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Rapid Fluorescence-Based Screening for *Wolbachia* Endosymbionts in *Drosophila* Germ Line and Somatic Tissues▽†

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***Wolbachia* is a globally distributed bacterial endosymbiont present in arthropods and nematodes. The advent of sensitive PCR-based approaches has greatly facilitated the identification of *Wolbachia*-infected individuals and analysis of population infection levels. Here, a complementary visual fluorescence-based *Wolbachia* screening approach is described. Through the use of the fluorescent dye Syto-11, *Wolbachia* can be efficiently detected in various *Drosophila* tissues, including ovaries. Syto-11 also stains *Wolbachia* in other insects. Because *Wolbachia* is inherited through the maternal germ line, bacteria reside in the ovaries of flies in infected populations. An advantage of this staining approach is that it informs about *Wolbachia* titer as well as its tissue and cellular distribution. Using this method, the infection status of insect populations in two central California locations was determined, and variants with unusually low or high *Wolbachia* titers were isolated. In addition, a variant with ovarioles containing both infected and uninfected egg chambers was identified. Syto-11 staining of *Cardinium*- and *Spiroplasma*-infected insects was also analyzed.**

Wolbachia species are obligate intracellular, bacterial endosymbionts present in over 60% of all insect species (10). Manipulation of host reproduction and efficient maternal transmission have facilitated the global spread of *Wolbachia* in arthropods. Under optimal laboratory conditions, *Wolbachia* transmission in *Drosophila melanogaster* (11) and *Drosophila simulans* (10a) is 100%, but infection rates in the field can be highly variable with location, season, and host species (10a, 11). In addition, different *Wolbachia* strains that have various effects on host reproduction and fecundity seem to exist in single collection sites (24). Determining the *Wolbachia* status quickly and reliably is important for ecological work, as well as for biochemical and genetics studies of host-pathogen interaction. It has been estimated that 30% of the strains in the Bloomington Stock Center are *Wolbachia* infected (4). Because *Wolbachia* is present in a number of somatic tissues (6), including the adult brain (1, 17), an infection may influence fly behavior and metabolism (12, 14, 18), thus having implications for the larger *Drosophila* research community.

The infection status of fly stocks is commonly determined by PCR (2, 11a, 18), which involves a 3-step process to first prepare the DNA, followed by the actual PCR step and gel electrophoreses (19, 25). The PCR method is efficient, especially when many samples are analyzed. However, the infection status of some species has been underestimated with conventional PCR and was revealed only after "long PCR" involving 2 polymerase enzymes (13). On the other hand, too-sensitive PCR conditions may produce false positives. Standard PCR methods do not reveal the degree of *Wolbachia* infection, tissue of origin, and variations in *Wolbachia* localization or titers in the host. To complement the PCR detection, a quick method to visualize *Wolbachia* in live insect tissue was developed, using the fluorescent nucleic acid stain Syto-11 (1). *Wolbachia* visualization offers a semiquantitative analysis of the infection level. In addition, variants that differ in their *Wolbachia* distribution patterns or other characteristics can be detected. Variants with novel phenotypes are of great interest to studies of the host-bacterium interaction. Because *Wolbachia* cannot be cultured and genetically modified, natural genetic variants are currently the only way to examine the effect of the bacterium genotype on the interaction with the host.

The Syto-11 nucleic acid stain was used to determine the infection status of *D. simulans* flies from the isolated Big Creek reserve in coastal central California and to analyze the *Wolbachia* distribution in fly ovaries with respect to *Wolbachia* density and localization. Finally, this method was used to analyze other *Drosophila* tissues and mosquito and wasp ovaries, including strains that were also infected with maternally transmitted bacteria of the genera "*Cardinium*" and *Spiroplasma*.

MATERIALS AND METHODS

PCR. Flies were crushed in PCR buffer (20) containing proteinase K (0.8 mg/ml) and heated to 60°C for 45 min, followed by a 10-min incubation at 95°C to inactivate the proteinase. One microliter of this crude DNA prep was used to amplify the *wsp* sequence using the following primers: AACGCTACTCCAGC TTCTGC (reverse) and GATCCTGTTGGTCCAATAAGTG (forward). The cycle parameters were 92°C for 2 min; 92°C for 15 s, 58°C for 40 s, and 72°C for 40 s, repeated 34 times; and 72°C for 10 min. The PCR product was separated on a 1% agarose gel.

