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Evolutionary Dynamics of *w*Au-Like *Wolbachia* Variants in Neotropical *Drosophila* spp.

Wolfgang J. Miller[1]* and Markus Riegler[2]

Laboratories of Genome Dynamics, Center of Anatomy and Cell Biology, Medical University of Vienna, Vienna, Austria,[1] *and School of Integrative Biology, University of Queensland, St. Lucia, Queensland 4072, Australia*[2]

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***Wolbachia* bacteria are common intracellular symbionts of arthropods and have been extensively studied in *Drosophila*. Most research focuses on two Old World hosts, *Drosophila melanogaster* and *Drosophila simulans*, and does not take into account that some of the *Wolbachia* associations in these species may have evolved only after their fast global expansion and after the exposure to *Wolbachia* of previously isolated habitats. Here we looked at *Wolbachia* of Neotropical *Drosophila* species. Seventy-one lines of 16 Neotropical *Drosophila* species sampled in different regions and at different time points were analyzed. *Wolbachia* is absent in lines of *Drosophila willistoni* collected before the 1970s, but more recent samples are infected with a strain designated *w*Wil. *Wolbachia* is absent in all other species of the willistoni group. Polymorphic *w*Wil-related strains were detected in some saltans group species, with *D. septentriosaltans* being coinfected with at least four variants. Based on *wsp* and *ftsZ* sequence data, *w*Wil of *D. willistoni* is identical to *w*Au, a strain isolated from *D. simulans*, but can be discriminated when using a polymorphic minisatellite marker. In contrast to *w*Au, which infects both germ line and somatic tissues of *D. simulans*, *w*Wil is found exclusively in the primordial germ line cells of *D. willistoni* embryos. We report on a pool of closely related *Wolbachia* strains in Neotropical *Drosophila* species as a potential source for the *w*Au strain in *D. simulans*. Possible evolutionary scenarios reconstructing the infection history of *w*Au-like *Wolbachia* in Neotropical *Drosophila* species and the Old World species *D. simulans* are discussed.**

Wolbachia strains are intracellular gram-negative, vertically transmitted *Alphaproteobacteria* that infect at least 20% of all insects (24, 47). In *Drosophila*, *Wolbachia* infections are capable of inducing cytoplasmic incompatibility (CI) or male killing (34). The CI phenotype increases the fitness of *Wolbachia*-infected females relative to uninfected females and drives *Wolbachia* through host populations. In recent years scientific interest has broadly focused on the evolutionary and functional interactions between *Wolbachia* and genetic model systems such as *D. melanogaster* and *D. simulans*, two well-studied Old World species belonging to the melanogaster group (42). In *D. melanogaster*, a single infection variant, *w*Mel (50), had been described until not long ago (36). This infection is associated with variable levels of CI in its natural host. In *D. simulans*, five *Wolbachia* variants have been described: *w*Ri, *w*Ha, and *w*No, which can induce CI, and *w*Ma and *w*Au, which generally do not (29). Strains *w*Mel of *D. melanogaster* and *w*Au of *D. simulans* are closely related in respect to the most sensitive molecular gene marker sets of *wsp* (50) and *ftsZ* (9, 35). There is a complete lack of *wsp* sequence polymorphism within *w*Mel (36) and *w*Au (2, 23), which suggests either a strict clonality of the parasite or a recent acquisition by their host species. The phylogenetic relationship of these two *Wolbachia* strains has previously been analyzed (see, e.g., references 9, 21, and 50); however, the evolutionary origins of both the *w*Au and *w*Mel associations remain unclear, including a possible recent acquisition from other host species after the global expansion of both Old World *Drosophila* species.

In contrast to the well-studied *Wolbachia* associations in *D. melanogaster* and *D. simulans*, little is known about the occurrence of *Wolbachia* among American Neotropical *Drosophila* strains comprising two groups of species, the saltans group and the willistoni group (Fig. 1). There are presently two conflicting reports about the occurrence of *Wolbachia* in Neotropical *Drosophila*: Bourtzis et al. (4) screened a broad range of *Drosophila* species derived from various labs and from the Drosophila Species Stock Center (DSSC) in Bowling Green, Ohio (now held at the University of Arizona, Tucson). In their survey only two species out of the 41 stocks comprising 30 species were infected with *Wolbachia*. Interestingly, none of the analyzed DSSC fly lines was infected. The six Neotropical *Drosophila* species surveyed, including *D. willistoni*, *D. prosaltans*, and *D. sturtevanti*, were uninfected (4). The Neotropical samples surveyed originated from iso- or oligofemale lines kept at the DSSC since the 1950s. In contrast, Werren et al. (46) reported that a natural population of *D. willistoni* collected in the early 1990s in Panama was infected with *Wolbachia*. Its presence in *D. willistoni* was recently confirmed by discovering partial fragments of a *Wolbachia* genome in the Trace Archive of the *D. willistoni* genome sequencing project (37; J. Brownlie, personal communication). The genome sequence was derived from an isofemale line collected in the early 1990s in Guadeloupe (L. Ehrman, personal communication).

Here we reevaluated the *Wolbachia* infection status of Neotropical *Drosophila* species by conducting a large-scale survey. Seventy-one lines of 16 Neotropical *Drosophila* species belonging to the willistoni and saltans groups were searched for *Wolbachia*. We compared the occurrence of infection in old versus

* Corresponding author. Mailing address: Laboratories of Genome Dynamics, Center of Anatomy and Cell Biology, Medical University of Vienna, Währingerstr. 10, A-1090 Vienna, Austria. Phone: 43-1-4277-60624. Fax: 43-1-4277-60690. E-mail: wolfgang.miller@meduniwien.ac.at.

