Localization of endosymbionts of *Bemisia tabaci* (Gennadius) using double-fluorescence *in situ* hybridization approach

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The bacterial endosymbionts are integral to the physiology of sucking insect pests like whitefly *Bemisia tabaci*, as they contribute to the nutrition and fitness traits of their host insects. While the primary endosymbiont *Porteira* aids nutritionally, the secondary endosymbionts play additive roles such as increased fitness, thermal tolerance and host-plant plasticity. We have deployed double fluorescent *in situ* hybridization (FISH) technique to detect endosymbionts of *B. tabaci* using 16srRNA-based FISH probes targeting both primary endosymbiont, *Portiera* and secondary endosymbionts *Rickettsia* and *Hamiltonella*. Our results have shown that *Portiera* and *Hamiltonella* are confined in bacteriocytes with higher concentrations, whereas *Rickettsia* is found to have a scattered distribution pattern outside the bacteriocytes. FISH is particularly useful in understanding the colocalization pattern of the endosymbionts and their interactions in the whitefly *B. tabaci*.

Keywords: *Bemisia tabaci*, fluorescent *in situ* hybridization, *Hamiltonella*, *Portiera*, *Rickettsia*.

THE sweet-potato whitefly, *Bemisia tabaci* (Gennadius) (Aleyrodidae: Hemiptera) is a complex of cryptic species distributed globally in tropical, subtropical and temperate regions[1–3]. Due to its unusual capacity to move to new regions, evolve, establish and dominate, *B. tabaci* is recognized as one of the world's 100 worst invasive alien species[4]. It is a phloem-feeding polyphagous insect that attacks more than 100 species of plants belonging to 89 families. It is also a devastating vector which transmits more than 111 viruses such as Begomoviruses, Carlaviruses, Clostero viruses, Criniviruses and Ipomoviruses[5–8]. *B. tabaci* is labelled as a cryptic species complex because of its high level of genetic diversity in the worldwide distributed genetic groups. The nucleotide sequence of the mitochondrial cytochrome oxidase subunit I (mtCOI) gene can be used as a barcode to classify *B. tabaci* populations[9]. Forty-five cryptic species have been identified globally based on a 3.5% pairwise divergence in COI sequences within *B. tabaci*[10]. To date, 13 *B. tabaci* genetic groups have been identified in India, which include Asia I, Asia I India, Asia II 1, Asia II 5, Asia II 6, Asia II 7, Asia II 8, Asia II 11, Asia II 13, Middle East Asia Minor (MEAM) 1, MEAM K, China 3 and China 7 (refs 10, 11).

Many of the sucking insect pests in nature have symbiotic relationships with various microbes, including endosymbiotic bacteria[12]. Like many other sucking insects, *B. tabaci* is reported to have a close association with select bacterial symbionts, which are categorized into primary and secondary on the basis of their functional role. Primary endosymbionts provide nutrients that are lacking in the main insect diet. They are called obligatory endosymbionts and possess a mutualistic relationship with the host[13–15]. Primary endosymbionts are maternally inherited and are generally located in specialized cell structures within the insect called bacteriocytes[16–18]. However, secondary endosymbionts could reside in various host tissues, including hemolymph, gut, malphigian tubules, salivary glands and ovaries, and are also called facultative endosymbionts[19–22].

B. tabaci is reported to harbour a primary endosymbiont, *Portiera aleyrodidarum* and seven other secondary endosymbionts, viz. *Arsenophonus*, *Cardinium*, *Fritschea*, *Hamiltonella*, *Hemipteriphilus*, *Rickettsia* and *Wolbachia*[23–26]. The secondary endosymbionts are reported to influence various biological and ecological characteristics of *B. tabaci* like reproductive potential[27–29], survival[30–33], insecticide resistance[32], plant diseases transmission capacity[31,34,35], resistance against natural enemies such as parasitic wasps[36], faster developmental time[37], skewed sex ratio[27] and increased thermal tolerance[38]. All the secondary endosymbionts of *B. tabaci* co-localize with the primary endosymbiont inside the bacteriocytes, ensuring vertical transmission. Furthermore, some *Rickettsia* strains are located outside the bacteriocytes and occur in most body cavity[39–41], implying that *Rickettsia* may undergo occasional horizontal transfer[37,42]. Infection profiles, diversity and patterns of endosymbionts are greatly influenced by geographic regions and modes of transmission, viz. horizontal, vertical or maternal[43,44]. Previous fluorescence *in situ* hybridization (FISH) studies have reported that *Portiera*, *Arsenophonus* and *Hamiltonella* are housed in the bacteriocytes, whereas *Rickettsia* is localized inside as well as outside the bacteriocytes[31,38]. Although distribution patterns of secondary symbionts,

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viz. *Fritschea*, *Hamiltonella* and *Hemipteriphilus* are restricted to bacteriocytes and can co-occur with *Portiera* in the bacteriome[34], recent studies have reported the localization of *Arsenophonus*, *Cardinium*, *Rickettsia* and *Wolbachia* in various tissues outside the bacteriocytes[21,34].

