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Cytological and Molecular Evidence for *Wolbachia* Infection in Uzi Flies of *Exorista* Species

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Summary The cytoplasmically inherited bacterial symbiont, *Wolbachia* is well known for inducing a variety of reproductive abnormalities in the diverse arthropod hosts it infects. *Wolbachia* has been implicated in causing cytoplasmic incompatibility, parthenogenesis, and the feminization of genetic males in different hosts. In the present investigation, electron microscopy and PCR technology was applied on uzi flies of *Exorista* species (Diptera: Tachinidae), serious pests of silkworm *Bombyx mori* L. TEM examination of uzi flies of *Exorista* species showed *Wolbachia* in the reproductive tissues. Details of the ultrastructure of *Wolbachia* are described. The application of PCR technique revealed that the *Wolbachia* is present in uzi fly populations of *Exorista* species collected from different localities. Thereby it creates a lot of scope for future research on the management of uzi flies by means of *Wolbachia*.

Key words *Wolbachia, Exorista,* Cytoplasmic incompatibility, TEM, PCR, Uzi flies.

Wolbachia are maternally (=vertically or transovarially) transmitted tiny alpha proteobacteria (Rickettsiales) infecting principally the gonadal tissues of invertebrate hosts. The type species *Wolbachia pipientis* was described from the gonads of the mosquito *Culex pipiens* (Hertig 1936). *W. pipientis* bacteria can induce cytoplasmic incompatibility (Yen and Barr 1973, Wade and Stevens 1985, O'Neill and Karr 1990, Bourtzis *et al.* 1996, Breeuwer 1997).

Basically, *Wolbachia*-induced cytoplasmic incompatibility may give rise to reproductive isolation of host subpopulations (Breeuwer and Werren 1990, Giordano *et al.* 1997). Thus, *Wolbachia* play an important role in the evolution and speciation of the latter. Moreover, the interesting range of effects they may exert on their hosts inspires promising applications in biological control of agricultural pests (Bourtzis and O'Neill 1998).

Sericulture is an agro-based industry which involves mainly the rearing of silkworms on their host plants for the production of raw silk. Among the insect pests that attack silkworms, the important one is uzi fly, a serious endoparasite which causes considerable damage to the sericulture industry (Siddappaji and Channabasavanna 1990, Narayanaswamy and Devaiah 1998, Singh *et al.* 2000). It is in this context, electron microscopy and PCR technology was applied on uzi flies of *Exorista* species, (Diptera: Tachinidae), serious pests of silkworm *Bombyx mori* L. in order to determine the presence and ultrastructure of *Wolbachia*.

Material and methods

The uzi fly populations of *Exorista* sp. screened for *Wolbachia* infection are listed in Table 1.

Electron microscopy

Ovaries from one day old adult uzi flies of *Exorista* sp. were dissected in cold (4°C) 3% glutaraldehyde at pH 7.4 and fixed in fresh glutaraldehyde for 24 h at 4°C. Following fixation in the

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primary fixative, the ovaries were washed twice with 15 min interval in 0.1 M sodium cacodylate buffer (pH 7.4). After washing, it was fixed in 1% osmium tetroxide for 1.5 h. Again, the tissues were washed with 0.1 M buffer, they were dehydrated in graded ethanol upto 90% for 30 min in each grade. The ovaries were stained with 2% uranyl acetate in 95% ethanol at 4°C for 1 h. The material was further dehydrated twice in absolute alcohol exposing for 30 min each time. Thereafter 2 changes with propylene oxide given, keeping for 30 min during each change. After clearing in propylene oxide, the ovaries were left overnight in a fixative of 1 : 1 propylene oxide : araldite on a rotator at room temperature. After 12 h with 2 changes in pure embedding medium, the materials were kept again in rotator for 6 h for infiltration. Then the ovaries were embedded in araldite CIY 212. Flat embedding moulds were used for proper orientation of the tissue. The liquid embedding medium containing the tissues was polymerized in an oven at 60°C for 48 h.

