Development of primary cell cultures from the adult xylem-feeding leafhopper, *Kolla paulula*, as a tool for studying *Wolbachia* biology

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**A B S T R A C T**

Xylophagous leafhoppers are vectors of xylem-limited plant pathogens such as citrus variegated chlorosis (CVC) and Pierce’s disease, which cause *Xylella* diseases. Currently, no cure for *Xylella* diseases exists. The objective of endosymbiont control using *Wolbachia pipientis* is to reduce the populations of insect vectors, potentially preventing the expansion of *Xylella* diseases. The purpose of this study was to establish primary cell cultures from adult xylem-feeding leafhoppers to study *Wolbachia* biology. Cells from adult male and female *Kolla paulula* (Walker) (Hemiptera: Membracoidea: Cicadellinae) were successfully cultured in Dulbecco’s Modified Eagle Medium containing 20% fetal bovine serum and were maintained for more than 6 months. Cells of both male and female adults are round and semi-attached. The doubling times for male and female *K. paulula* cells are approximately 8 and 10 days, respectively. The presence of *Wolbachia in K. paulula* cell cultures was detected by polymerase chain reaction (PCR) amplification of *Wolbachia* surface protein (*wsp*) gene. The cell cultures developed in this study may be useful in studying interactions between *Wolbachia* and its hosts of different genders.

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**Introduction**

Leafhoppers are sap sucking insects that feed on leaf tissue, phloem sap, or xylem sap. This feeding behavior causes leaves to curl and is responsible for the transmission of numerous harmful pathogens (Purcell, 1982; Ng and Falk, 2006; Weintrab and Bealnand, 2006). Xylophagous leafhoppers, for example, are vectors of Xylella fastidiosa, a xylem-limited gamma-proteobacterium. *X. fastidiosa* causes serious plant diseases such as citrus variegated chlorosis (CVC) and Pierce’s disease in grapevines (Redak et al., 2004). These diseases have caused considerable agricultural losses in the United States and in some South American countries (Redak et al., 2004; Curley et al., 2007). Currently, no effective treatment for *Xylella* diseases exists. To prevent the expansion of *Xylella* diseases, new strategies for pest management must be developed. Previous studies have proposed that symbiotic control using *Wolbachia pipientis* is a possible strategy to reduce the size of insect vector populations (Dobson, 2003; Cook et al., 2008; Marshall et al., 2011). *W. pipientis* is a maternally transmitted alpha-proteobacterium, which inhabits a variety of filarial nematodes and insect species (Werren, 1997). Based on the sequence data of *Wolbachia* surface protein (*wsp*), *Wolbachia* strains can be grouped into six supergroups (A–F). Strains belonging to supergroups A and B are frequently found in insect species (Werren et al., 1995; Zhou et al., 1998). *Wolbachia* infection results in reproductive abnormalities, including cytoplasmic incompatibility (CI), parthenogenesis, feminization, and male killing (Stouthamer et al., 1999). Among *Wolbachia*-mediated reproductive defects, CI has been successfully applied to reduce the populations of medflies, mosquitoes, and planthoppers in laboratory studies (Noda et al., 2001; Zabalou et al., 2004; Xi et al., 2005).

Insect cell lines perform a vital function in *Wolbachia* applications. To apply the *Wolbachia*-mediated endosymbiont control successfully, *Wolbachia* strains must adapt and proliferate in cells within target insects. Cell lines cultured in vitro, therefore, can serve as temporary hosts for the maintenance and amplification of *Wolbachia* strains (O’Neill et al., 1997; Dobson et al., 2002; McMeniman et al., 2008; Kawai et al., 2009). Insect cell lines are also used to study *Wolbachia*–host interactions. For example, by comparing proteomics from *Wolbachia* infected cell lines and from *Wolbachia*-free cell lines, researchers identified several host genes that might be involved in the processes of *Wolbachia* infection (Dangi et al., 2009; Fallon and Witthuhn, 2009; Hughes et al., 2011). Currently, insect cell lines are...
frequently used to study Wolbachia biology (Brennan et al., 2008; Frentiu et al., 2010; Kambris et al., 2010).