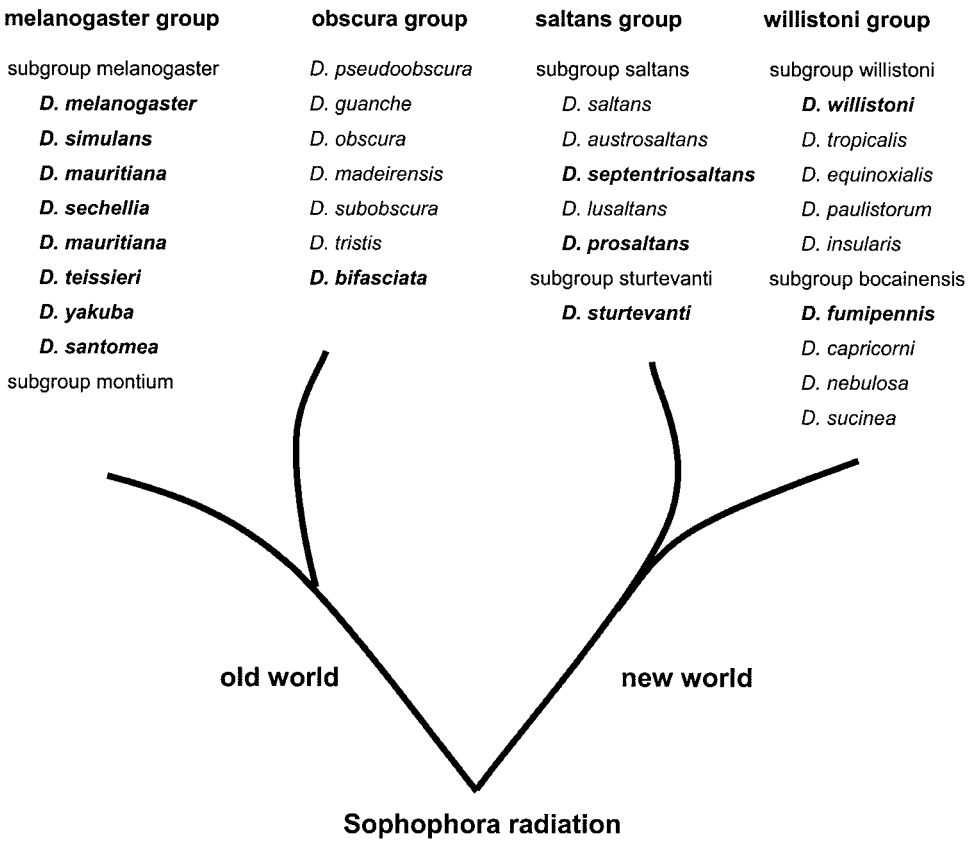

FIG. 1. Phylogenetic relationship of the *Sophophora* radiation (41). The *Wolbachia* infection statuses of the *Drosophila* species shown were deduced from data published previously (melanogaster and obscura groups) and from this study (saltans and willistoni groups). In boldface are species that have been found to be infected with various *Wolbachia* strains based on *wsp* sequences (see Materials and Methods).

recent population samples of different geographic origins and increased replicate numbers of analyzed lines per species, as analysis of only one or a few iso- or oligofemale lines would not detect low infection frequencies in species. Different diagnostic tools such as *Wolbachia*-specific *wsp* and *ftsZ* PCR, Southern blot hybridizations, and immunological diagnostic methods were applied for this purpose.

MATERIALS AND METHODS

***Drosophila* strains.** Fly samples were kindly provided by colleagues Margaret Kidwell, Egon Bartel, Kim van der Linde, Francesco Ayala, Jeff Powell, and Peter Chabora and by the DSSC, Tucson, Ariz. (For details about geographic origin, collector's name, and date of collection, see Tables 1 to 3.) All strains were kept on standard fly food in vials at a constant temperature of 21°C.

PCR diagnostics, cloning, sequencing, and strain typing. Genomic DNAs derived from single adult female flies were extracted according to the single-fly PCR protocol (14), and the quality of fly genomic DNAs was tested by control PCR experiments carried out with primers binding to conserved segments in exon 2 and exon 3 of the *Adh* gene (15). *Wolbachia*-specific PCRs were performed as previously described (24). In brief, 2 μl of the 50-μl single-fly sample was added to 20 μl of PCR mix (0.75 U *Taq* DNA polymerase [Promega] in 1× reaction buffer, 0.10 μM of each primer, and 75 μM of each deoxynucleoside triphosphate). PCR primer sets were used as described previously (24). The *Wolbachia* infection of *D. willistoni* was discriminated from *w*Au infection of *D. simulans* by the hypervariable VNTR-141 locus in *w*Mel (primer set VNTR-141F-R), isolated by Riegler et al. (36). At least two independent PCRs were analyzed per sample. PCR fragments of the expected size were gel eluted, cloned into the pGEM-T Easy vector, and transformed into JM109 (Promega). Both strands of each clone were sequenced by GENterprise GmbH, Mainz, Germany. *Wolbachia* strain names were assigned to *wsp* sequence variants deriving from different hosts according to current standards (34, 50). This is important in order to keep the ecological origin of the *Wolbachia* symbiosis transparent. The highly polymorphic *wsp* gene undergoes homologous recombination among strains, which is problematic for an evolutionary analysis of the symbiosis (1). Therefore, we used a multilocus approach, including *wsp* and *ftsZ* genes as well as the VNTR-141 locus.

Phylogenetic analysis. Multiple *wsp* sequence alignments, including the hypervariable regions (bases 217 to 252 and 520 to 582), were generated using the Clustal X program (40). Alignments were based on amino acid translations followed by manual modifications. A base substitution was included in the analysis if it occurred in two or more plasmid clones obtained from independent PCRs. Other substitutions were eliminated. The final alignment is available at ftp://ftp.ebi.ac.uk/pub/databases/embl/align/under accession number ALIGN_000917. Phylogenetic trees were constructed by applying PAUP* (39) in the absence of an available outgroup. Neighbor-joining analyses after midpoint rooting and unweighted-pair group method with arithmetic mean analyses yielded similar phylogenies, supporting the close relationship of *w*Au-like *Wolbachia* variants.

Single-fly Southern hybridization. DNA extraction from individual 10-day-old female flies, restriction digestion with HindIII, vertical agarose gel separation, and membrane blotting were performed according to the protocol described by Junakovic (26). Nylon membranes were probed with the eluted *wsp* PCR fragment of *w*Wil derived from the *D. willistoni* strain Pan 02 (Table 1) cloned into the pGEM-T Easy vector.

Semiquantitative genomic *wsp* PCRs. The density of *Wolbachia* in *D. willistoni* was determined by semiquantitative *wsp* PCRs on 10 individual adult females of staged ages. After gel separation and SYBR Green I staining (Roche), the emission intensities of the obtained *wsp* fragments were determined and compared to *wsp* signal intensities derived from individual *D. simulans* (from a Coffs Harbor line) infected with *w*Au and *D. simulans* (from a Riverside line) infected with *w*Ri.