Table 1. List of uzi fly populations of *Exorista* sp. and their *Wolbachia* infection status as assayed by the PCR using *wsp* primers

Species	Locality*	PCR results (+/−)
Exorista sp.	Ramanagar (Karnataka)	+
Exorista sp.	Sira (Karnataka)	+
Exorista sp.	Madhugiri (Karnataka)	+
Exorista sp.	Mandya (Karnataka)	+
Exorista sp.	Chitradurga (Karnataka)	+
Exorista sp.	Ananthpur (Andhra pradesh)	+
Exorista sp.	Hosur (Tamilnadu)	+

* All locality belong to south India.

Before proceeding to ultra-thin sectioning, 1 μm thick semi-thin sections, which floated on the distilled water were lifted with a eye lash on to a clean glass slide. This slide was placed on a hot plate set at 80°C and dried. The sections were stained using 1% toluidine blue for 1 min followed by washing in running water. Dried slides were mounted with DPX for light microscopy.

After the semi-thin sections were scanned under light microscope, the ultra-thin sections were scanned under electron microscope. The ultra-thin sections were stained in uranyl acetate for 2 h, washed well in water, dried and then stained in 0.2% lead citrate for 3–5 min followed by a wash. The grids were dried and preserved in grid box. The sections were scanned and photographed using Jeol 100 CX Transmission Electron Microscope at the National Institute of Mental Health and Neuro Sciences, Bangalore.

Template preparation

Total genomic DNA of uzi fly populations of *Exorista* sp. used for PCR amplification was prepared by following the standard phenol chloroform procedures (Sambrook *et al.* 1989) in Seribiotech Research Laboratory (CSB), Bangalore.

PCR amplification

Polymerase chain reaction (PCR) was done in 25 μl reaction volume: 1 μl DNA sample, 2.5 μl 10× buffer (Genei), 0.5 μl dNTPs (10 mM each), 1 μl 26 μM forward primer and 1 μl 35 μM reverse primer, 0.5 μl *Taq* DNA polymerase (Genei) and distilled, deionized water was added to a final volume of 25 μl. The PCR reaction mix was prepared in one batch and then added to each sample. PCR amplification was done under the following thermal profile: 92°C 30 s, 52°C 30 s and 75°C 30 s per cycle for 30 cycles (MJ Research PTC-200). The PCR products were electrophoresed in a 1.5% agarose gel to determine the presence and size of amplified DNA, then photographed on a UV transilluminator.

The general *wsp* primers used in PCR amplification are as follows: *wsp* 81F (5′TGGTCCAATAAGTGATGAAGAAAC) *wsp* 691R (5′AAAAATTAAACGCTACTCCA).

As negative controls, PCR reactions were also performed on blanks.

Results and discussion

Electron microscopy

Under the electron microscope, we found pleomorphic microorganisms in the cytoplasm of the oocyte of *Exorista* sp. (Figs. 1, 2). The microorganisms are vary in shape from spherical to elongate. Most of the longer microorganisms appeared to be dividing. Late stages of cell division were represented as 2 cells connected by an isthmus of cytoplasm (Fig. 1, W-1).