The present study was conducted to culture cells from three leafhoppers, *Kolla paulula* (Walker, 1858) (Hemiptera: Cicadellinae), *Idioscopus niveosparsus* (Walker, 1870) (Cicadellidae: Idiocerinae), and *Empoasca sonani* (Matsumura, 1931) (Cicadellidae: Typhlocybinae). *K. paulula* is a widespread xylem-feeding leafhopper in Taiwan (Shih et al., 2009) and is the most common xylem feeder in orchards in central Taiwan (Shih et al., unpublished data). Therefore, we hypothesize that *K. paulula* is a vector of *Xylella* diseases. *I. niveosparsus* is a phloem sap feeder that is a serious pest of mango (Chou and Chou, 1990; Backus et al., 2005). *E. sonani* feeds on leaf tissue (Gitau et al., 2009). Because these three species use different feeding behaviors and because all of these leafhoppers can be reared in the laboratory, we decided to develop cell cultures from these three leafhoppers as tools for further studies on the relationship between *Wolbachia* and its leafhopper hosts. Although cell lines are easier to establish from embryos and larvae, such cell lines have both male and female genetic backgrounds. In this study, we established cell lines from male or female adults to obtain unique sex backgrounds. Such cell lines derived from adults might be useful for studying the regulation of *Wolbachia* sex-specific expression genes such as *pk2* and the phage-related DNA methylase (Duron et al., 2007; Walker et al., 2007; Yamada et al., 2011).

**Materials and methods**

**Primary cell culture**

*K. paulula* adults were captured from fields in Douliou City, Taiwan, and *I. niveosparsus* and *E. sonani* adults were captured from fields in Wufeng City, Taiwan. All leafhoppers were reared in laboratory cages. Ten male or female adults were surface sterilized using a 75% ethanol solution for 10 min, followed by a 3% bleach solution for 5 min. The sterilization procedure was repeated three times. The insects were then washed three times in sterile phosphate-buffered saline (PBS). The heads and wings were removed and the rest of bodies were homogenized in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 20% heat-inactivated fetal bovine serum (FBS). Tissue debris was removed by centrifugation and the supernatant was transferred to a tissue culture flask (Nunc, 25 cm²) containing DMEM and 20% FBS. The flasks were maintained at 28 °C in a humidified

![Fig. 1](image1.png) **Fig. 1.** Morphology of cells established from female *K. paulula* adults. Light microscopy on day 21 of culture.

![Fig. 2](image2.png) **Fig. 2.** Primary cultures from adult leafhoppers. Cells isolated from adult leafhoppers were cultured in DMEM supplemented with 20% FBS. Cell growth was monitored using light microscopy observation every week. The pictures of cells were taken with a digital camera in Weeks 1, 3, 8, and 9.
incubator. The culture medium was changed once per week. Cell morphology was observed by light microscope and pictures were taken using a digital camera. To measure the growth rates of primary cell lines, the cells (1.5 × 10^6 cells/ml) were seeded in a tissue culture flask (Nunc, 25 cm^2) containing 10 ml DMEM supplemented with 20% FBS. Ten microliters of cells were taken from the flask, stained with trypan blue, and counted using a hemocytometer under a light microscope every week.

PCR-based detection of wsp gene

Total DNA was extracted from adults or cell cultures using Gene-Spin™-V2 Genomic DNA Isolation Kit (Bio-Protech, Taiwan) according to the manufacture protocol. PCR was performed using total DNA as the template. The presence of Wolbachia was detected by PCR amplification using a fragment of wsp gene. The forward primer B81F (5′-TGGTCAATAAGTGATGAAGAAAC) and reverse primers A691R (5′-AAAAATTAACGCTACTCCA) or B522R (5′-ACCAGCTTTTGCTTGATA) were designed according to a previous study (Braig et al., 1998). The PCR conditions were as follows: 95 °C for 1 min; 95 °C for 30 s, 55 °C for 1 min, 72 °C for 3 min (35 cycles); and 72 °C for 10 min. The amplicons were ligated into pGEM-T Easy cloning vectors (Promega) according to the instructions of the manufacturer. Clones containing the number of female cells.

