolution, we assessed the "presence" or "absence" of 24 ISWpi1 copies at orthologous genomic sites in 16 A-supergroup *Wolbachia* strains. Nucleotide sequences of 24 different ISWpi1 copies identified from the *w*Mel, *w*Ana, *w*Sim, and *w*Wil *Wolbachia* genomes (Wu et al. 2004; Salzberg, Dunning Hotopp, et al. 2005; Salzberg, Hotopp, et al. 2005; Cordaux 2008) were downloaded from GenBank along with 500 bp of genomic sequence flanking each element on both sides (when available). Specific oligonucleotide primers were designed in the flanking sequences of each ISWpi1 copy, using the program Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The presence or absence of the 24 ISWpi1 copies was investigated in 12 A-supergroup *Wolbachia* strains from table 1 (strains from *Delia radicum*, *Drosophila suzukii*, and *Pachycrepoideus dubius* were excluded because of insufficient amounts of DNA) using locus-specific PCR assays and confirmed by sequencing of the resulting PCR products, as described above. PCR conditions for each locus, including primer sequences and expected PCR product sizes, are shown in supplementary table S1 (Supplementary Material online). Two loci (*w*Mel#4 and *w*Mel#9 in supplementary table S1, Supplementary Material online) had to be discarded for further analyses because PCR amplification was successful only in the *w*Mel sample. No case of double amplification of expected PCR products for both presence and absence alleles was observed, suggesting homogeneity of the *Wolbachia* population within individual hosts. Sequences were deposited in GenBank under accession numbers EU714507–EU714683. In addition, we performed in silico PCR for 4 A-supergroup *Wolbachia* strains for which genome sequence is available: *w*Mel, *w*Ana, *w*Sim, and *w*Wil (Wu et al. 2004; Salzberg, Dunning Hotopp, et al. 2005; Salzberg, Hotopp, et al. 2005; Cordaux 2008).

## Southern Blotting

To assess ISWpi1 copy number variation among *Wolbachia* strains, approximately 5 μg of total DNA from various samples were digested with *Hin*dIII at 37 °C overnight. *Hin*dIII was chosen because in silico digestion of the *w*Mel genome predicted the 13 *w*Mel ISWpi1 copies to be located on different digested genomic fragments of relevant sizes. Digested DNA was size fractionated on 1% agarose gels

and Southern blotted to nylon membranes. Probes were prepared as internal portions of ISWpi1 amplified by PCR using the aforementioned primers ISWpi1-F and ISWpi1-R. PCR products were labeled using [α-$^{32}$P]-deoxycytidine triphosphate by the random primer method and hybridized overnight to membranes. The final wash was at 52 °C in $0.1 \times$ standard saline citrate. Hybridized blots were imaged and analyzed using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

## Sequence Analyses

Sequences were aligned using ClustalW as implemented in the software Bioedit version 7.0 (Hall 1999), followed by manual adjustments. MEGA version 4 (Tamura et al. 2007) was used to calculate nucleotide sequence divergence and build A- and B-supergroup *Wolbachia* phylogenetic trees using distance-based (Neighbor-Joining [NJ] and unweighted pair group method with arithmetic mean) and character-based (maximum parsimony [MP]) methods. The different methods yielded largely congruent phylogenies, and we show in the paper the trees that displayed the highest confidence levels in branching patterns, as detailed below.

Due to low genetic differentiation among strains (Werren, Zhang, and Guo 1995), distance-based methods yielded A-supergroup *Wolbachia* trees with mostly short branches and low confidence in the branching patterns (i.e., low bootstrap scores). By contrast, MP yielded only 5 equally most parsimonious trees (tree length: 875 steps) that differed only in the branching patterns of the 4 highly closely related *Wolbachia* strains from *Drosophila simulans* (*w*Ri and *w*Sim variants) and *Drosophila ananassae* (2 *w*Ana variants). Overall, this suggested high support for the branching patterns of the MP inference. Based on prior knowledge on strain origins, the most parsimonious tree linking the 2 *D. simulans Wolbachia* variants, on the one hand, and the 2 *D. ananassae Wolbachia* variants, on the other hand, was considered as the most biologically relevant tree. The high consistency index (0.875) provided further support for the MP tree shown in figure 1.

Regarding B-supergroup *Wolbachia* strains, distance-based and MP trees essentially differed on the position of the *Reticulitermes santonensis Wolbachia* strain. However, the MP analysis yielded as many as 190 equally parsimonious trees (tree length: 440 steps), with a consistency index of only 0.745. By contrast, the 2 distance-based methods (which agreed on the branching pattern of the *R. santonensis Wolbachia* strain) were characterized by high bootstrap scores. Hence, 10 out of 14 nodes displayed bootstrap values >95% in the NJ tree, thus providing strong support for the NJ topology.