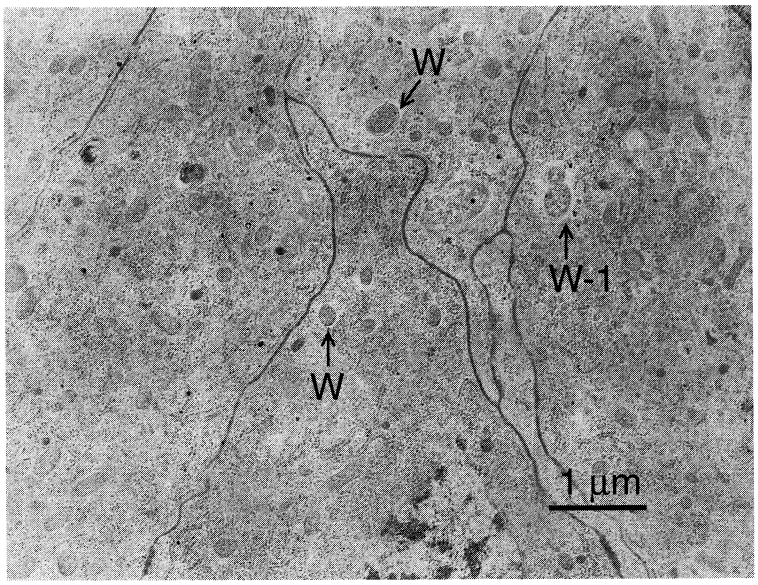

Fig. 1. Section through adult ovary of *Exorista* sp. showing infected oocyte with many *Wolbachia* (W) lying in membrane-bound vacuoles. W-1): Late stage of cell division of *Wolbachia.*

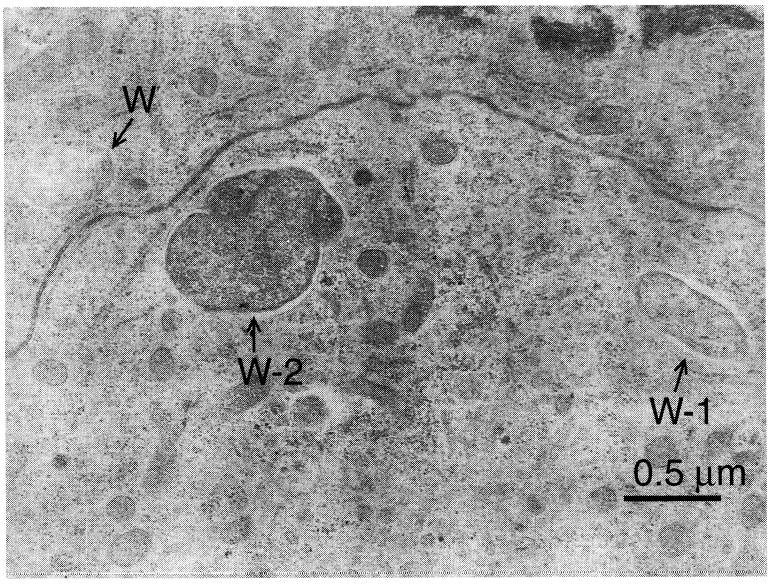

Fig. 2. Enlarged view of *Wolbachia* (W) in cytoplasm of a oocyte of an adult of *Exorista* sp. W-1 and W-2): *Wolbachia* surrounded by 3 enveloping membranes.

The cytoplasm of these procaryotes consisted of a granular, cortical region in which densely staining ribosomes aggregated along the inner surface of the cell envelope and projected into the medullary region. These procaryotes were surrounded by 3 enveloping membranes. The first membrane, close to the procaryote body, represented the plasma membrane of the procaryote. The second membrane irregularly distant from the plasma membrane, was often difficult to distinguish. It represented the outer part of the cell wall of the microorganism. The third membrane was closely related to the cytoplasm of the host cell. It was frequently distant from the cell wall, particularly at division. It consisted a membrane of the cytoplasm of the host cell, forming a kind of vacuole devoted to each single microorganism (Fig. 2, W-1, 2).

The procaryotes were usually seen singly within a given vacuole, despite tight spacing between them. The components of the envelope often stained more intensely than did the vacuole membrane, they were roughly parallel. Occasionally the vacuole membrane was seen closely appressed to the cell envelope.

Procaryotes found in the uzi flies of *Exorista* species were morphologically very close to Gram-negative bacteria, showing a plasma membrane surrounding the cytoplasm and a cell wall with an outer membrane, resembling Rickettsiales (Family Rickettsiaceae, tribe Wolbachieae).