Syto-11 staining. Flies, wasps, or mosquitoes were dissected in a depression slide in ice-cold phosphate-buffered saline (PBS). Individual ovarioles should be separated, and best staining is achieved when the actin sheath around the ovariole is removed. Ovarioles were then placed onto a microscope slide or coverslip (traditional or inverted microscope, respectively), into a drop of Syto-11 (Molecular Probes, Invitrogen; 1:100 dilution of the manufacturer's 5 mM stock in

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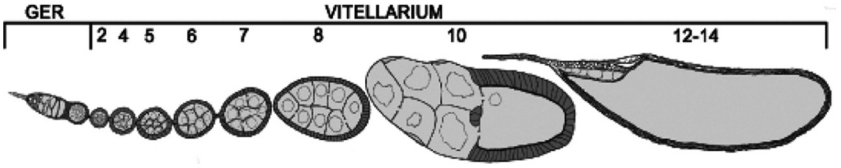


FIG. 1. Schematic of oocyte development inside one ovariole (adapted from reference 8). The germarium (GER) and the different egg chamber development stages are indicated above the schematic.

PBS), and placed into a small box on wet tissue paper. Samples need to incubate in Syto-11 for 20 min before visualization (room temperature or on ice). Maximal incubation time before microscope analysis is 50 min at room temperature or 145 min at 4°C, after which the live samples start to deteriorate. Immediately prior to microscope observation, the ovarioles were overlaid with a second, smaller coverslip. Brains were dissected and incubated the same way as ovarioles were. If brain structures were being analyzed rather than overall infection, the coverslips were separated with small balls of modeling clay.

MitoTracker staining. Ovarioles were dissected and incubated in a drop of PBS containing both Syto-11 (1:100) and MitoTracker (Molecular Probes M-7512, 1:1000 of the 1 mM stock in dimethyl sulfoxide [DMSO]). Incubation was on ice for 20 min.

Confocal microscopy. Slide analysis was performed on a Leica DM IRB inverted microscope equipped with the TCS SP2 confocal system. Syto-11 fluorescence was generated with a 488-nm excitation beam, and emission was captured between 585 and 690 nm. *Wolbachia* can be seen best with a 64× objective at a 1.5 to 4× zoom.

PI staining. Ovaries were fixed and stained as described previously (8). Briefly, dissected ovaries were fixed in 0.5% paraformaldehyde-60% heptane for 20 min. Fixed ovaries were treated with RNase (15 mg/ml) overnight. For actin staining, ovaries were incubated with Alexa Fluor 488 phalloidin (Invitrogen, 1:100) for 1 h at room temperature. Then ovaries were rinsed and stained in propidium iodide (PI)-containing mounting medium (10 μg/ml).

Insect strains. *D. simulans* flies were collected at Big Creek Reserve in Big Sur, CA, and on the Channel Islands near Santa Barbara, CA. Fertilized females were used to start isogenetic lines that were kept in the laboratory. In addition, we used lab stocks of *D. simulans* infected with *Wolbachia* Riverside as positive controls and stocks that where cured by tetracycline about 8 years ago as negative controls. Wasp strains of *Encarsia inaron*, either uninfected or infected with *Wolbachia*, *Cardinium*, or both, were generously provided by M. S. Hunter (University of Arizona). *Spiroplasma*-infected *D. melanogaster* was generously provided by M. Mateos (Texas A&M University). Female mosquitoes were caught after biting (to initiate egg development). The species is not known, and the samples were taken in Duluth, MN.