# Results and Discussion
## Widespread Distribution of ISWpi1 among *Wolbachia* Strains

The taxonomic distribution of ISWpi1 is apparently restricted to *Wolbachia* bacteria, as found earlier by Blast
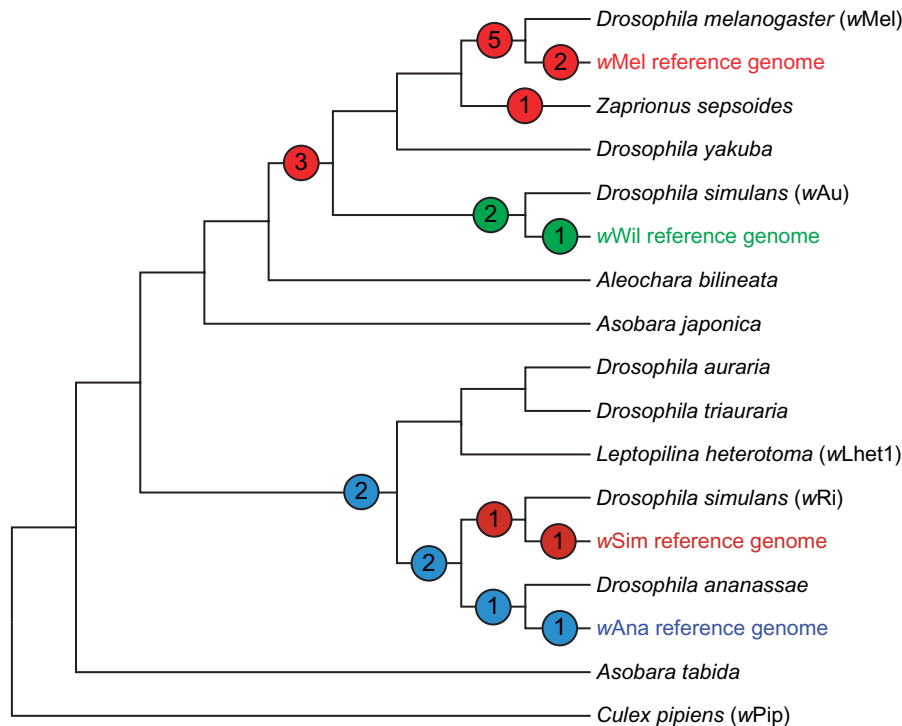
FIG. 1.—Distribution of 22 ISWpi1 copies isolated from the wMel (red), wWil (green), wSim (brown), and wAna (blue) reference genome sequences. Colored circles highlight the numbers of inferred absence/presence transitions of ISWpi1 copies in different branches of the phylogenetic tree of 16 A-supergroup *Wolbachia* strains. The tree was reconstructed by MP (based on 9,782 bp of sequence flanking the 22 ISWpi1 loci and the *wsp* gene) and rooted using the B-supergroup *Wolbachia* strain from *Culex pipiens* (Sanger Institute, http://www.sanger.ac.uk/Projects/W_pipientis/). Branch length is arbitrary. *Wolbachia* strains are identified by the host species from which they were isolated.

searches against the entire GenBank database and all prokaryote genomes listed in the "microbial genomes" section of GenBank (Cordaux 2008). In this study, we confirm ISWpi1 restricted distribution even though new sequence data have been added to GenBank since previous searches. Using a PCR-based ISWpi1 detection assay, we screened a panel of 40 diverse *Wolbachia* strains belonging to the A, B, and G *Wolbachia* supergroups (table 1). A PCR fragment of the expected size (681 bp) was obtained in 22 out of the 40 tested *Wolbachia* strains. Absence of the expected 681-bp long PCR fragment in some strains is unlikely to be caused by systematic PCR failure due to primer mismatches because average ISWpi1 sequence divergence across 22 *Wolbachia* strains is only 0.22% (see below), indicating that 2 full-length ISWpi1 sequences are expected to differ by only 2 substitutions on average. Moreover, *Wolbachia* strains inferred to lack ISWpi1 based on the first PCR assay were subjected to a second ISWpi1 detection assay, which confirmed the initial results.