FISH has been used to locate and recognize viruses and bacteria from a variety of samples; it can also be extended to other cell components. Spatial localization and visualization of endosymbionts are vital steps which help in better understanding of expression profiling studies. Comprehending spatio-temporal infections of symbiotic microbiota aids in understanding their interactions with host cellular components[45,46]. It is essential to visualize the particular symbionts and their respective nucleic acids *in situ* while studying the interactions between *B. tabaci* and its endosymbionts, regardless of whether they cause negative effects on the whitefly. This is important while studying the movement of endosymbionts within and between host cells of *B. tabaci*. The double-FISH approach is robust and involves simple steps like sample preparation as whole mounts or individual dissected organs, sample fixation, hybridization and visualization under a microscope to locate various bacteria and viruses in plant and insect tissues. The double-FISH method is efficient and simple compared to the single-FISH method in hybridizing a single sample with two different fluorescent dyes of 20 base pairs of short-DNA probes on the 3′- or 5′-ends. This technique has been used successfully on a variety of insect and plant tissues. It can also be used to study the expression of mRNAs, other RNAs or DNAs in the cells or tissues[45]. The localization of endosymbionts in *B. tabaci* is necessary to fully understand the transmission pathway and potential interaction sites inside the host. In the present study, we focus on localization of primary endosymbiont *Portiera*, and secondary endosymbionts *Rickettsia* and *Hamiltonella* of *B. tabaci*, which influence numerous aspects of the whitefly. So spatial localization of the symbionts within *B. tabaci* is of vital interest.

Material and methods

Whitefly rearing for FISH analysis

Rearing of B and Q biotypes of whiteflies was carried out in insect-proof cages placed separately in growth chambers on cotton plants (*Gossypium hirsutum* cv. Acala) maintained at the following conditions; temperature 25° ± 2°C, 60–70% relative humidity and 14 h light/10 h dark photoperiod. This particular experiment was conducted in the Department of Entomology, The Volcani Center, Israel.

Insect collection, fixation, hybridization, and visualization of endosymbionts using FISH

Ten iso-female adult whiteflies of B and Q biotypes maintained in cages were collected separately using an aspirator. The collected whiteflies were immersed in Carnoy's fixative, which is a mixture of chloroform–ethanol–glacial acetic acid in the ratio 6 : 3 : 1 (vol/vol) inside micro-centrifuge tubes and allowed for fixation overnight. The fixative was removed followed by decolorization using 6% H_2O_2 in ethanol for at least 2 h. The specific oligonucleotide florescent probes, BTP1 5′-Cy5-TGTCAGTGTCAGCCCA-GAAG-3′, Rb1 5′-Cy3-TCCACGTCGCCGTCTTGC-3′ and BTH 5′-Cy3-CCAGATTCCCAGACTTTACTCA-3′ were used to target *Portiera*, *Rickettsia* and *Hamiltonella* respectively. Samples were allowed for hybridization using hybridization buffer containing 20 mM *Tris*-HCl at pH 8.0, 0.9 M NaCl, 0.01% (w/v) of sodium dodecyl sulphate and 30% (v/v) formamide containing 10 pmol fluorescent probe/ml. Double-FISH was carried out by combining more than one specific probe with different fluorescent dyes and no-probe treated samples were used as a negative control. Samples were mounted on a glass slide along with a hybridization buffer containing a liquid blocker and finally covered with a cover slip. The sides of the cover slip were sealed with nail polish and allowed to dry for 5–10 min. Samples on the glass slide were visualized using a confocal microscope (IX81, Olympus FluoView FN 500 confocal microscope, Olympus Optical Co, Tokyo, Japan)[45].