RESULTS AND DISCUSSION

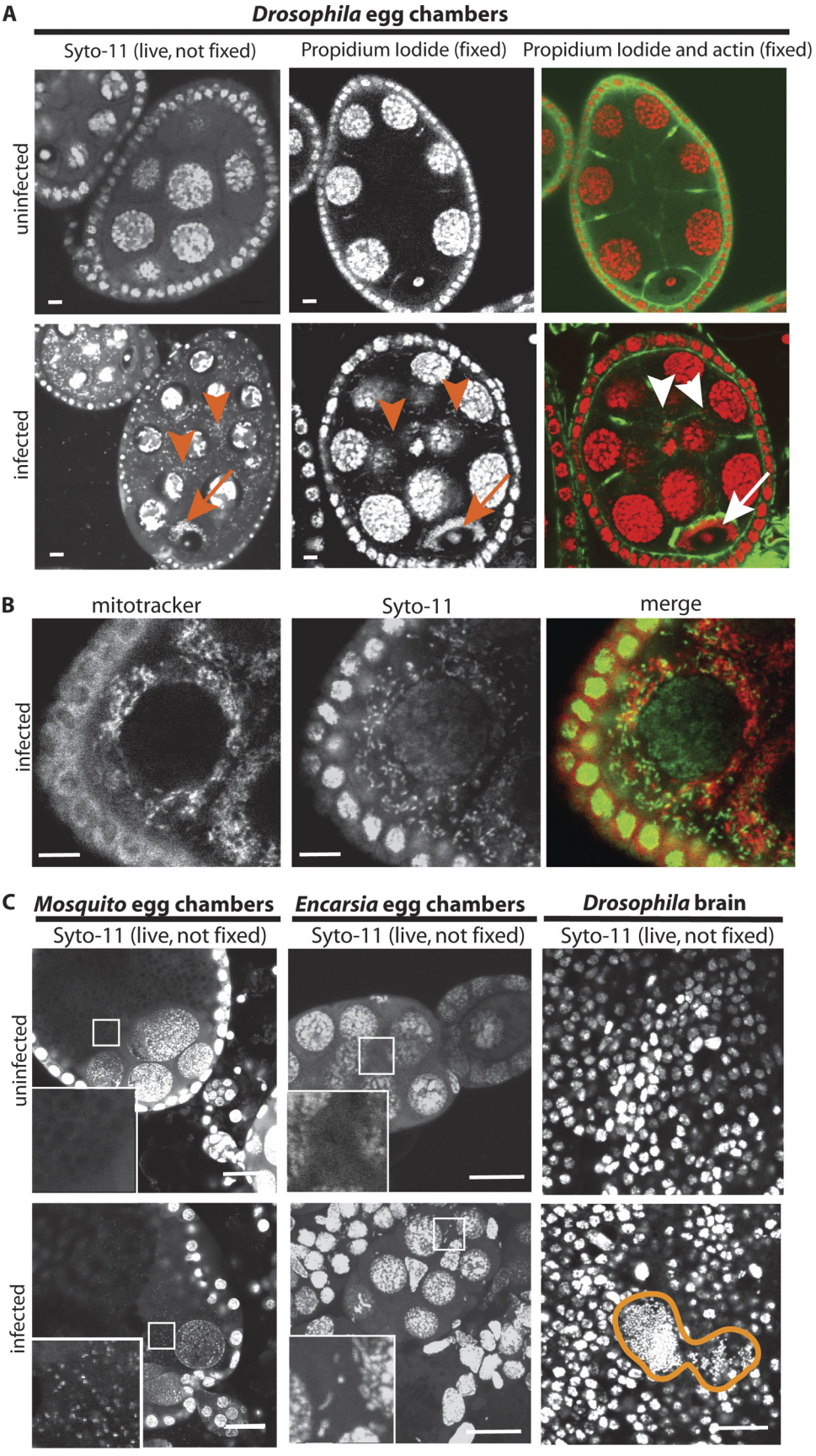
Live Syto-11 staining provides a rapid and sensitive *Wolbachia* assay. We have developed an assay to determine the *Wolbachia* infection status by fluorescent imaging. The focus is on *Drosophila*, which is known only to be potentially infected by two inherited bacteria, *Wolbachia* and *Spiroplasma* (16). Oocytes can easily be dissected from individual female insects, and since *Wolbachia* infection is passed on through the female germ line, this tissue is arguably the most significant one to identify the infection status of a fly line. The staining also works well on other tissues. Once the insects are dissected, Syto-11 is directly applied and ready for observation after a 20-min incubation (see Materials and Methods). Because the samples are not fixed, they must be visualized within 50 min after the incubation if maintained at room temperature. Placing the samples at 4°C allows visualization up to 145 min after dissection. Using the Syto-11 staining, one person can easily process 6 to 8 insects/hour by oocyte staining. Due to background fluorescence, live imaging of Syto-11-stained *Wolbachia* cells requires confocal microscopy.