ISWpi1 was not uniformly distributed among *Wolbachia* supergroups ($P < 10^{-5}$, Fisher's exact test). It was present in all 15 A-supergroup *Wolbachia* strains screened (table 1), in agreement with its presence in all A-supergroup *Wolbachia* strains for which genomic information is available (Cordaux 2008). By contrast, ISWpi1 was found in only 32% (7/22) of B-supergroup and none (0/3) of the G-supergroup *Wolbachia* strains tested (table 1). Overall, these results indicate that ISWpi1 is widespread among

*Wolbachia* endosymbionts because it is present in the genomes of 55% of all *Wolbachia* strains tested.

### Extreme ISWpi1 Sequence Homogeneity within and among *Wolbachia* Strains

To investigate ISWpi1 nucleotide variation, we compared the ISWpi1 sequences obtained from the 22 *Wolbachia* strains identified above as possessing ISWpi1. PCR products were directly sequenced to simultaneously sequence homologous regions from multiple ISWpi1 copies possibly occurring within a single *Wolbachia* genome. Lack of ambiguous sites in the sequence trace files suggested extremely low to no nucleotide divergence among the different ISWpi1 copies occurring within each *Wolbachia* genome. This result is consistent with the virtual lack of nucleotide variation previously recorded among the ISWpi1 copies present within various sequenced *Wolbachia* genomes (Cordaux 2008). However, some private substitutions might have remained undetected with this sequencing strategy. Thus, the 22 ISWpi1 sequences can actually be viewed as consensus sequences of all individual ISWpi1 copies inserted within each of the analyzed *Wolbachia* genomes, making them useful for comparisons among strains. Overall, nucleotide divergence of the 22 ISWpi1 sequences from the various A- and B-supergroup *Wolbachia* strains was only 0.22%. This virtual lack of ISWpi1 sequence variation among *Wolbachia* genomes is in sharp

contrast with the ~3.7% average nucleotide divergence among *Wolbachia* supergroups A and B recorded for 8 highly conserved housekeeping genes (range: 2.2–4.9%) and even much lower than the divergence (~0.7%) observed for the extremely conserved 16S rRNA gene (Paraskevopoulos et al. 2006).

Purifying selection acting on ISWpi1 transposase genes is unlikely to account for this extreme ISWpi1 sequence homogeneity because it would imply that selection for transposition is stronger than selection constraining housekeeping genes essential for *Wolbachia* metabolism. Maintaining such intense levels of purifying selection on ISWpi1 sequences seems further implausible given the elevated evolutionary rates and relative inefficiency of natural selection in endosymbiotic bacteria with reduced effective population sizes, such as *Wolbachia* (Wu et al. 2004). Gene conversion (i.e., the nonindependent evolution of repetitive DNA sequences) could explain the homogeneity of ISWpi1 copies within *Wolbachia* genomes, but it cannot account for the homogeneity of ISWpi1 among *Wolbachia* genomes. Therefore, the most likely explanation for the presence of highly homogeneous ISWpi1 sequences in *Wolbachia* strains as divergent as those belonging to different supergroups is that ISWpi1 has been transpositionally active and laterally acquired by diverse *Wolbachia* strains during very recent evolutionary times (Wagner 2006).

## Recent and Intense ISWpi1 Transpositional Activity

To evaluate the timing of ISWpi1 transpositional activity during *Wolbachia* evolution, we analyzed the phylogenetic distribution of 22 individual ISWpi1 copies in 16 A-supergroup *Wolbachia* strains. This approach allowed us to pinpoint transitions between absence and presence of individual ISWpi1 copies, which are signatures of transpositional activity, during A-supergroup *Wolbachia* evolutionary history. Some transitions might have been overlooked because ISWpi1 status could not be determined for some loci in some taxa. We emphasize, however, that it would not affect our conclusions based on a conservative set of unambiguously determined transitions.

We were able to map presence/absence transitions to the *Wolbachia* phylogeny for 11 *w*Mel ISWpi1 copies. Our results indicated that none of the ISWpi1 copies is shared by all A-supergroup *Wolbachia* strains (fig. 1 and supplementary table S2 [Supplementary Material online]). Instead, all copies showed very narrow strain distributions. Hence, 7 ISWpi1 copies identified from the *w*Mel genome sequence were apparently specific to *w*Mel. The other copies were shared with just a few closely related *Wolbachia* strains that exhibit >99% nucleotide sequence identity with *w*Mel based on the hypervariable *Wolbachia*-specific *wsp* gene (Charlat et al. 2003, 2004). In fact, 2 copies have presumably been transpositionally active so recently in *w*Mel that they are polymorphic for insertion presence or absence among different geographic *w*Mel variants. Specifically, ISWpi1 copies at loci *w*Mel#6 and *w*Mel#12 isolated from the sequenced *w*Mel genome (Wu et al. 2004; Cordaux 2008) were absent from our *w*Mel sample originating from France. Although *w*Mel#6 (WD0516-0517 in the original

*w*Mel genome annotation) has previously been shown to be polymorphic (Riegler et al. 2005), we identified here *w*Mel#12 as a novel polymorphic marker that may prove useful for studies of *Wolbachia* diversity and evolution in *D. melanogaster*.