TABLE 1. Distribution of *Wolbachia* in natural populations and stocks of *D. willistoni*

Region and fly line	Location; source[a]	Collection yr	PCR[b]	Southern blotting[c]
American continental				
Pan 02	Panama City, Panama; KL	2002	+	+
Lag	Laguna Negra, Rocha, Uruguay; LB	2000	+	+
Apa 5.1	Veracruz, Mexico; JS	1998	+	+
Apa 8.2	Veracruz, Mexico; JS	1998	+	+
Pan 98	Panama; EB	1998	+	+
JS 6.3	Jaton Sacha near Tena, Ecuador; PO	1997	+	+
JS 1	Jaton Sacha near Tena, Ecuador; PO	1997	+	+
Para 3	Belem, Pará, Brazil; MM	1997	+	+
Para 4	Belem, Pará, Brazil; MM	1997	+	+
RIP	Ribeirao Preto, Sao Paulo, Brazil; CR	1995	+	ND[d]
Pan 92	BCI, Panama; EB	1992	+	ND
Manaus	Manaus, Brazil; MM	1986	−	−
wilB6	Belize; FA	1974	+	+
wilC	Costa Rica; FA	1971	−	−
SP	Sao Pedro, Rio Grande do Sul, Brazil	1965	−	ND
WIP4	Ipitanga, Bahia, Brazil; HW and AC	1961	−	−
14030-0811.6	Fairchild Gardens, FL; WH	1959	−	−
14030-0811.1	San Salvador, El Salvador; WH	1955	−	−
14030-0811.0	San Maria d'Ostuna, Nicaragua; WH	1954	−	−
14030-0811.3	Atlixco, Veracruz, Mexico; WH	1947	−	−
14030-0811.2	Royal Palm Park, FL; WH	1941	−	−
Caribbean				
wilG1-FWI	Basse Terre, Guadeloupe; PC	2000	+	+
LAntilles 6	St. Vincent and Grenadines; HH	1997	+	+
LAntilles 3	Grand Etang, Grenada; HH	1997	−	−
LAntilles 4	St. Vincent and Grenadines; HH	1997	−	−
LAntilles 1	Toro Negro, Puerto Rico; HH	1994	+	+
wilG2	Guana Island, Virgin Islands; PC	1991	+	+
wilG1	Basse Terre, Guadeloupe; PC	1991	+	+
wilH	Grande-Terre, Guadeloupe; PC	1991	−	−

[a] Collectors: CR, C. Rohde; EB, E. Bartel; FA, F. Ayala; HH, H. Hollocher; HW, H. Winge; KL, K. van der Linde; LB, L. Basso da Silva; MM, M. Martins; PC, P. Chabora; PO, P. O'Grady; WH, W. Heed.
[b] Results obtained per line on individual flies from independent genomic PCRs with *ftsZ* and *wsp* primer sets ($n = 6$ adult females per line).
[c] Results derived from genomic single-fly Southern blot hybridizations probed with the *wsp* fragment ($n = 5$ adult females per line).
[d] ND, not determined.

Immunological studies. *Wolbachia* density and tissue tropism of *w*Wil in *D. willistoni* were determined using the polyclonal *Wolbachia* surface protein (WSP) antibody (11). WSP protein expression was analyzed via Western blotting of protein extracts derived from individual adult flies in independent replicates as well as whole-mount immunostainings on adult tissues and staged embryos (44). Rabbit anti-*wsp* antibody was used at a 1:500 dilution overnight at 4°C and detected after incubation with a 1:500 dilution of Alexa Fluor 488 goat anti-rabbit immunoglobulin G-labeled secondary antibody (Molecular Probes) at room temperature for 1 h. The total number of primordial germ line cells (PGCs) in stage 10 and later embryos of *D. willistoni* was determined using the pole cell-specific polyclonal rabbit anti-VASA antibody at a dilution of 1:1,000. Slides were stained for 3 min with 1 μg/ml DAPI (4′,6′-diamidino-2-phenylindole) (Molecular Probes), rinsed, stained with 5 μg/ml propidium iodide (Molecular Probes) for 20 min, rinsed again, and mounted with ProLong antifade medium (Molecular Probes).

Fluorescence microscopy. Immunostainings of embryos and ovaries were examined by using a Zeiss Axiomot 2 Epifluorescence microscope. Images were processed using Photoshop 6.0 (Adobe).

Nucleotide sequence accession numbers. The *wsp* sequence data derived from Neotropical *Wolbachia* strains were deposited in GenBank under accession numbers AY620207 to AY620229 and DQ118779, as well as AY858801 for the respective sequence from *D. ananassae* collected in 2002 in Sao Tome. Sequences of the diagnostic VNTR-141 loci of *D. simulans* (Coffs Harbor) and *D. willistoni* were deposited in GenBank under accession numbers DQ118777 and DQ118778, respectively.

RESULTS

Isolation of *Wolbachia* from *D. willistoni*. Twenty-one continental American and eight Caribbean lines of *D. willistoni* were screened for *Wolbachia* by using *wsp* PCR and single-fly Southern hybridization. Based on both molecular methods, 12 continental and 5 Caribbean lines tested positive. All five lines originating from the DSSC as well as most lines derived from collections before the 1980s were devoid of *Wolbachia* (Table 1). The five DSSC-derived fly lines collected in Central America and Florida in the 1940s and 1950s and the Brazilian and Costa Rican lines collected in the 1960s and 1970s lack *Wolbachia* (Table 1 and Fig. 2A). The oldest sample of *D. willistoni* infected with *Wolbachia* originates from a line of flies collected in Belize in 1974 (sample wilB6). The second-oldest infected line was collected in Panama in 1992. While the Brazilian line "Manaus" originating from a collection in 1986 is uninfected, all continental lines, ranging from Mexico to Uruguay, collected in the 1990s and later harbor *Wolbachia* infections (Table 1). Whereas older continental lines are devoid of *Wolbachia*, more recent samples are universally infected. Caribbean samples of *D. willistoni* show a more heterogeneous infection pattern. For example, recent collections from Grenada and St. Vincent (line L'Antilles 4) in 1997 are not infected. A line collected from Grand Terre, Guadeloupe (wilH), in 1991 is uninfected, whereas another one collected on the neighboring island Basse Terre (wilG1) in the same year is infected with *Wolbachia* (Table 1).