Oogenesis is divided into early stages inside the germarium and later stages in the vitellarium that correlate with the position of the egg chamber in the ovariole (Fig. 1). The germ line stem cells reside in the germarium at the anterior apex of the oocyte. They undergo asymmetric divisions to produce self-renewing stem cells and a differentiating daughter cell. This daughter cell will undergo four rounds of mitotic division with incomplete cytokinesis, forming an interconnected cyst of 16 nuclei. During midoogenesis in the vitellarium, the cyst differentiates into 15 nurse cells and one posteriorly localized oocyte nucleus. During late oogenesis, nutrients and cytoplasm provided by the nurse cells allow rapid oocyte growth.

Live Syto-11 staining of early and midoogenesis egg chambers produces *Wolbachia* images at a resolution comparable to those observed for the more extensive, time-consuming fixed protocol (Fig. 2A). Fixed fluorescent analysis of *Wolbachia* relies on immunofluorescence or the nucleotide stain propidium iodide (stains *Wolbachia* and host DNA) and phalloidin (stains actin to visualize the cell outlines). The panels in Fig. 2A show uninfected (top) or infected (bottom) stage 6/7 *D. simulans* egg chambers. With both staining methods, the absence of *Wolbachia* infection is evident in the top row. In the infected samples (Fig. 2A, bottom row), the crescent-shaped *Wolbachia* mass around the nucleus of the future oocyte can clearly be identified (arrows), as has been described (8, 20a). *Wolbachia* cells in the nurse cells are also visible throughout the egg chamber (arrowheads) and clearly distinguishable from the uninfected egg chambers. Images of fixed ovaries with additional actin staining (Fig. 2A, right) also show the cell outlines and the ring canals in green. Similar to propidium iodide, Syto-11 also stains host nuclei.

Although Syto-11 is a general nucleic acid stain, it does not significantly mark mitochondria at the concentration and incubation time used. While Syto-11 sometimes causes a green halo in areas of high mitochondria concentration, these areas can clearly be distinguished from the brightly stained bacteria. Figure 2B shows mitochondria marked with MitoTracker (left panel and red in merged image) and *Wolbachia* stained with Syto-11 (middle panel and green in merged image). Mitochondria and *Wolbachia* stainings do not overlap, as seen by the distinct red and green dots in the merged images. At a 10-times-higher concentration (1:10 instead of 1:100 dilution of the manufacturer's stock), Syto-11 still does not mark mitochondria significantly, but there is more background and the stain seems to become cytotoxic, and oocytes begin to die within 10 min (see Fig. S1 in the supplemental material). At a lower concentration (1:300), the staining of nurse cells can be irregular in some ovaries.

Staining *Wolbachia* cells with Syto-11 also works well for


A
Drosophila egg chambers
Syto-11 (live, not fixed)
Propidium Iodide (fixed)
Propidium Iodide and actin (fixed)
uninfected
infected
B
mitotracker
Syto-11
merge
infected
C
Mosquito egg chambers
Encarsia egg chambers
Drosophila brain
Syto-11 (live, not fixed)
Syto-11 (live, not fixed)
Syto-11 (live, not fixed)
uninfected
infected