To test if the very recent ISWpi1 transpositional activity suggested by the transition patterns of ISWpi1 copies isolated from *w*Mel can be generalized to other ISWpi1 copies, we extended our analysis to 11 additional ISWpi1 copies isolated from the partial genome sequences of *w*Ana (6 loci), *w*Sim (2 loci), and *w*Wil (3 loci). Again, all ISWpi1 copies exhibited very narrow strain distributions (fig. 1 and supplementary table S2 [Supplementary Material online]). Even the 2 most widely distributed ISWpi1 copies isolated from *w*Ana were found in closely related *Wolbachia* strains that are identical based on the hypervariable *Wolbachia*-specific *wsp* gene (Miller and Riegler 2006).

Next, we assessed ISWpi1 copy number variation among A-supergroup *Wolbachia* strains by Southern blotting. Results indicated that the number of distinct bands (i.e., putative distinct copies) for A-supergroup *Wolbachia* strains varies from 7 to 13 copies (fig. 2). These figures are in line with the copy numbers estimated from genome sequence data for other A-supergroup *Wolbachia* strains (Cordaux 2008). Interestingly, there are approximately twice as many ISWpi1 copies in *w*Mel compared with the closely related *w*Au, whereas there are similar copy numbers between *w*Mel and the distantly related *w*Ana (fig. 2 and Cordaux 2008).

Overall, extensive heterogeneity in ISWpi1 copy numbers among *Wolbachia* strains, along with very narrow distribution of 22 individual ISWpi1 copies identified from 4 different host genomes and extreme ISWpi1 sequence homogeneity, provides compelling evidence for intense ISWpi1 transpositional activity during recent *Wolbachia* evolution. We emphasize that the extensive polymorphism observed, both in terms of overall copy numbers and patterns of presence or absence of individual copies among *Wolbachia* strains, may result from a combination of insertion events and secondary excisions. In any event, this testifies to the intense transpositional activity that *Wolbachia* endosymbionts have recently experienced and may continue to currently experience. ISWpi1 recent transposition in various *Wolbachia* strains is further supported by the fact that the 2 overlapping open reading frames constituting ISWpi1 transposase are intact in all sequenced portions, suggesting that there are sources of functional transposases in all A- and B-supergroup *Wolbachia* genomes containing ISWpi1 we analyzed. If so, our results provide strong evidence that the genomes of ancient obligate endosymbionts can carry high loads of functional and active transposable elements.

## Frequent ISWpi1 Horizontal Transfers during Recent *Wolbachia* Evolution

The ubiquitous presence of ISWpi1 in the *Wolbachia* A supergroup, coupled with reduced levels of sharing of individual copies among *Wolbachia* strains, suggests that some *Wolbachia* strains may have independently acquired
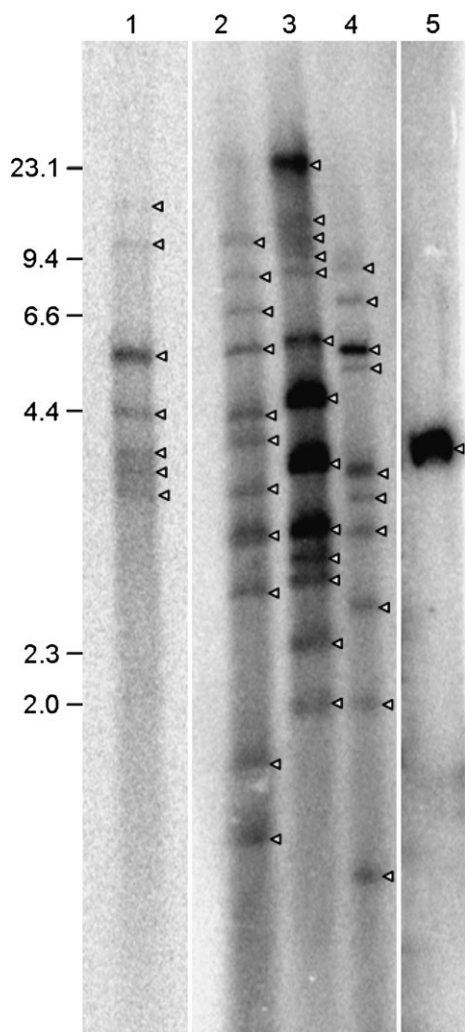
FIG. 2.—Southern blotting of *Hind*III-digested DNA. Lanes: *Wolbachia* strains from *Drosophila simulans* *w*Au (1), *Drosophila melanogaster* (2), *Asobara japonica* (3), *Drosophila ananassae* (4), and *Armadillidium vulgare* *w*VulC (5). Figures on the left indicate fragment sizes (kb). White triangles highlight the positions of the fragments. A single band was detected in *w*VulC, which presumably corresponds to the ISWpi1 relic identified by PCR and sequencing (see main text). Other *Wolbachia* strains exhibit from 7 to 13 distinct bands.