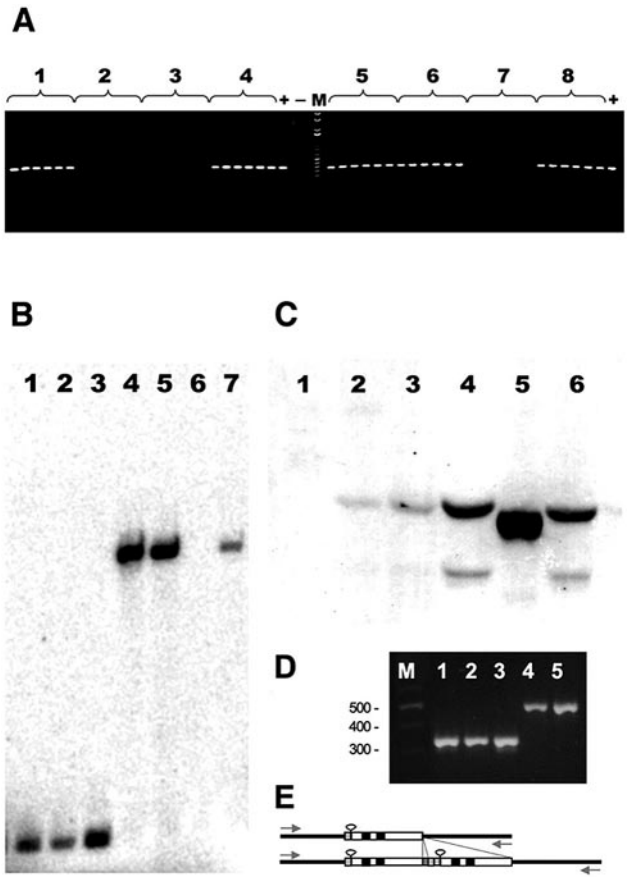

FIG. 2. Intra- and interspecific distributions of *Wolbachia* in Neotropical *Drosophila* species. (A) Single-fly *wsp* PCR on eight strains of *D. willistoni* collected at different American locations in different years (see Table 1). For each *D. willistoni* strain tested, PCRs were performed separately on six individual 2-day-old female flies. Lines are as follows: 1, Pan 02; 2, wilC; 3, wilH; 4, wilB6; 5, Apa 5.1; 6, Para 4; 7, WIP4; 8, wilG1. (B) Genomic single-fly Southern blot hybridization probed with the *wsp* plasmid of *w*Wil on individual 10-day-old females of *D. melanogaster*/*w*Mel CS (lane 1), *D. melanogaster*/*w*Mel yw^{c67} (lane 2), *D. simulans*/*w*Ri (lane 3), *D. simulans*/*w*Au (lanes 4 and 5), *D. willistoni*/*w*Wil treated with tetracycline (lane 6), and *D. willistoni*/*w*Wil strain Pan 02 (lane 7). (C) Western immunoblotting using the anti-WSP antibody (1:1,000) on single-fly protein extracts derived from *D. willistoni*-T, the tetracycline-treated control line of JS 6.3 (lane 1), *D. willistoni*/*w*Wil (lane 2), *D. septentriosaltans*/*w*Spt (lane 3), *D. simulans*/*w*Au (lane 4), *D. simulans*/*w*Ri (lane 5), and *D. melanogaster*/*w*Mel (lane 6). (D) VNTR-141 specific PCR on *D. willistoni*/*w*Wil Pan 02 (lane 1), *D. willistoni*/*w*Wil JS6.3 (lane 2), *D. willistoni*/*w*Wil Para 4 (lane 3), *D. simulans*/*w*Au Coffs Harbor (lane 4), and *D. simulans*/*w*Au Yaounde 6 (lane 5). (E) Schematic comparison between the VNTR-141 loci (34) of *w*Wil (top) and *w*Au (bottom). The basic unit is composed of a 15-bp repeat (stippled), a 23-bp hairpin (loop), an 18-bp insertion (hatched), and a 15-bp repeat (black). The size difference is caused by a 141-bp duplication in VNTR-141 of *w*Au.

Multiple *wsp* PCRs on individual flies from lines of *D. willistoni* confirmed the complete absence of *Wolbachia* in uninfected lines. Within infected fly lines, each individual tested was positive for *Wolbachia* (Fig. 2A and data not shown). These 100% infection frequencies suggest a close-to-complete vertical transmission efficiency of *Wolbachia* in *D. willistoni* hosts. This is corroborated by our observations that flies from naturally *Wolbachia*-infected populations of *D. willistoni* kept in our lab maintained a stable 100% infection frequency in the 3 years since collection.

Molecular characterization of the *D. willistoni*-specific *Wolbachia* strain *w*Wil. We sequenced fragments of two genes, *wsp* and *ftsZ*, from 12 *Wolbachia*-infected lines covering continental and Caribbean populations of *D. willistoni* in order to characterize the molecular structure and phylogenetic relationship of this *Wolbachia* association with other *Wolbachia* variants. Until recently these two diagnostic marker genes were regarded as the most informative for molecular *Wolbachia* variant classification (34). All isolated *Wolbachia* clones of *D. willistoni* were identical in their sequence. Below we refer to the strain as *w*Wil. With respect to the *wsp* sequence of *w*Wil obtained from the 12 infected lines (accession numbers AY620218 to AY620229, no sequence polymorphism could be detected. Moreover, all *wsp* and *ftsZ* sequences of *D. willistoni* were 100% identical to the respective *wsp* and *ftsZ* genes (accession numbers AF020067 and AY227739) of the *Wolbachia* variant *w*Au. As deduced from comparative Southern blots (Fig. 2B), the close relationship between *w*Au and *w*Wil is corroborated by the conservation of the two HindIII restriction sites flanking the *wsp* locus.

In contrast to the identity of *w*Wil and *w*Au at the *wsp* and *ftsZ* sequence level, comparative genomic single-fly Southern blots (Fig. 2B) and semiquantitative PCRs (data not shown) of infected individuals of *D. willistoni* and *D. simulans* showed clear quantitative differences. Strong signals comparable to those of *w*Ri were obtained from *w*Au-infected *D. simulans* adults, and the intensity of *w*Wil in similar-sized *D. willistoni* clearly showed a 70% reduction compared to that of *w*Au (Fig. 2B, lanes 4, 5, and 7). This quantitative effect was also detected at the WSP protein expression level by Western blots derived from single-fly protein extracts with the polyclonal anti-*wsp* antibody (Fig. 2C, lanes 2 and 4). The WSP proteins of *w*Au and *w*Wil have the same molecular weight, whereas, for example, the homologues of two other *Wolbachia* variants that infect *D. melanogaster* and *D. simulans (w*Mel and *w*Ri, respectively) differ significantly (Fig. 2C, lanes 5 and 6).

In contrast to the *wsp* and *ftsZ* sequence identity between *w*Wil and *w*Au, we were able to discriminate both strains at the genomic level by applying the recently isolated polymorphic marker VNTR-141 (36). This diagnostic marker covers the noncoding polymorphic VNTR-141 locus in *w*Mel (positions 89003 to 90332 in the *w*Mel chromosome). By performing VNTR-141-specific PCRs (Fig. 2D), we have obtained a 528-bp fragment from *w*Au (accession number DQ11877) and a 387-bp fragment from *w*Wil (accession number DQ118778). The length difference is caused by a 141-bp duplication in *w*Au that is not present in *w*Wil (Fig. 2E). Hence, *w*Wil is closely related but not identical to *w*Au of *D. simulans*.