Whitefly guts dissection, fixation, hybridization and visualization for Rickettsia

The adult whiteflies were captured with an aspirator and anaesthetized using an acetone-impregnated paper towel. No-probe samples were used as a control to confirm the specific hybridization. The anesthetized whiteflies were mounted on a depression glass slide for gut dissection under a stereo-microscope. Due to their small size, 1X phosphate buffered saline (PBS) was mixed with 1% toluidine blue stain for enhanced visualization. The samples were incubated for at least 2–3 min for better absorption of the stain. The head of the insect was removed, and the thorax and abdomen were gently pulled together to examine and expel the gut. Later, the contents of the abdomen were pushed out using forceps.

PBS and toluidine blue from the successful dissections were drained out followed by the addition of 300 µl of Carnoy's fixative to fix the guts for 5 min. The fixative was added from one side of the depression well rather than the top to prevent uncontrolled displacement of the dissected guts. The gut clung to the glass slide once it came into contact with the fixative, and did not move during the process. Later, the fixative was removed followed by the addition 500 µl hybridization buffer. At the final step, 10 pmol of the fluorescent DNA probe Rb1 5′-Cy3-TCCACGTCGC-CGTCTTGC-3′ was added, complementary to a sequence in *Rickettsia*.

Slides containing the specimen were allowed for hybridization by incubating them overnight in a small humid

chamber made up of a small plastic box with a moistened paper towel at the bottom, at room temperature in the dark. After hybridization, the gut was picked carefully with a sharp dissection needle and quickly transferred to a new microscope slide with new hybridization buffer supplemented with DAPI (0.1 mg/ml in 1X PBS) and liquid blocker. Before mounting and viewing, 4,6-diamidino-2-phenylindole (DAPI), a blue-fluorescent DNA stain that exhibits a 20-fold increase in fluorescence was added. Specimens were observed under a confocal microscope after covering them with a cover slip and sealing them with nail polish[45].

Results and discussion

The localization trends of primary endosymbiont *Portiera* in both the biotypes B and Q, and the secondary endosymbionts, *Rickettsia* in Q biotype and *Hamiltonella* in B biotype were visualized using the double-FISH approach, which uses the two target-specific probes to trace two endosymbionts within *B. tabaci* nymphs, adults and gut simultaneously. All the localization studies reveal that *Portiera* is regularly found within the bacteriome, whereas the secondary endosymbionts show varied localization patterns[24,45].

Localization pattern in nymphs of Q biotype

The use of a *Portiera*-specific probe (red) coupled to Cy5 and a *Rickettsia*-specific probe (blue) conjugated to Cy3 in double-FISH of *Portiera* and *Rickettsia* on *B. tabaci* nymph showed that *Portiera* is confined to bacteriocytes, while *Rickettsia* represents scattered distribution (Figure 1). Previous FISH studies on the nymphs and adults revealed that *Portiera* and *Rickettsia* were found in the bacteriocytes of cassava whitefly, where *Rickettsia* exhibited dual localization patterns too[24,26,46]. The present localization patterns are at par with previous findings. *Rickettsia* was found only in a constricted pattern in nymphal instars and pseudo pupae, restricted to the bacteriocytes in the abdomen. The confined and scattered patterns of *Rickettsia* were detected in *B. tabaci*[47].

Localization pattern in nymphs of B biotype

Similarly, for double-FISH of *Portiera* and *Hamiltonella* on *B. tabaci* nymph, a *Portiera*-specific probe (blue) conjugated to Cy5 and a *Hamiltonella*-specific probe (red) coupled to Cy3 identified co-localization of both endosymbionts in bacteriocytes (Figure 2). A previous study which localized five of the seven known *B. tabaci* secondary endosymbionts in distinct life stages of the Middle East Asia Minor 1 (MEAM 1) and mediterranean (MED) populations using FISH revealed that *Portiera* and *Hamiltonella* were detected in patches colonizing only the bacteriome in all observed life stages in both MEAM 1 and MED populations (eggs, nymphs and adults)[39].