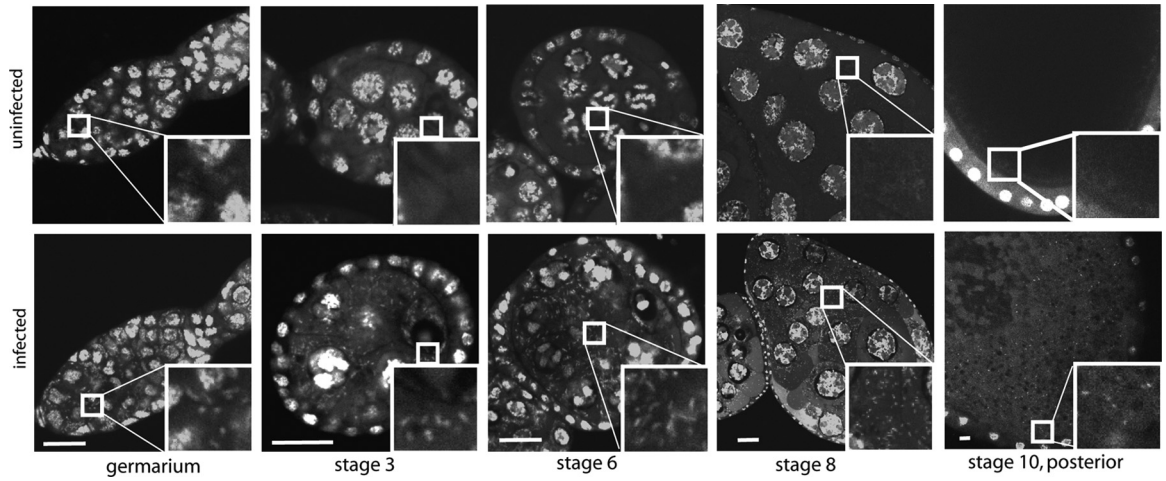


FIG. 3. Syto-11-stained infected and uninfected germaria and egg chambers at different stages. In the infected strain, *Wolbachia* can be seen in the germarium and in egg chambers up to stage 10. Insets show the boxed area enlarged to visualize the absence/presence of *Wolbachia*. (Scale bars = 10 μm.)

other insects, such as mosquitoes and wasps (*Encarsia* is shown) (Fig. 2C), and for *Wolbachia*-infected nematodes (15). *Wolbachia* imaging in other insect tissues by Syto-11 is also possible, such as *Drosophila* brain (1) (Fig. 2). As opposed to the ovaries, *Wolbachia* often occurs in large clusters in the adult *Drosophila* brain (see outlines in Fig. 2).

Syto-11 staining of *Cardinium* and *Spiroplasma*. *Drosophila* has been found to carry only *Wolbachia* or *Spiroplasma* as heritable bacterial endosymbionts (16, 22), whereas wasps, spiders, and mites are also known to carry *Cardinium*, a bacterium belonging to the *Bacteroidetes*, which is less widespread than *Wolbachia* and has so far been found only in *Hymenoptera* and *Hemiptera* (26). *Spiroplasma* is as long as *Wolbachia* but only 1/100th of the width (5). In double-infected *D. simulans*, *Spiroplasma* infection does not interfere with *Wolbachia* detection (see Fig. S2A in the supplemental material). *Spiroplasma* leads to a slightly higher Syto-11 background, and the bacteria can sometimes be seen as a rim on the anterior of the developing oocyte in stage 7 to 9 egg chambers (see inset).

In contrast to *Spiroplasma*, *Cardinium* has a size range similar to *Wolbachia* (0.5 to 1 um) (27). Therefore, in *Encarsia* samples that are doubly infected with *Wolbachia* and *Cardinium*, it is not possible to distinguish between these bacteria (see Fig. S2B in the supplemental material). In single infections, *Wolbachia* is less abundant in developing egg chambers than is *Cardinium*, but the numbers are very similar in the mature egg. Therefore, Syto-11 cannot be used to distinguish between these two endosymbionts in species in which both occur, which are most often spiders (7).

One advantage of assaying *Wolbachia* infection in *Drosophila* by using Syto-11 is that the images provide information on *Wolbachia* localization throughout oogenesis. Figure 3 illustrates the Syto-11 stain in egg chambers of a variety of stages, from the germ cells in the germarium to stage 10 ovaries. Uninfected oocytes are in the top row, and infected oocytes are in the bottom row. The insets show areas between host nuclei where *Wolbachia* cells are clearly absent (top) or present (bottom). The left panels show that *Wolbachia* cells can be visualized in the germarium, where the stem cells reside. In stage 3 egg chambers, in the vitellarium, *Wolbachia* cells are observed in both the nurse cells and the future oocyte, which can be recognized by the smaller, more compact nucleus. In stage 6, *Wolbachia* cells are visible throughout the nurse cells and in the oocyte, where they are localized to the anterior of the oocyte nucleus (Fig. 2 and 3). In stage 8, *Wolbachia* cells are distributed throughout the egg chamber. *Wolbachia* visualization becomes more difficult in infected stage 10 oocytes due to increasing amounts of oocyte yolk autofluorescence and decreasing Syto-11 absorption throughout the egg chamber. In later stages, *Wolbachia* cannot be identified unequivocally. Stages 2 through 6 are best suited to identify the infection status of a *Drosophila* oocyte.