Extreme pole cell tropism of *wWil in D. willistoni* embryos. Whole-mount immunostainings were performed on early embryos and ovaries of both fly species, using the anti-WSP antibody. In early embryos of *D. simulans*, *w*Au bacteria were detected in somatic and germ line tissues during all stages of embryonic development (Fig. 3A). Nuclei of earlier blastodermal stages were infected with *Wolbachia*, with some significant enrichment in the posterior pole cell region in both *D. simulans* and *D. willistoni*. Such posterior accumulations of *w*Au in *D. simulans* blastodermal embryos were reported recently (44). In

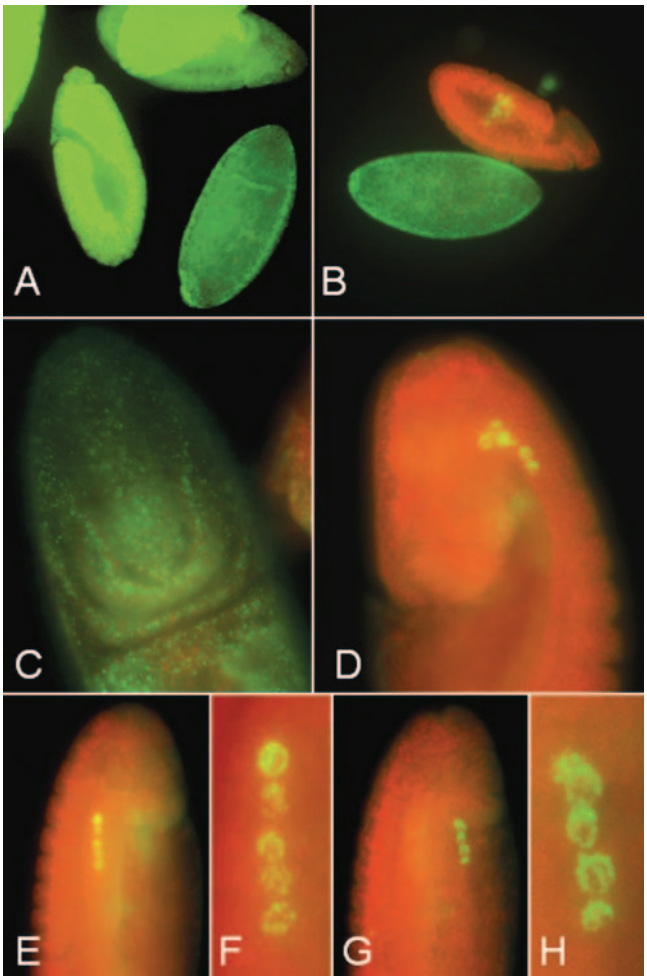

FIG. 3. Distribution of *Wolbachia* in *Drosophila* embryos. (A and B) Whole-mount immunostainings with rabbit anti-WSP antibody (green) on early-stage embryos of *D. simulans* infected with *w*Au (A) and *D. willistoni* JS 6.3 infected with *w*Wil (B). (C and D) Stage 9 to 10 embryos of *D. simulans* (C) and *D. willistoni* (D) are shown in detail. Whereas *w*Au in *D. simulans* uniformly infects both somatic and germ line cells, *w*Wil selectively targets a very limited number of primordial germ cells. In *D. willistoni*, *w*Wil is not detectable at the immunological level in somatic cells at embryonic stage 9 or later. (E to H) Lateral views of a stage 12 embryo of *D. willistoni* infected with *w*Wil (E and G) and their enhanced magnifications show a row of five heavily infected primordial germ cell nuclei on both lateral sides of the embryo (F and H).

contrast to this, during stages 9 and 10 of embryonic development of *D. willistoni*, *w*Wil specifically targets the germ line (Fig. 3B). In early gastrulating embryos, shortly after pole cell invagination, somatic tissues of *D. simulans* were heavily infected by *w*Au (Fig. 3C). At this developmental stage, *w*Wil bacteria in *D. willistoni* are selectively targeting a small number of primordial germ line cells, whereas somatic tissues are devoid of bacteria (Fig. 3D). Later, during stages 12 to 14, in the course of germ band retraction, only one lateral pair of five or six PGC nuclei was infected by *w*Wil (Fig. 3E to H). Control immunostainings with the *Drosophila* germ line-specific VASA antibody (28) showed that, in contrast to *D. melanogaster*, the Neotropical species *D. willistoni* harbors a reduced number of PGCs which perfectly colocalize with *w*Wil (data not shown). Based on the tight temporal and spatial association between the host-encoded VASA protein and WSP-expressing *w*Wil, we assume that this intracellular parasite possesses a molecular association with the host-expressed, pole cell-specific *vasa* RNA or with its encoded protein.

Natural polymorphism of *w*Au-like *Wolbachia* in other Neotropical *Drosophila* species. We have expanded our survey into species of the willistoni group in order to search for a potential origin of the *w*Wil detected in recent collections of *D. willistoni*. Besides *D. willistoni*, 21 fly lines derived from eight species of this group, covering both the willistoni and bocainensis subgroups (Fig. 1), were screened for the presence of *Wolbachia* by using the *wsp* primer set. With the exception of *D. fumipennis*, a strain kept at the DSSC since 1958, all willistoni group species sampled were negative for *wsp* and *ftsZ* PCR (Table 2). On the basis of its *wsp* sequence, the infection in *D. fumipennis* (*w*Fum; accession number AY620207) shows only a distant relationship to *w*Wil (Fig. 4), similar to the A subgroup *Wolbachia* infection of *Pegoscapus longiceps* (accession number AF521161).

In contrast to the absence of *w*Wil infections in the willistoni group, three out of the seven tested species belonging to the saltans group harbor *Wolbachia* (Table 3). The two saltans subgroup members *D. septentriosaltans* and *D. prosaltans* are infected with *w*Wil-related *Wolbachia* strains, designated *w*Spt and *w*Pro, respectively. The *wsp* sequence of the *Wolbachia* strain *w*Pro SG1 (accession number AY620208) isolated from *D. prosaltans* shows 97.9% homology to *w*Wil of *D. willistoni* and is almost identical (99.0%) to the *w*Spt PNM2 strain (AY622214) of *D. septentriosaltans*. Below we refer to these Neotropical strains *w*Wil, *w*Spt, and *w*Pro (Fig. 4) as *w*Au-like *Wolbachia* because of their close phylogenetic relationship with *w*Au of *D. simulans*.

Six *w*Spt *wsp* sequences were isolated from three different *D. septentriosaltans* lines collected in Panama between 1998 and 2002 (Table 3). At least four different *w*SPT subtypes can be distinguished according to their *wsp* sequences (Fig. 4): *w*SPT BCI1 (accession number AY620209) is identical to *w*Cer2 (accession number AF418557) of the cherry fruit fly *Rhagoletis cerasi* (33) and to *w*Tei (accession number AY291347) and *w*Yak (accession number AY291348) of *D. teissieri* and *D. yakuba*, respectively (8). The variant *w*Spt PLR1 (accession number AY620211) clusters with *w*Spt PLR2 (accession number AY620212), BCI2 (accession number AY620210), and PNM1 (accession number AY620213). The latter three *wsp* clones are identical at the sequence level but stem from three different Panamanian *D. septentriosaltans* populations (Table 3). The fourth subtype, *w*Spt PNM2 (accession number AY620214), is the most divergent variant positioned between *w*Mel (accession number AF020072) of *D. melanogaster* and the *w*Au-like *Wolbachia* clade (Table 4). All lines of *D. septentriosaltans* tested are multiply infected with *wsp* variants of *w*Spt. For example, individual flies from the PNM strain from Panama City harbor at least two different types of *wsp* sequences. Each *wsp* variant sequenced seems to be part of an intact open reading frame encoding a 196-amino-acid (aa) section of the WSP protein. The observed *wsp* sequence polymorphism of *w*Spt variants within *D. septentriosaltans* is manifested even at the protein level (Table 4). With respect to the