The tribe Wolbachieae contains only the genus *Wolbachia*. The closest species to the procaryotes we observed in the uzi flies of *Exorista* species seems to be *Wolbachia pipientis*, responsible for maternally inherited incompatibility in other dipterans like mosquitoes and *Drosophila* species (Yen and Barr 1971, 1973, Wright and Wang 1980, Wright and Barr 1981, Meek 1984, Louis and Nigro 1989). The fine structure of *W. pipientis* is also typical of Gram-negative bacteria and the cell shape is very similar to the shape of procaryotes of mosquito and *Drosophila* species. Hence, based on the site of infection, ultrastructure, and evidence of a cell wall, we tentatively assigned the microorganisms of the uzi flies of *Exorista* species to the genus *Wolbachia*.

PCR assay

Braig *et al.* (1998) found that the primer combination of *wsp* 81F and *wsp* 691R was able to amplify *wsp* gene fragments from different strains of *Wolbachia*. By using the same general *wsp* primers, Zhou *et al.* (1998) amplified fragment of the *wsp* gene from 28 *Wolbachia* strains. They reported that these primers amplify a DNA fragment ranging from 590 to 632 bp depending on the individual *Wolbachia* strain and these primers were only able to amplify fragments from infected insects and not from uninfected hosts. A similar results are obtained in the present study using *wsp* primers (81F, 691R) in a polymerase chain reaction. A fragment of the *wsp* gene was detected in different uzi fly populations of *Exorista* sp. (Table 1). The mobilities of DNA fragments that were amplified using DNA from different uzi fly populations of *Exorista* sp. are shown in Figs. 3 and 4. The prominent reaction products are represented by single bands of the expected size, around 600 bp. Hence, from the PCR assay, it is evident that *Wolbachia* is present in endoparasitic uzi fly populations of *Exorista* sp. collected from different localities of south India.

There is a considerable interest in using CI (Cytoplasmic Incompatibility) *Wolbachia* in biological control (Aeschilimann 1990, Beard *et al.* 1993, Bourtzis and O'Neill 1998, Caspari and Watson 1959, Curtis 1976, Curtis and Adak 1974, Girin and Bouletreau 1995, Krishnamurthy and Laven 1976, Laven 1967, Sinkins *et al.* 1997, Stouthamer 1993, Werren 1997). Early studies considered use of *Wolbachia* to eradicate host populations in a method analogous to sterile-male release (Laven 1967). Even before the etiology of cytoplasmic incompatibility was determined in mosquitoes, attempts were made to exploit it as a method of genetic pest control. These early attempts focused on the use of cytoplasmic incompatibility to introduce sterility into wild populations of mosquitoes. Similarly, laboratory and field trials can be conducted to investigate the use of cytoplasmic incompatibility for the suppression of uzi fly population. CI strains with lower transmission rates that achieve polymorphic equilibria within host populations might be employed with effect.