ISWpi1 via lateral transfers. To estimate the number of independent ISWpi1 acquisitions in the *Wolbachia* B supergroup, we analyzed ISWpi1 distribution according to bacterial strain phylogenetic relationships (fig. 3). At this level of resolution, the presence of ISWpi1 in B-supergroup *Wolbachia* strains putatively results from at least 4 independent acquisitions (fig. 3). This may be an underestimate because 1) a higher phylogenetic resolution in the *Lomaspilis marginata*/*Talitrus saltator*/*Amaurobius ferox* group of closely related *Wolbachia* strains might result in the inference of additional independent ISWpi1 acquisitions, 2) a larger screening of *Wolbachia* strains for ISWpi1 presence might uncover additional acquisition events, and 3) one cannot formally exclude that ISWpi1 has been transferred several times to individual *Wolbachia* strains. In any event, these results suggest that horizontal transmission may be a major determinant of the current ISWpi1 distri-

bution among *Wolbachia* strains. Only limited cases of horizontal transfers of mobile DNA in obligate endosymbiotic bacteria have been reported previously, including a plasmid in *Buchnera* (Van Ham et al. 2000), a bacteriophage in *Wolbachia* (Bordenstein and Wernegreen 2004; Gavotte et al. 2007), and a putative conjugative element in *Rickettsia* (Blanc et al. 2007). ISWpi1 from *Wolbachia* is the first transposable element unambiguously shown to horizontally transfer in obligate endosymbiotic bacteria.

Frequent ISWpi1 transfers among different *Wolbachia* strains could be facilitated by the occasional co-occurrence of divergent *Wolbachia* strains within the same host cells (Vavre et al. 1999; Bordenstein and Wernegreen 2004), as well as the presence of bacteriophage WO in many *Wolbachia* genomes (Bordenstein and Wernegreen 2004; Wu et al. 2004; Braquart-Varnier et al. 2005; Gavotte et al. 2007) that could serve as a shuttle for efficiently transferring genetic material among strains. Consistently, the *w*Bm *Wolbachia* genome from the nematode *Brugia malayi* that lacks bacteriophage WO (Foster et al. 2005) also lacks recent ISWpi1 copies (Cordaux 2008). On the other hand, bacteriophage WO distribution seems restricted to *Wolbachia*, and it has never been found in other bacteria to date (Bordenstein and Wernegreen 2004; Gavotte et al. 2007), which could also contribute to explain why ISWpi1 taxonomic distribution also appears restricted to *Wolbachia* (Cordaux 2008). If so, *Wolbachia* bacteria may constitute a highly dynamic system for genetic exchanges among strains (Bordenstein and Wernegreen 2004), whereas at the same time being less prone to exchanges with other bacterial species, perhaps as a result of the specialization of vectors involved in IS horizontal transfer.