TABLE 2. Distribution of *Wolbachia* in the willistoni group

Species	Fly line, location, source[a]	Collection yr	PCR[b]
willistoni subgroup			
D. tropicalis	PNM; Panama City, Panama; KL	2002	−
	Panama; JS	1998	−
	BCI; Panama City, Panama; EB	1997	−
D. insularis	St. Kitts, St. Lucia; HH	ND[c]	−
D. equinoxialis	Apazapan, Veracruz, Mexico; JS	1998	−
	Gigante, Panama; EB	1997	−
	PLR; Gamboa, Panama; KL	2002	−
	FS; Colon, Panama; KL	2002	−
D. paulistorum	JS 5.2; Jaton Sacha, Tena, Ecuador; PO	1997	−
	Interior; LE	1970	−
	Central americas; LE	1959	−
	14030-0771.6; San Salvador, El Salvador	1955	−
	14030-0771.2; Mesitas, Mexico; LE	ND	−
	A28; LE	ND	−
bocainensis subgroup			
D. capricorni	14030-0721.1; Canal Zone, Panama	1961	−
D. sucinea	Xalapa Botanical Gardens; Mexico, JS	1998	−
	14030-791.0; Medellin, Colombia	1958	−
D. nebulosa	Apazapan, Veracruz, Mexico; JS	1998	−
	14030-0761.0; Palmira, Columbia	ND	−
	14030-0761.1; San Jose, Costa Rica	ND	−
D. fumipennis	14030-0751.1; Arima Valley, Trinidad	1958	+

[a] Collectors: EB, E. Bartel; HH, H. Hollocher; JS, J. Silva; KL, K. van der Linde; LE, L. Ehrman; PO, P. O'Grady.
[b] Results obtained per line on individual flies from independent genomic PCRs with *ftsZ* and *wsp* primer sets ($n = 6$ adult females per line).
[c] ND, not determined.

WSP consensus sequence *w*BCL1 (accession number AY620209), two amino acid substitutions are found, i.e., in the sequence of *w*Spt PLR1, PLR2, BCI2, PNM1, and PNM2 at consensus position aa 24 (Tyr to His) and in the variant PLR1 (accession number AY620211) at position 126 (Asp to Gly).

At least two *wsp* variants of *w*Pro were isolated from the *D. prosaltans* SG line from Panama. Both *w*Pro variants share a host species diagnostic substitution at aa 23 (Thr to Ser), and *w*ProSG1 has a substitution at aa 88 (Table 4).

***w*Stv *Wolbachia* in *D. sturtevanti*.** Our survey yielded another new *Wolbachia* variant, *w*Stv, which was isolated from *D. sturtevanti*, a member of the sturtevanti subgroup (Fig. 1). The distribution pattern of the *w*Stv infection within its host species is patchy; e.g., *w*Stv is present in the isofemale line Pan 6 (accession number AY620216) but is absent from Pan 12 (Table 3). As deduced from *wsp* sequence data *w*Stv belongs to A-group *Wolbachia* but is distantly related to the *w*Au-like variants (Fig. 4). Three closely related but distinctive variants of *w*Stv were isolated as singly occurring infections from three Panamanian populations (accession numbers AY620215, AY620216, and AY620217) (Fig. 4). Interestingly, the *wsp* sequence of *w*Stv MI (accession number AY620215) collected in Maria Eugenia, Panama, is identical to that of *w*Whi (accession number AF237886) isolated from the phlebotomine sand fly *Lutzomyia shannoni* in Colombia (31). Those authors proposed, based on an extensive data set showing that other non-American populations of *L. shannoni* are free of *Wolbachia*, that *L. shannoni* probably acquired *w*Whi recently from another host in America.

DISCUSSION

***w*Wil infection of D. *willistoni*.** Our survey shows that Neotropical *Drosophila* species belonging to the willistoni and saltans groups are infected with various A-group *Wolbachia* strains. In *wsp* and *ftsZ* sequence analysis, *w*Wil of *D. willistoni* is identical to *w*Au of *D. simulans*. However, *w*Wil can be discriminated from *w*Au by the VNTR-141 polymorphism and the strict pole cell tropism in its natural host. Hence, *w*Wil is closely related but not identical to *w*Au of *D. simulans*. Our biogeographic analysis suggests that the infection is absent in *D. willistoni* stocks collected before the 1970s. Two alternative hypotheses may explain this result, i.e., a stochastic loss in the stocks or a recent invasion in the field. All five DSSC-derived *D. willistoni* samples tested negative for *w*Wil (Table 1) and were kept under artificial lab conditions since the 1940s and 1960s. The DSSC collection was moved first from Texas to Ohio and then to Arizona. We cannot exclude the possibility that the *Wolbachia* infection was present in all lines but was then stochastically lost in independent lines in the course of their long-term stock maintenance due to stress factors, starvation, dramatic reduction of population size, or application of antibiotics. This hypothesis cannot completely be dismissed; however, we have three arguments against it: (i) *w*Wil infec-