Analysis of *Wolbachia* distribution and abundance in the oocyte. To verify Syto-11 staining as an alternative to PCR for

FIG. 2. Syto-11 and propidium iodide-Alexa Fluor 488 phalloidin stains of uninfected and infected insect tissue. (A) Staining of stage 6 *Drosophila simulans* egg chambers shows that both Syto-11 (staining of live tissue) and iodide (staining of fixed tissue) result in similar *Wolbachia* labeling in nurse cells (arrowheads) and highlights the anterior *Wolbachia* crest in the oocyte (arrows). The additional actin stain by Alexa Fluor 488 phalloidin shows the cell outlines and ring canals between cells in the egg chambers. (B) Syto-11 does not stain mitochondria at the concentration used, as indicated by the lack of overlap between mitochondria staining with MitoTracker (left panel and red in merged panel) and Syto-11 staining (middle panel and green in merged panel). (C) Syto-11 also stains *Wolbachia* in live mosquito and wasp (*Encarsia*) egg chambers (left and middle). *Wolbachia* infection status is clearly seen in the larger-magnification insets. The right panels show Syto-11 staining of uninfected and infected *Drosophila simulans* adult brains. The orange outline shows a *Wolbachia* cluster in the brain from the infected fly. (Scale bars = 10 μm.)