## Why So Many ISs in *Wolbachia* Genomes?

While investigating ISWpi1 distribution by PCR in 40 *Wolbachia* strains, we amplified ISWpi1 "relics" from the genomes of 5 B-supergroup *Wolbachia* strains: a 312-bp fragment in 4 *Wolbachia* strains (including *w*VulC) and a 550-bp fragment in 1 *Wolbachia* strain (table 1). DNA sequencing revealed that the shorter and longer fragments exhibited 12.3% and 10.4% nucleotide divergence with ISWpi1, respectively, and 20.1% with each other. In addition, both fragments were severely truncated compared with ISWpi1 due to multiple internal deletions and both were lacking any significant coding capacity. Southern blotting of *w*VulC *Wolbachia* strain DNA against an ISWpi1 probe identified a single band (fig. 2), suggesting that the ISWpi1 relic identified above is the only ISWpi1 copy currently inserted in the *w*VulC genome. Other highly divergent copies have also been reported from the B-supergroup *w*Pip and D-supergroup *w*Bm *Wolbachia* strains (Duron et al. 2005; Cordaux 2008), suggesting an ancient presence of ISWpi1 in *Wolbachia* genomes. Because our PCR-based strategy was designed to preferentially detect ISWpi1 copies closely related to the ISWpi1 consensus sequence (i.e., presumably recent copies), it is possible that some ISWpi1 relics have remained undetected in our screening. Thus, the distribution of ISWpi1 relics among *Wolbachia* genomes may be underestimated.
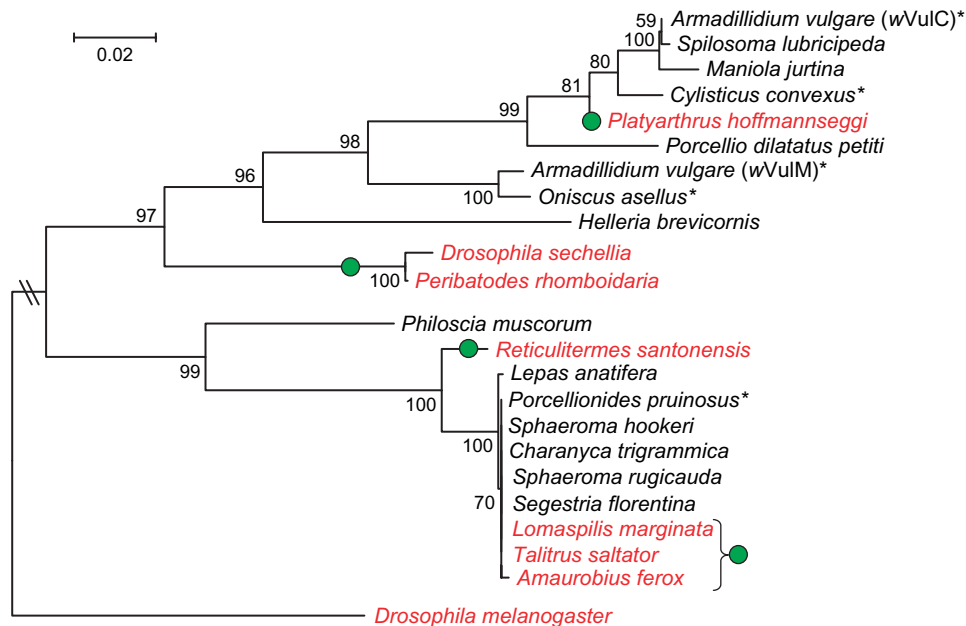
Fig. 3.—NJ tree of 22 B-supergroup *Wolbachia* strains for which ISWpi1 presence or absence is known, based on *wsp* sequences and the Kimura 2-parameter substitution model. Bootstrap values (based on 1,000 replicates) are shown on branches (%). The tree was rooted using the A-supergroup *Wolbachia* strain from *Drosophila melanogaster*. The broken line indicates that the branch is not drawn to scale. *Wolbachia* strains are identified by the host species from which they were isolated. Strains possessing (red) or lacking (black) ISWpi1 copies are shown. Stars indicate strains possessing ISWpi1 relics. Green circles indicate the putative independent ISWpi1 acquisitions.

Overall, our results are consistent with a scenario in which ISs recurrently invade and then go extinct in bacterial genomes (Wagner 2006) so that ancient relics and recent ISWpi1 copies represent temporally distinct ISWpi1 invasions of *Wolbachia* genomes. It has been proposed that IS could be maintained in *Wolbachia* genomes because they confer a selective advantage to their bacterial hosts (Brownlie and O'Neill 2005; Foster et al. 2005). Alternatively, it is possible that ISs are maintained in *Wolbachia* simply as a consequence of the inefficiency of host genomes to eliminate them (Wu et al. 2004). The rationale underlying this hypothesis is that symbiotic bacteria tend to have small effective population sizes, thus rendering selection against deleterious mutations and transposable element insertions less efficient (Wu et al. 2004). The evolutionary history and dynamics of ISWpi1 suggest yet another explanation: *Wolbachia* genomes are recurrently exposed to new IS invasions (Bordenstein and Wernegreen 2004).