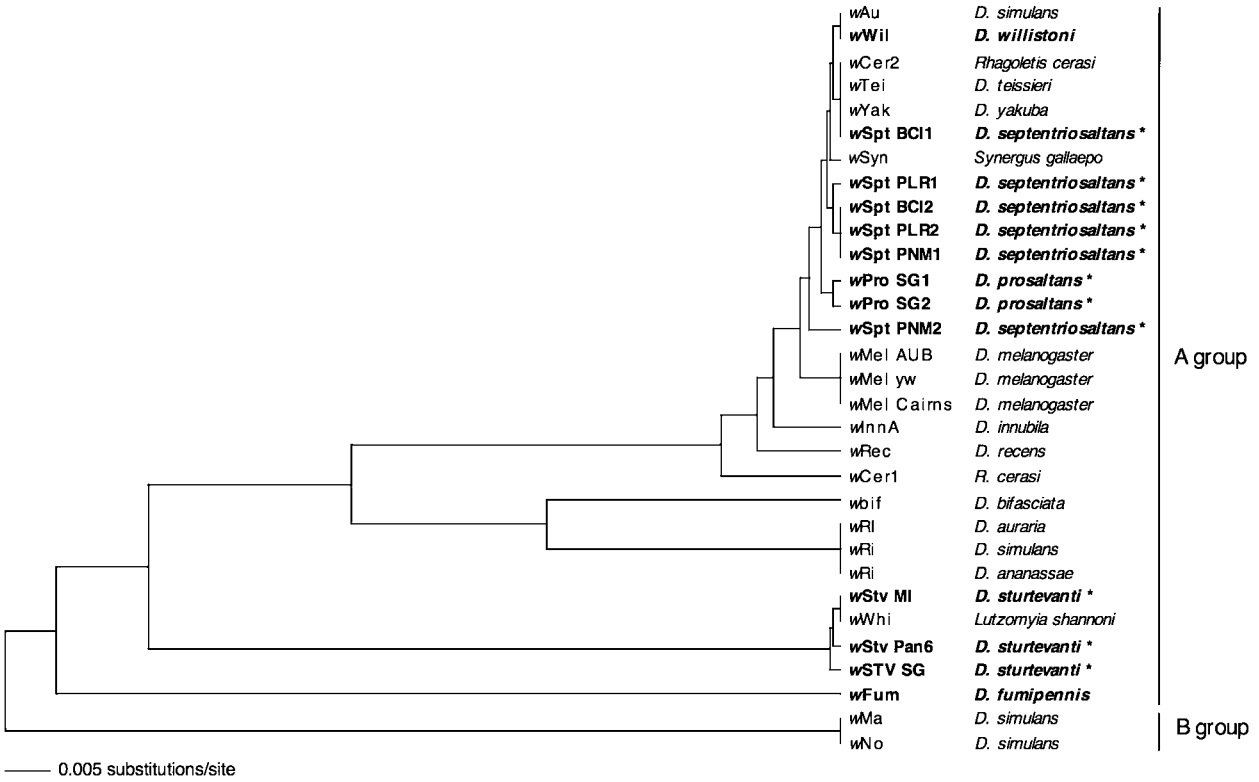

FIG. 4. Unweighted-pair group method with arithmetic mean cladogram based on *wsp* sequence alignment, including the hypervariable region of the *Wolbachia* strains (50) derived from Neotropical *Drosophila* species (boldface) and from earlier reported host species (lightface). Host species found to harbor polymorphic *Wolbachia* variants are indicated by asterisks.

tions in *D. willistoni* lines were completely stable under our lab conditions for more than 3 years, (ii) the DSSC contains infected *Drosophila* lines originating from equally old collections (e.g., *D. fumipennis*), and (iii) overall ratios of infected versus uninfected *D. melanogaster* fly lines in several other stock centers stayed constant over the last 80 years (36). Hence, we are in favor of the hypothesis of recent spreading, for which we can add three supporting observations: (i) the lack of sequence variation of all available *w*Wil markers obtained from our samples suggests clonality of the infection and recent acquisition by horizontal transfer from an external source; (ii) individual adult flies of two alcohol samples of *D. willistoni* (DSSC stock numbers 14030–0811.4 and 14030-0811.5; kindly provided by S. J. Castrezana, *Drosophila* Species Stock Center, Tucson, Ariz.) collected in Mexico in the 1950s were uninfected, whereas control PCRs with *Adh*-specific primer sets were successful; and (iii) the two old strains wilC and wilB6, collected by F. Ayala in Central America in the 1970s, are uninfected and infected, respectively (Table 1). Additional analysis of *D. willistoni* populations collected between the 1970s and 1990s would doubtlessly improve our data set, although these strains would be difficult or impossible to obtain.

The complete absence of *w*Au-like *Wolbachia* in the related willistoni group species tested (Table 2) supports the idea that *D. willistoni* was infected after its speciation. Since all recently collected continental samples of *D. willistoni* are infected with *w*Wil, we assume that this *Wolbachia* strain reached fixation in continental populations of *D. willistoni. w*Wil's pole cell tropism and its 100% transmission rate, seen in lab lines, were probably crucial factors. A recent *Wolbachia*-driven process should also be detected in the biogeographic distribution of mitochondrial variation, but this has not yet been looked at in the context of *Wolbachia* infections. A departure from an expected ratio of mitochondrial versus nuclear DNA polymorphism has been reported when comparing different populations of *D. willistoni*, and a selective mitochondrial sweep has been suggested as one plausible reason (38; J. Silva and M. Kidwell, personal communication).

***w*Au-like *Wolbachia* originated in saltans group species.** We found polymorphic but closely related *wsp* sequences of *w*Pro and *w*Spt in the host species *D. prosaltans* and *D. septentriosaltans*, respectively. This implies that these *Wolbachia* variants are an outcome of old associations with Neotropical *Drosophila* species. Independent multiple horizontal transfers with closely related *Wolbachia* strains are less likely. The progenitor of *w*Pro and *w*Spt presumably infected the common ancestor of both host species before speciation and subsequently diverged at the *wsp* sequence level in the course of long-term vertical transmission. Host-specific diagnostic sites within *wsp* correspond with our hypothesis (Table 4). Therefore, we suggest that *w*Au-like variants evolved in the American Neotropical saltans group species and are potential donors for the horizontal transmission to *D. willistoni*. A similar event has been suggested for *Wolbachia* associations among the Old World sibling species *D. simulans* and *D. sechellia*, where original *Wolbachia* infections in an original species have not yet yielded a sequence divergence in *wsp* in the sibling species (6).

TABLE 3. Distribution of *Wolbachia* in the saltans group

Species	Fly line, location/source[a]	Collection yr	PCR[b]
saltans subgroup			
D. saltans	PNM; Panama City, Panama; KL	2002	−
	PLR; Gamboa, Panama; KL	2002	−
	FS; Colon, Panama; KL	2002	−
	BCI; Panama; KL	1998	−
D. austrosaltans	14030-0771.0; Pirassununga, Brazil	1959	−
D. lusaltans	14045-0891.0; Petionville, Haiti	ND[c]	−
D. septentriosaltans	PLR; Gamboa, Panama; KL	2002	+
	PNM; Panama City, Panama; KL	2002	+
	FS; Colon, Panama; KL	2002	+
	BCI; Panama; EB	1998	+
D. subsaltans	14044-0872.0; Balem, Brazil	1959	−
D. prosaltans	SG; Summit Gardens, Panama; EB	1998	+
	14045-0901.3; Balboa, Panama	1958	−
sturtevanti subgroup			
D. sturtevanti	PNM; Panama City, Panama; KL	2002	−
	PLR; Gamboa, Panama; KL	2002	−
	Barb 1; Turner's Hall, Barbados; HH	1999	+
	Barb 2; Turner's Hall, Barbados; HH	1999	−
	Pan 6; Panama; TM	1999	+
	Pan 12; Panama; TM	1999	−
	MI; Maria Eugenia, Panama; EB	1998	+
	SG; Summit Gardens, Panama; EB	1998	+

[a] Collectors: EB, E. Bartel; HH, H. Hollocher; KL, K. van der Linde; TM, T. Markow.
[b] Results obtained per line on individual flies from independent genomic PCRs with *ftsZ* and *wsp* primer sets ($n = 6$ adult females per line).
[c] ND, not determined.