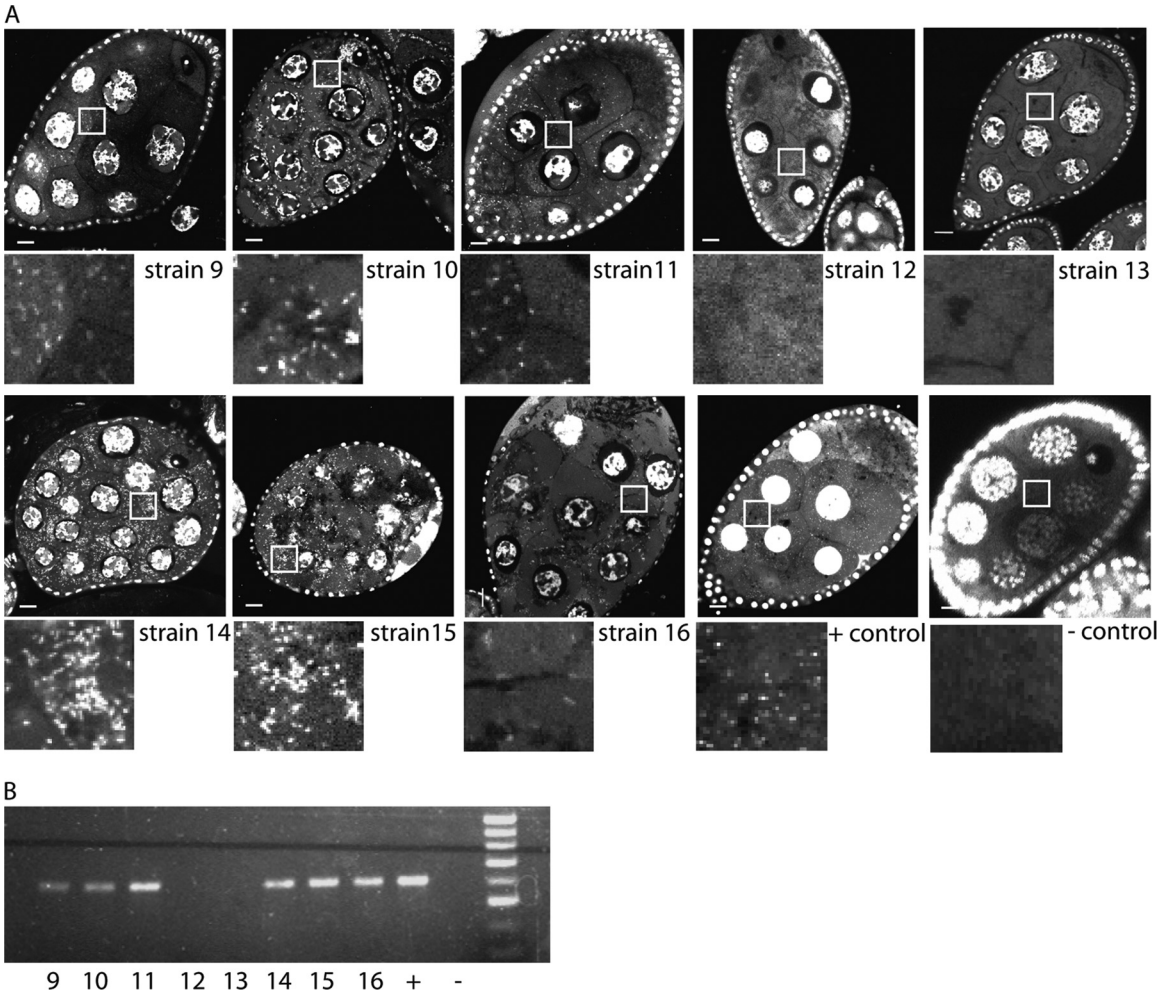


FIG. 4. Syto-11 and PCR analysis of *Wolbachia* infection in inbred *D. simulans* lines established from individual females collected at Big Creek, CA. (A) Images of Syto-11-stained stage 5 and 6 egg chambers. Insets show a magnification of the indicated boxes to better visualize *Wolbachia* presence/absence. Scale bars indicate 10 μM. Strains 9, 10, 11, 14, 15, and 16 are *Wolbachia* positive, while strains 12 and 13 are negative. (B) PCR products of *wsp* amplification from strains 9 through 16, and the infected and cured laboratory *D. simulans* strains as positive and negative controls. (Scale bars = 10 μm.)

Wolbachia detection in fly populations, lines were established from individual *D. simulans* females collected at the Big Creek Reserve in Southern Big Sur. Since *Wolbachia* is strictly maternally transmitted, this allows the generation of *Wolbachia* lines originating from single females. *Wolbachia* is readily visualized in the nurse cells during midoogenesis. Figure 4 depicts images of infected (strains 9, 10, 11, 14, 15, and 16) and uninfected (strains 12 and 13) egg chambers. In the infected strains, Syto-11-stained *Wolbachia* puncta are visible in the nurse cell and oocyte cytoplasm (see insets). No Syto-11-stained puncta are observed in the nurse cells of the uninfected egg chambers. The presence/absence of *Wolbachia* was similarly identified by PCR amplification of the *Wolbachia* surface protein (*wsp*). In addition, Syto-11 can reveal bacterium load in a semiquantitative manner, and Fig. 4 shows that strains 9, 10, 11, and 16 have a low *Wolbachia* titer, and strains 14 and 15 have a high titer.

Detection of natural variants. A valuable feature of *Wolbachia* detection by Syto-11 staining is the discovery of natural variants. One strain was identified that shows infection in some egg chambers but has others that are free of *Wolbachia*, even within individual ovarioles (Fig. 5A). In the shown ovariole, *Wolbachia* was present in the anterior part of the germarium, where the germ line stem cells are located (arrow in inset). No bacteria resided in the older, more posterior part of the germarium, where the first 16-cell cyst is located (orange boxes in the schematic and confocal images). As shown in the schematic and confocal images, *Wolbachia* infection was observed in vitellarium stages 2, 5, 6, 8, and 9 (not all stages are present in one ovariole at a time). *Wolbachia* infection was absent in the stage 7 egg chamber. In other ovarioles, different compositions of infected/uninfected egg chambers were observed. Interestingly, so far we have not detected any uninfected offspring. This strain will be further analyzed to find out if this variation is caused by the host genotype or by the bacterial genotype. Either outcome will be very interesting for the analysis of *Wolbachia* partitioning at the germ line stem cell versus secondary infection by *Wolbachia* tropism (9). Figure 5B illustrates other examples of variant strains that were identified to