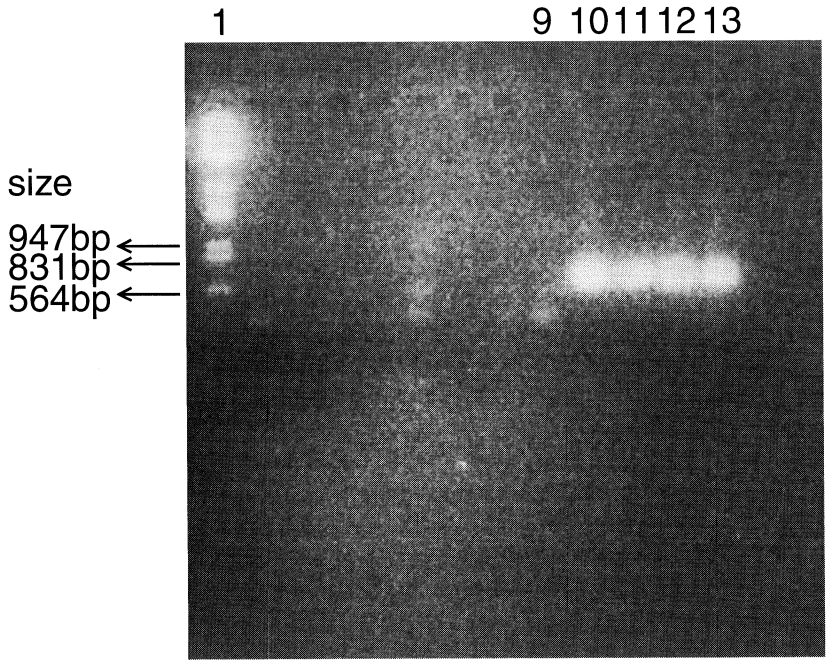

Fig. 3. PCR analysis of *Wolbachia* infection in uzi fly populations of *Exorista* sp. using *wsp* primers. Lanes: 1) λ hind III ECORI, 9) Negative control, 10, 11, 12 and 13) *Exorista* sp. of Ramanagar, Sira, Madhugiri and Mandya.

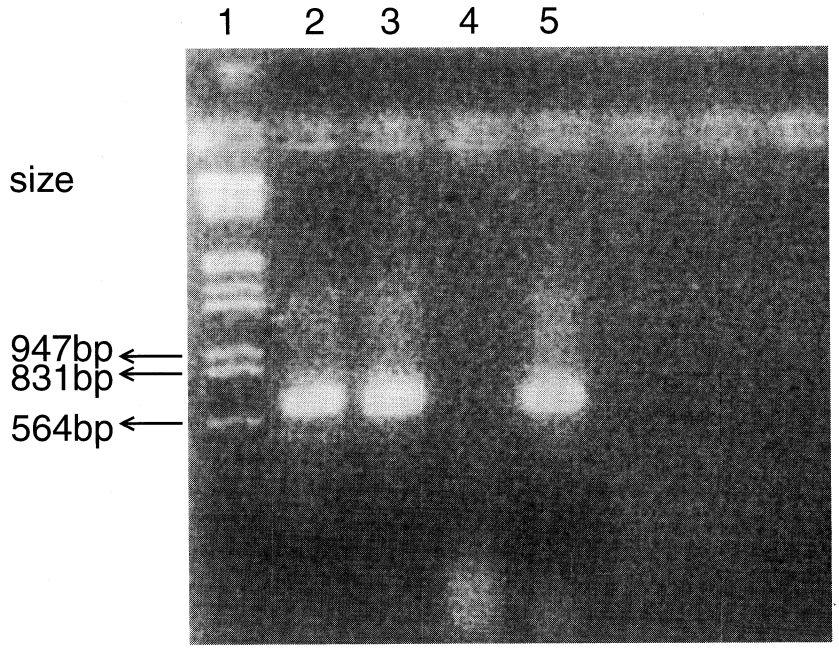

Fig. 4. PCR analysis of *Wolbachia* infection in uzi fly populations of *Exorista* sp. using *wsp* primers. Lanes: 1) λ hind III ECORI, 2 and 3) *Exorista* sp. of Chitradurga and Ananthpur, 4) Negative control, 5) *Exorista* sp. of Hosur.

A more ambitious use of *Wolbachia* involves genetically engineered organisms. Several projects are underway to genetically engineer vector arthropods for refractoriness to disease agents (Beard *et al.* 1993). The ability of a CI *Wolbachia* strain to sweep through a population, bringing along with it other maternally inherited factors (such as genetically altered endosymbionts) could be an effective mechanism for genetic replacement (Beard *et al.* 1993, Caspari and Watson 1959, Curtis 1976, Curtis and Adak 1974). It may also be possible to use *Wolbachia* to spread other chromosomally located insect genes into the desired insect species. This approach can be performed once the *Wolbachia* genes that are responsible for cytoplasmic incompatibility are isolated and introduced into an insect chromosome. Based on theoretical modelling, if these genes are expressed appropriately, then they will spread into an insect population along with any linked chromosomal gene(s) (Sinkins *et al.* 1997). Bidirectional cytoplasmic incompatibility and/or the establishment of double infections of 2 mutually incompatible strains provide the tools for repeated *Wolbachia* sweeps to replace a target insect population. In the present investigation, as the electron microscopy and PCR assay revealed that *Wolbachia* is present in uzi fly populations of *Exorista* species, it opens a new arena for research on control aspect of uzi flies of *Exorista* species through *Wolbachia*.

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