Recent horizontal transfer into *D. similans*: origin of the *w*Au infection. Non-CI-inducing *w*Au of *D. simulans* (17) is found worldwide, including in Australia, Madagascar, Cameroon, parts of Europe and Japan, Ecuador, Jamaica, and the southern United States (2, 3, 7, 23). The overlapping geographic distribution of populations of *D. simulans*, *D. willistoni*, and other Neotropical *Drosophila* species in Central America, together with *wsp* and *fstZ* sequence identity of the two *Wolbachia* variants *w*Au and *w*Wil, strongly suggests a recent horizontal transfer of *Wolbachia* from an original native Neotropical *Drosophila-Wolbachia* guild to the immigrating Old World species *D. simulans*. To date *D. willistoni* can be regarded as the most likely donor species of this transfer. Recent transfers of transposable elements between *D. willistoni* and another immigrating Old World *Drosophila* species, *D. melanogaster*, have been shown for the canonical *P* transposon (10, 18), and for the retrotransposon *copia* (13, 25). Furthermore, the male-killing bacterium *Spiroplasma poulsonii* of the *D. wil-*

TABLE 4. Variable nucleotide and amino acid sites in the *wsp* sequence of the closely related *w*Au-like *Wolbachia* strains of *Drosophila*

Strain	Nucleotide at variable position in *wsp* DNA consensus[a]													Strain	Amino acid at variable position in *wsp* amino acid sequence								
	68	70	258	263	333	340	363	377	426	520	529	536	538		23	24	88	114	126	174	177	179	180
Consensus	C	T	T	G	T	G	A	A	T	G	A	T	A	Consensus	T	Y	G	A	D	D	R	V	T
*w*Au						A								*w*Au				T					
*w*Wil						A								*w*Wil				T					
*w*Pro SG1	G	C		A										*w*Pro SG1	S	H	E						
*w*Pro SG2	G	C												*w*Pro SG2	S	H							
*w*Spt PLR1		C						G						*w*Spt PLR1		H			G				
*w*Spt PLR2		C												*w*Spt PLR2		H							
*w*Spt BCI2		C												*w*Spt BCI2		H							
*w*Spt PNM1		C												*w*Spt PNM1		H							
*w*Spt PNM2		C	C		C		G							*w*Spt PNM2		H							
*w*Spt BCI1														*w*Spt BCI1									
*w*Tei														*w*Tei									
*w*Yak														*w*Yak									
*w*Cer2														*w*Cer2									
*w*Mel										A	G	C	G	*w*Mel						N	G	A	A

[a] Position 1 of the consensus sequence corresponds to position 164 in the *wsp* sequence of *w*Au of *D. simulans* (accession number AF020067).

listoni group species *D. nebulosa* has recently infected immigrating *D. melanogaster* populations in Brazil (30). Extensive phylogenetic studies of hosts and their parasites suggest horizontal transmission of *Wolbachia* variants between distantly related insect species (5, 16, 43, 46). Furthermore, it has been experimentally demonstrated that *Wolbachia* can be shuffled horizontally within and between Trichogramma parasitoid species (19, 20).

In agreement with the hypothesis of an American origin of *w*Au and opposed to an African origin (7) is the extensive analysis of mitochondrial variation in *D. simulans*. *w*Au is globally associated with the mitochondrial-*si*II haplotype of *D. simulans* (23). However, some African populations of *D. simulans* harboring the *si*II haplotype are uninfected. Ballard proposed recently that uninfected flies migrated to Ecuador and acquired *w*Au in a horizontal transmission event from an unknown host source (2). Subsequently, *w*Au spread throughout natural populations of *D. simulans* worldwide. The infection model outlined by Ballard, based on mitochondrial haplotypes and geographic distribution of *w*Au-infected *D. simulans*, is in line with our hypothesis that a Neotropical species such as *D. willistoni* could be the donor species of *w*Au.

In summary, we suggest a potential evolutionary scenario: *w*Au-like variants evolved in the guild of the Neotropical saltans group, being vertically transmitted and/or horizontally shuffled between related host species over a long period of time. More recently, a proto-*w*Au-like strain, the ancestor of *w*Wil, infected horizontally a locally isolated population of *D. willistoni*, most likely in Central America. In this population, *w*Wil evolved perfect maternal transmission through an extreme tissue tropism towards the germ line of *D. willistoni*. Within the last 300 years, immigrating *D. simulans* flies from Africa may have become infected by *w*Wil or by another *w*Au-like strain from infected Neotropical *Drosophila* species through vectors such as parasitoid wasps (20, 43). *w*Au-infected *D. simulans* has then spread worldwide (2). An alternative source for *w*Au is an acquisition from outside the closely related *Wolbachia* pool of Neotropical *Drosophila* species, but if so, the fact that Neotropical *Drosophila* species are infected with closely related *Wolbachia* strains will need to be explained. It is unclear how *w*Wil and *w*Au drove themselves through host populations. Presently, neither *w*Au in *D. simulans* nor *w*Wil in *D. willistoni* is able to induce measurable levels of CI (17; W. J. Miller, unpublished data). The possibility that they did so in the past cannot be excluded. As reported by Ballard and coworkers, *w*Au seems to induce weak levels of CI in some infected populations of *D. simulans* from Florida (3, 23, 29). Alternatively, the driving force for the spreading of *w*Au-like strains could be a positive fitness contribution to their hosts that remains to be elucidated. The phenotypes of the Neotropical *Wolbachia* strains still need to be elucidated. The *w*Au-like strains are nested within the *Mel* cluster (50) of closely related *Wolbachia* strains that have a variety of phenotypic effects in other host species. Based on the *wsp* sequence, the variant *w*Spt BCI1 of *D. septentriosaltans* is identical to the infection of the African species *D. yakuba* and *D. teissieri* (27) and to *w*Cer2 of *R. cerasi* (33). Whereas *w*Cer2 causes strong CI in *R. cerasi* and in transinfected *Ceratitis capitata* (49) and intermediate CI in transinfected *D. simulans* (35), *w*Tei and *w*Yak do not induce CI but are able to fully rescue the *w*Ri *mod* function in their original host (48). *Wolbachia* infections of *D. melanogaster* (32, 50) and the quinaria group member *D. recens* induce CI (45). The strain *w*InnA causes male killing in the related *D. innubila* (22), where it is regarded as an ancestral infection (12).

The present paper shows the complexity of evolutionary dynamics of *Wolbachia* in Neotropical *Drosophila* species and its success in colonizing the Old World species *D. simulans*. Both *w*Wil and *w*Au successfully colonized natural populations of *D. willistoni* in America and of *D. simulans* globally. The detailed understanding of the evolutionary "jump-and-go" dynamics of *Wolbachia* will have important implications for practical applications of this symbiont as a vector system and in biological pest control management.

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