## Conclusion

It is generally considered that IS proliferation characterizes lineages that have recently evolved toward an obligate endosymbiotic lifestyle (Moran and Plague 2004; Plague et al. 2008). By contrast, ancient obligate endosymbionts typically lack IS because of degradation of old insertions and absence of exposure to new transposition events (Moran and Plague 2004). Unexpectedly, our results show that at least a subset of all IS copies of the obligate endosymbiont *Wolbachia* are not remnants of ancient IS proliferation following the shift to endosymbiotic lifestyle at an earlier stage of *Wolbachia* evolution. Instead, *Wolbachia* experi-

ence recurrent invasions by new IS, which may explain, at least partly, the unusually high density of transposable elements found in the genomes of these endosymbionts.

## Supplementary Material

Supplementary tables S1 and S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

## Literature Cited

Bandi C, Anderson TJ, Genchi C, Blaxter ML. 1998. Phylogeny of Wolbachia in filarial nematodes. Proc Biol Sci. 265:2407–2413.

Blanc G, Ogata H, Robert C, Audic S, Claverie JM, Raoult D. 2007. Lateral gene transfer between obligate intracellular

bacteria: evidence from the Rickettsia massiliae genome. Genome Res. 17:1657–1664.

Bordenstein SR, Reznikoff WS. 2005. Mobile DNA in obligate intracellular bacteria. Nat Rev Microbiol. 3:688–699.

Bordenstein SR, Wernegreen JJ. 2004. Bacteriophage flux in endosymbionts (Wolbachia): infection frequency, lateral transfer, and recombination rates. Mol Biol Evol. 21:1981–1991.

Bouchon D, Rigaud T, Juchault P. 1998. Evidence for widespread Wolbachia infection in isopod crustaceans: molecular identification and host feminization. Proc Biol Sci. 265:1081–1090.

Bourtzis K, Miller TA. 2003. Insect symbiosis. Boca Raton (FL): CRC Press.

Braquart-Varnier C, Greve P, Felix C, Martin G. 2005. Bacteriophage WO in Wolbachia infecting terrestrial isopods. Biochem Biophys Res Commun. 337:580–585.

Brownlie JC, O'Neill SL. 2005. Wolbachia genomes: insights into an intracellular lifestyle. Curr Biol. 15:R507–R509.

Chandler M, Mahillon J. 2002. Insertion sequences revisited. In: CR, Craig NL, Gellert M, Lambowitz AM, editors. Mobile DNA II. Washington (DC): ASM Press. p. 305–366.

Charlat S, Ballard JW, Mercot H. 2004. What maintains noncytoplasmic incompatibility inducing Wolbachia in their hosts: a case study from a natural Drosophila yakuba population. J Evol Biol. 17:322–330.

Charlat S, Le Chat L, Mercot H. 2003. Characterization of noncytoplasmic incompatibility inducing Wolbachia in two continental African populations of Drosophila simulans. Heredity. 90:49–55.

Cordaux R. 2008. ISWpi1 from Wolbachia pipientis defines a novel group of insertion sequences within the IS5 family. Gene. 409:20–27.

Cordaux R, Lee J, Dinoso L, Batzer MA. 2006. Recently integrated Alu retrotransposons are essentially neutral residents of the human genome. Gene. 373:138–144.

Cordaux R, Michel-Salzat A, Bouchon D. 2001. Wolbachia infection in crustaceans: novel hosts and potential routes for horizontal transmission. J Evol Biol. 14:237–243.

Cordaux R, Michel-Salzat A, Frelon-Raimond M, Rigaud T, Bouchon D. 2004. Evidence for a new feminizing Wolbachia strain in the isopod Armadillidium vulgare: evolutionary implications. Heredity. 93:78–84.

Darby AC, Cho NH, Fuxelius HH, Westberg J, Andersson SG. 2007. Intracellular pathogens go extreme: genome evolution in the Rickettsiales. Trends Genet. 23:511–520.

Duron O, Lagnel J, Raymond M, Bourtzis K, Fort P, Weill M. 2005. Transposable element polymorphism of Wolbachia in the mosquito Culex pipiens: evidence of genetic diversity, superinfection and recombination. Mol Ecol. 14:1561–1573.

Filee J, Siguier P, Chandler M. 2007. Insertion sequence diversity in archaea. Microbiol Mol Biol Rev. 71:121–157.

Foster J, Ganatra M, Kamal I, et al. (23 co-authors). 2005. The Wolbachia genome of Brugia malayi: endosymbiont evolution within a human pathogenic nematode. PLoS Biol. 3:e121.