Results and discussion

Cell morphology and growth curve

Morphology of adult male and female K. paulula cells was observed under a light microscope. The shape of both male and female cells was round. Cells were 3 to 7 μm in diameter and all cells were semi-attached (Fig. 1).

Fig. 2 shows that both male and female adult K. paulula cells grow rapidly in DMEM supplemented with 20% FBS. To test whether this medium can also support the growth of cells from other leafhoppers, we prepared cell cultures from I. niveosparsus, a phloem sap feeder, and E. sonani, a leaf tissue feeder. We found that cells derived from I. niveosparsus started to die in Week 3. By the end of Week 9, only a few cells were living in the medium. In contrast, E. sonani cells increased rapidly at the beginning. However, by the end of Week 9, the cell numbers had decreased significantly.

The growth curves of cells derived from male and female K. paulula adults were similar. K. paulula adult females had a faster growth rate. Both male and female cells reached their highest densities in Week 4 (5.6 × 10^6 cells/ml for male cells and 6.12 × 10^6 cells/ml for female cells) after which cell densities decreased. The doubling times for male and female cells were approximately 8 and 10 days, respectively (Fig. 3).

Several reports describe the preparation of cell lines from leafhoppers. For example, Kimura (1984) established primary cells from embryos of Nephotettix cincticeps, a vector that transmits the rice dwarf virus. The cells were maintained in Liu and Black’s medium. Kamita et al. (2005) established cell lines from Homalodisca coagulate (Germar, 1821), the glassy-winged sharpshooter (GWSS), a vector of X. fastidiosa. H. coagulate cells were cultured in Ex-Cell 401 medium containing 10% FBS or in LH medium containing 20% FBS. (H. coagulate was renamed Homalodisca vitripennis in 2006 (Takiya et al., 2006)). We established cell lines from K. paulula adults using DMEM supplemented with 20% FBS. This is the first report concerning primary cultures of K. paulula adults. Currently, we do not know why primary cell cultures could be established from K. paulula adults, but not from I. niveosparsus or E. sonani adults. One possibility might be due to the different food sources. I. niveosparsus feeds on phloem sap and E. sonani feeds on leaf tissue. This study sought to collect other
Xylem feeding leafhoppers and test whether their cells can survive and proliferate in DMEM supplemented with 20% FBS.

**Detection and identification of Wolbachia strains associated with *K. paulula***

To determine whether *K. paulula* is infected by single or multiple Wolbachia strains, two sets of primer pairs (B81F/A691R and B81F/B522R) were used in the reactions. As shown in Fig. 4, fragments of wsp can be amplified using both sets of primer pairs. The observed sizes of PCR products were similar to the predicted sizes, which were 610 bp and 440 bp, respectively. Results from the BlastN search showed that all PCR products were closely related to wholes, except the non-specific amplicon (Fig. 4B). The fragments of wsp amplified using B81F and A691R primers were similar to *wAlbA* (97% identities). The amplicons that were amplified using B81F and B522R primers were similar to *wCon* (98% identities) (data not shown). These two Wolbachia strains associated with *K. paulula* were designated as *wKpauA* and *wKpauB*. Previous studies have shown that *wCon* can cause CI in its insect host (Kondo et al., 2002). Therefore, it is necessary to test whether *wKpauB* can also induce CI in *K. paulula*. Moreover, the phylogenetic analysis based on the sequences of wsp showed that *wKpauB* was clustered with the Wolbachia strain associated with GWSS, but not with *wZpul*, the strain associated with *Zyginidia pullula* (Boheman; Hemiptera, Cicadellidae) (Fig. 5). Taken together, these results clearly demonstrated that *K. paulula* harbors at least two Wolbachia strains, and that these Wolbachia strains could be maintained in *K. paulula* cells cultured in vitro for a long period of time.

In conclusion, we successfully used commercially available DMEM supplemented with 20% FBS to culture primary cells from both adult and female *K. paulula* adults. It may be worthwhile to test whether this medium can be used to culture primary cells from other adult xylem feeding leafhoppers.

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**References**