Localization pattern in adults of Q biotype

Rickettsia FISH on *B. tabaci* adults using a *Rickettsia*-specific probe (red) coupled to Cy3 showed a scattered distribution

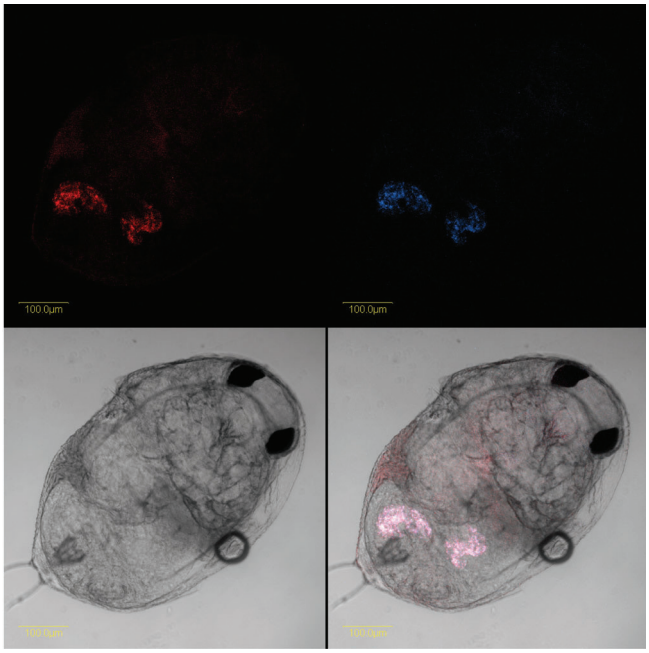

Figure 1. Double FISH of *Portiera* and *Rickettsia* on *Bemisia tabaci* nymph under IX81, Olympus FluoView FV500 confocal microscope (Olympus Optical Co, Tokyo, Japan). *Portiera*-specific probe (red) conjugated to Cy5 dye and *Rickettsia*-specific probe (blue) conjugated to Cy3 dye. Specimen at left corner in the bottom panel with no florescence served as control.

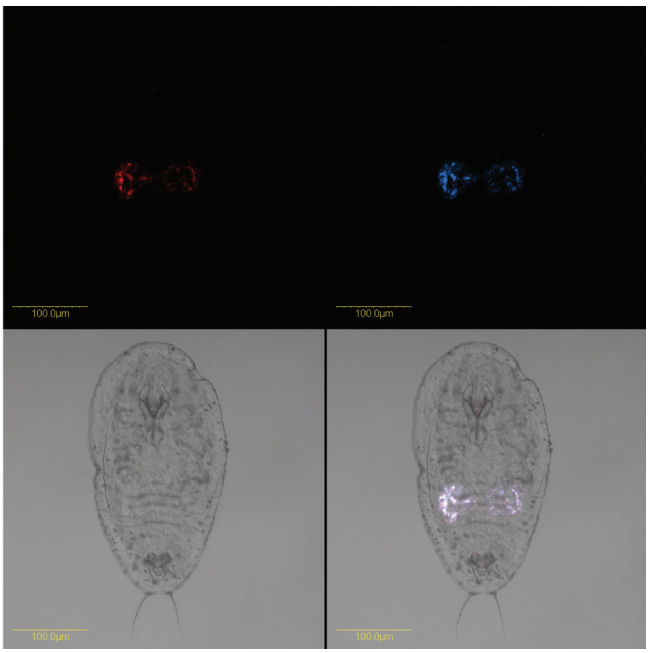

Figure 2. Double FISH of *Portiera* and *Hamiltonella* on *B. tabaci* nymph under IX81, Olympus FluoView FV500 confocal microscope. *Portiera*-specific probe (blue) conjugated to Cy5 dye and *Hamiltonella*-specific probe (red) conjugated to Cy3 dye. Specimen at left corner in the bottom panel with no florescence served as control.

of *Rickettsia* in the *B. tabaci* abdomen (Figure 3). Double-FISH of *Portiera* and *Rickettsia* on *B. tabaci* adults using a *Portiera*-specific probe (green) conjugated to Cy5 and *Rickettsia*-specific probe (red) conjugated to Cy3 revealed that *Portiera* was commonly found within bacteriocytes at higher concentration, whereas *Rickettsia* was found in lower concentration (Figure 4). *Rickettsia* was detected in the abdominal bacteriocytes in all females, but never in males. It was located in female wax glands and was observed in female colleterial glands[47]. *Rickettsia* was found in the major organs involved in the reproductive, digestive and secretion systems of *B. tabaci* in an experiment that used FISH to study its distribution in the whitefly body[38]. *Rickettsia* genomes from MEAM 1 and MED exhibited nearly full homology, implying that the same bacterium infects different *B. tabaci* species in various locations[47].