Gavotte L, Henri H, Stouthamer R, Charif D, Charlat S, Bouletreau M, Vavre F. 2007. A survey of the bacteriophage WO in the endosymbiotic bacteria Wolbachia. Mol Biol Evol. 24:427–435.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser. 41:95–98.

Iturbe-Ormaetxe I, Burke GR, Riegler M, O'Neill SL. 2005. Distribution, expression, and motif variability of ankyrin domain genes in Wolbachia pipientis. J Bacteriol. 187:5136–5145.

Miller WJ, Riegler M. 2006. Evolutionary dynamics of wAu-like Wolbachia variants in neotropical Drosophila spp. Appl Environ Microbiol. 72:826–835.

Moran NA, Plague GR. 2004. Genomic changes following host restriction in bacteria. Curr Opin Genet Dev. 14:627–633.

O'Neill SL, Hoffmann AA, Werren JH. 1997. Influential passengers: inherited microorganisms and arthropod reproduction. New York: Oxford University Press.

Paraskevopoulos C, Bordenstein SR, Wernegreen JJ, Werren JH, Bourtzis K. 2006. Toward a Wolbachia multilocus sequence typing system: discrimination of Wolbachia strains present in Drosophila species. Curr Microbiol. 53:388–395.

Plague GR, Dunbar HE, Tran PL, Moran NA. 2008. Extensive proliferation of transposable elements in heritable bacterial symbionts. J Bacteriol. 190:777–779.

Riegler M, Sidhu M, Miller WJ, O'Neill SL. 2005. Evidence for a global Wolbachia replacement in Drosophila melanogaster. Curr Biol. 15:1428–1433.

Rousset F, Bouchon D, Pintureau B, Juchault P, Solignac M. 1992. Wolbachia endosymbionts responsible for various alterations of sexuality in arthropods. Proc Biol Sci. 250:91–98.

Salzberg SL, Dunning Hotopp JC, Delcher AL, Pop M, Smith DR, Eisen MB, Nelson WC. 2005. Correction: serendipitous discovery of Wolbachia genomes in multiple Drosophila species. Genome Biol. 6:402.

Salzberg SL, Hotopp JC, Delcher AL, Pop M, Smith DR, Eisen MB, Nelson WC. 2005. Serendipitous discovery of Wolbachia genomes in multiple Drosophila species. Genome Biol. 6:R23.

Siguier P, Filee J, Chandler M. 2006. Insertion sequences in prokaryotic genomes. Curr Opin Microbiol. 9:526–531.

Tamas I, Klasson L, Canback B, Naslund AK, Eriksson AS, Wernegreen JJ, Sandstrom JP, Moran NA, Andersson SG. 2002. 50 million years of genomic stasis in endosymbiotic bacteria. Science. 296:2376–2379.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol. 24:1596–1599.

Touchon M, Rocha EP. 2007. Causes of insertion sequences abundance in prokaryotic genomes. Mol Biol Evol. 24:969–981.

Van Ham RC, Gonzalez-Candelas F, Silva FJ, Sabater B, Moya A, Latorre A. 2000. Postsymbiotic plasmid acquisition and evolution of the repA1-replicon in Buchnera aphidicola. Proc Natl Acad Sci USA. 97:10855–10860.

Vavre F, Fleury F, Lepetit D, Fouillet P, Bouletreau M. 1999. Phylogenetic evidence for horizontal transmission of Wolbachia in host-parasitoid associations. Mol Biol Evol. 16:1711–1723.

Verne S, Johnson M, Bouchon D, Grandjean F. 2007. Evidence for recombination between feminizing Wolbachia in the isopod genus Armadillidium. Gene. 397:58–66.

Wagner A. 2006. Periodic extinctions of transposable elements in bacterial lineages: evidence from intragenomic variation in multiple genomes. Mol Biol Evol. 23:723–733.

Wernegreen JJ. 2002. Genome evolution in bacterial endosymbionts of insects. Nat Rev Genet. 3:850–861.

Werren JH, Windsor D, Guo L. 1995. Distribution of Wolbachia among neotropical arthropods. Proc Biol Sci. 262:197–204.

Werren JH, Zhang W, Guo LR. 1995. Evolution and phylogeny of Wolbachia: reproductive parasites of arthropods. Proc Biol Sci. 261:55–63.

Wu M, Sun LV, Vamathevan J, et al. (30 co-authors). 2004. Phylogenomics of the reproductive parasite Wolbachia pipientis wMel: a streamlined genome overrun by mobile genetic elements. PLoS Biol. 2:E69.