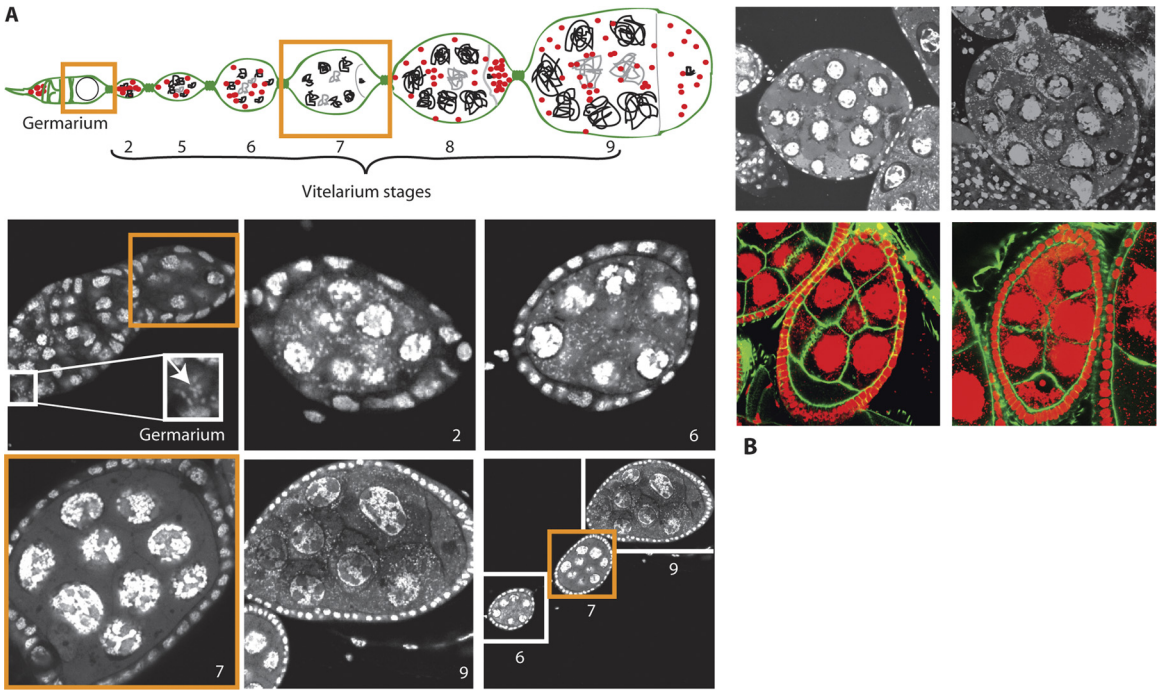


FIG. 5. Variants in *Wolbachia* localization and titer in *D. simulans*. (A) Schematic and images of a variant that has ovarioles with infected and uninfected egg chambers. In the confocal images of single egg chambers, the label in the lower right corner indicates the egg chamber stage, and the lower right panel is an overview of stages 6, 7, and 9. The germarium cyst and the stage 7 egg chamber are uninfected and marked by orange boxes in the schematic and the individual and overview confocal images. (B) Variants with low (left) and high (right) *Wolbachia* titer. Ovarioles are stained with either Syto-11 (top) or fixed and double stained with PI and Alexa Fluor 488 phalloidin (bottom row).

have an unusually low (left panels) or high (right panels) *Wolbachia* titer. This was visualized by Syto-11 (top) and PI-phalloidin (bottom) staining. All of these variants are stable in the lab through multiple generations. Whether these variants can be maintained as a stock has yet to be determined.

Infection status of the *D. simulans* population in isolated central California locations. Our monitoring of the infection levels of the *D. simulans* populations at Big Creek and the Channel Islands is ongoing. The infection levels were >90%, consistent with the infection levels in other California locations over the last decade (21). The infection level in 2007 at Big Creek was 92% (55/60). The infection levels in 2008 and 2009 at the Channel Islands were 100% (18/18) and 93% (26/28), respectively. These results illustrate that even in remote areas, like an isolated valley (Big Creek) and an island, the infection equilibrium of *Wolbachia* infection in *D. simulans* is the same as in nearby open areas.

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