Localization pattern in adults of B biotype

In the case of *Portiera*-specific probe (green) conjugated to Cy5 and *Hamiltonella*-specific probe (red) conjugated to Cy3 for double-FISH of *Portiera* and *Hamiltonella* on *B. tabaci* adult under a confocal microscope, *Portiera* and *Hamiltonella* were found in bacteriocytes and some specialized cell structures with higher concentration in the hindgut region of the abdomen of the whitefly (Figure 5). Our results are consistent with those of a previous study which depicted the co-occurrence of *Portiera* and *Hamiltonella* in the bacteriome[21]. From a single bacteriocyte of the MED population of *B. tabaci*, both the genomic sequences of the uncultured symbionts *Portiera* and *Hamiltonella* were recovered[21].

Localization pattern in guts of Q biotype

When FISH of *Rickettsia* on dissected gut was performed using *Rickettsia*-specific probe (red) conjugated to Cy3 and the gut was stained with DAPI before mounting and visualization, the gut of an adult female *B. tabaci* was found to have the highest bacterial load of *Rickettsia* with high-density distribution along the whole gut (Figure 6). Our results are in confirmation with an earlier study which reported similar infection pattern of *Rickettsia* in high concentration inside the digestive, salivary and reproductive organs of *B. tabaci*[38]. A recent study also revealed that expression levels of vitellogenin were lower in whole insects but higher in the midguts of *Rickettsia*-infected whiteflies[48]. This study indirectly depicted the localization of *Rickettsia* in the gut of whiteflies at higher concentration[48].

An earlier study on the localization of secondary endosymbionts in distinct life stages of *B. tabaci* MEAM 1 and MED populations using FISH revealed that *Arsenophonus* was found exclusively in the bacteriome in the MED population, although its distribution was more typically around the nuclei of bacteriocytes[34]. Another study reported that *Wolbachia* and *Rickettsia* had wider distribution trends than the other symbionts in different life stages of *B. tabaci* MEAM 1 and MED populations[39]. *Rickettsia* displayed two distinct distribution patterns: tightly within the bacteriome and concentrated around the periphery (confined phenotype), or spread throughout the body, excluding the bacteriome (scattered phenotype)[26]. In the Asia II 7 genotype of *B. tabaci*, *Wolbachia* had both confined and distributed phenotypes[47]. Similar patterns of endosymbiont localization have been observed in other whitefly species, including *Hamiltonella* and *Arsenophonus* in *Trialeurodes vaporariorum*[48] as well as *Cardinium*, *Rickettsia* and *Wolbachia* in *T. vaporariorum*[49]. *Fritschea* in MEAM 1 and New World 2 (NW2) genotypes[50], and *Hemipteriphilus* in China 1 genotype[24] have shown bacteriocyte restriction. For samples

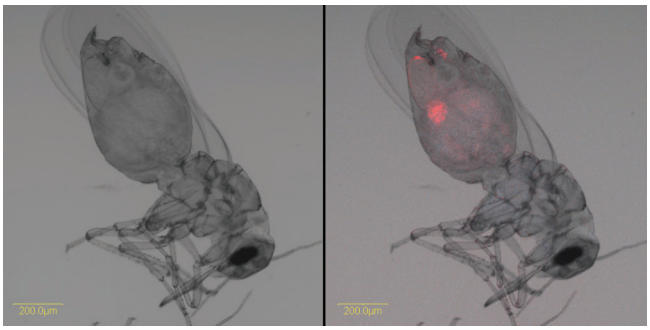

Figure 3. FISH of *Rickettsia* on *B. tabaci* adult under IX81, Olympus FluoView FV500 confocal microscope. *Rickettsia*-specific probe (red) conjugated to Cy3 dye. Specimen at left corner with no florescence served as control.

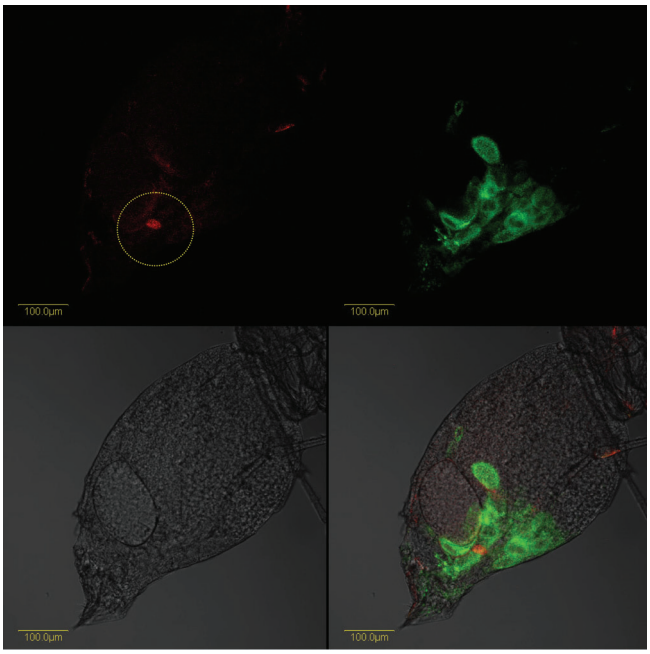

Figure 4. Double FISH of *Portiera* and *Rickettsia* on *B. tabaci* adult under IX81, Olympus FluoView FV500 confocal microscope. *Portiera*-specific probe (green) conjugated to Cy5 dye and *Rickettsia*-specific probe (red) conjugated to Cy3 dye. Specimen at left corner in the bottom panel with no florescence served as control.

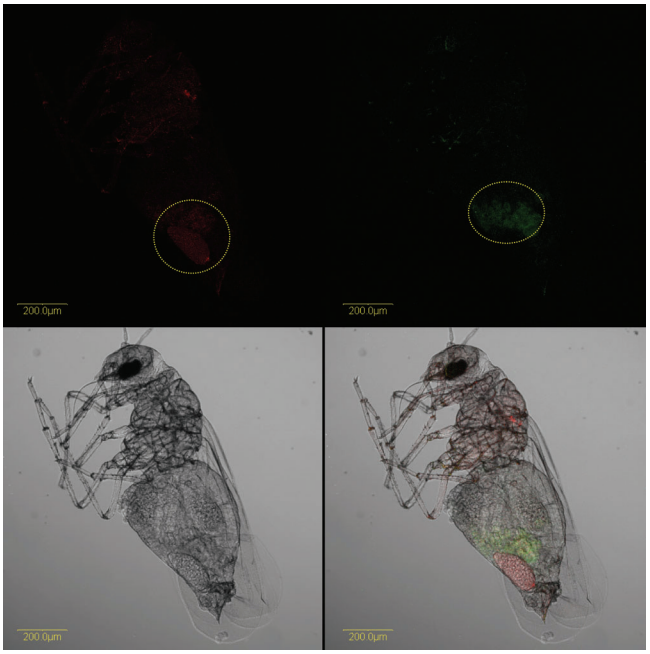

Figure 5. Double FISH of *Portiera* and *Hamiltonella* on *B. tabaci* adult under IX81, Olympus FluoView FV500 confocal microscope. *Portiera*-specific probe (green) conjugated to Cy5 dye and *Hamiltonella*-specific probe (red) conjugated to Cy3 dye. Specimen at left corner in the bottom panel with no florescence served as control.

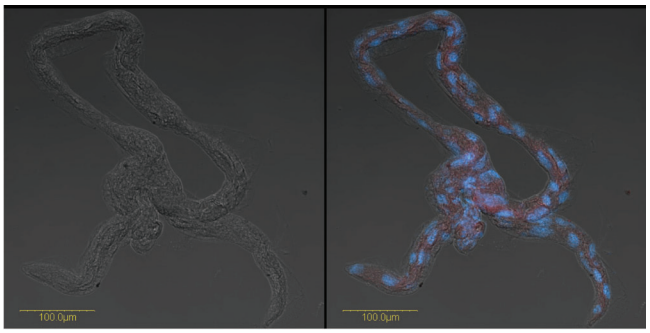

Figure 6. FISH of *Rickettsia* on dissected gut of an adult *B. tabaci* female. *Rickettsia*-specific probe (red) conjugated to Cy3 dye was used. The gut was stained with DAPI before mounting and visualization under Olympus FluoView FV500 confocal microscope. Specimen at left corner with no florescence served as control.

from the same population, the proportion of positive detection by FISH tends to be significantly higher than that by polymerase chain reaction[50,51].

Conclusion

The present study on the localization trends of the primary endosymbiont, *Portiera* and secondary endosymbionts *Rickettsia* and *Hamiltonella* using the double-FISH approach uncovers the confined distribution of *Portiera* and *Hamiltonella* in bacteriocytes with higher concentration, whereas *Rickettsia* was found with a scattered distribution pattern. We did not observe any significant difference between the localization patterns of endosymbionts between the B and Q genetic groups. However, there may exist variations in the titre of individual endosymbionts. Further studies on their expression levels may throw light on the physiological roles of the endosymbionts in *B. tabaci*.

Conflict of interest: The authors declare there is no conflict of interest.

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