Molecular evidence for *Wolbachia* in rice insects

Gram-negative endosymbiotic α-proteobacteria, *Wolbachia*, first reported in mosquitoes (*Culex pipiens*) in 1924 (ref. 1), are found in reproductive and some somatic tissues of many insects, isopods, mites and nematodes[2–4] and cause cytoplasmic incompatibility, sex-ratio distortion, parthenogenesis and feminization of genetic males of the host[5–7]. They are usually maternally transmitted through egg cytoplasm, although male transmission is reported in some laboratory strains[8]. Because of their obligate nature, they are not yet cultured on cell-free medium. But their presence in the host is known by DNA-based diagnosis using primers that specifically amplify the bacterial 16S ribosomal RNA gene[5]. *Wolbachia* has aroused much interest in biologists in recent years for its role in insect reproduction, evolution and parasitism and the possibility of using it as a vector to deliver insect or pathogen lethal gene products.

Rice is the most important food crop of the world, supporting more than half of the world population. More than 100 insect species thrive on rice and many cause potential damage. It is essential to understand the parasite genetic system for sustainable management of the pest population below economic threshold level. But little work has been done on the genetic and cytoplasmic factors of the rice-insects that have profound effect on population structure. The present paper describes heritable, extra chromosomal factors in rice-insects, which might have great bearing on the parasite reproduction system, evolution of parasitism, speciation and biocontrol.

Figure 1. *Wolbachia* 16S rRNA gene amplification in rice insects and mosquitoes. Lane M, Lambda DNA marker; lane 1, rice gall midge; lane 2, yellow stem borer (adult); lane 3, yellow stem borer (larvae); lane 4, white-backed planthopper; lane 5, brown planthopper; lane 6, rice leaffolder; lane 7, rice skipper; lane 8, rice mealy bug; lane 9, rice stink bug; lane 10, green leafhopper; lane 11, rice caseworm; lane 12, mosquito.

Genomic DNA from 10 rice-insects and mosquitoes (Table 1) was extracted[9]. *Wolbachia* was detected in a polymerase chain reactor (PCR)-based assay using the primers 5′-TTGT AGCCTGCTATGGTATAACT-3′ (forward) and 5′-GAATAGGTATG ATTTTCATGT-3′ (reverse) which were synthesized (Bangalore Genei, India) based on the published sequence information for the 16S rRNA gene of *Wolbachia pipientis*[5]. Amplification was done in a Perkin–Elmer Cetus, USA thermocycler using 80 ng of insect DNA in a 50 µl reaction mixture containing 1X PCR buffer, 200 µM of each of the four dNTPs, 150 ng of each primer and 1.5 units of *Taq* DNA polymerase (Bangalore Genei, India) for 35 cycles, each cycle consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and chain extension at 72°C for 2 min. The amplified products were size-fractionated on 1.6% agarose gel and visualized by ethidium bromide staining and UV fluorescence.

The primers amplified a 870 bp fragment in leaffolder, caseworm, gall midge, white-backed planthopper and mosquito (Figure 1). The size of the amplified product matches that of the 16S rRNA gene amplified from rice gall midge[10] and other insects[5] harbouring *Wolbachia*, using the same set of primers. No amplification of the 16S rRNA gene in yellow stem borer, brown planthopper, green leafhopper, rice

Table 1. Detection of *Wolbachia* in rice-insects and mosquitoes

Common name	Scientific name	Family/Order	Stage used	*Wolbachia*
Rice leaffolder	*Cnaphalocrosis medinalis*	Pyralidae/Lepidoptera	Larvae	+
Yellow stem borer	*Scirpophaga incertulas*	Pyralidae/Lepidoptera	Adult/larvae	–
Brown planthopper	*Nilaparvata lugens*	Delphacidae/Homoptera	Adult	–
White-backed planthopper	*Sogatella furcifera*	Delphacidae/Homoptera	Adult	+
Green leafhopper	*Nephotettix* spp.	Cicadellidae/Homoptera	Adult	–
Asian rice gall midge	*Orseolia oryzae*	Cecidomyiidae/Diptera	Adult	+
Rice caseworm	*Nymphulla depunctalis*	Pyralidae/Lepidoptera	Larvae	+
Rice skipper	*Parnara mathias*	Hesperiidae/Lepidoptera	Larvae	–
Rice mealy bug	*Brevennia rehi*	Pseudococcidae/Homoptera	Larvae	–
Rice stink bug	*Leptocorisa* spp.	Alydidae/Hemiptera	Adult	–
Mosquito	*Culex* spp.	Culicidae/Diptera	Adult	+

+, present; –, absent.

skipper, mealy bug and stink bug suggests absence of *Wolbachia* in these pests of rice. *Wolbachia* has recently been reported in white-backed planthopper in Japan[11]. Leaffolder and caseworm are new host records, while *Wolbachia* infection in white-backed planthopper and mosquito is reported for the first time from India.

Although *Wolbachia* is classified as monophyletic origin bacteria, recent molecular analysis using PCR primers that target faster-evolving and *Wolbachia*-specific genes such as *ftsZ*, a cell cycle gene[2] and *wsp*, a surface protein gene[12] has resolved variability existing among members of the *Wolbachia* genus and explained the role of the bacteria in host evolution and speciation. Further work on the horizontal and vertical distribution of *Wolbachia* in rice insects may help to understand the interaction and usefulness of the association.

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Selective loss of exocrine tissue during islet storage at –70°C

Since two and half decades[1] it has been shown that frozen–thawed–transplanted islets were able to normalize blood sugar in streptozotocin-diabetic rats. In experiments with adult rat islets, a number of different freezing protocols have been used, with viability demonstrated both *in vitro*[2] and *in vivo*[3]. Effective islet transplant programme promotion requires different forms of storage, such as cold storage of the pancreas during transport to a centre for islet isolation; tissue culture to reduce tissue immunogenicity and cryopreservation for long-term storage. It is also mandatory to have pure islet preparation without exocrine cell contamination for islet transplantation into a diabetic recipient.

In our ongoing project on islet isolation[4] and cryopreservation[5] we came across an interesting feature on differential susceptibility and sensitivity of pancreatic cell populations to cryo storage at –70°C. Islets were isolated from Balb/C mice of either sex following the protocol developed by us[4] and the resulting islets along with acinar cells were cryopreserved at –70°C (Deep Freezer, SO LOW, Cincinatti, Ohio, USA). After one month of storage at –70°C, islet preparations were rapidly thawed at 37°C and revived as described earlier[5]. Revived islets were stained for their viability and specificity employing trypan blue dye exclusion test and dithizone staining[6], respectively. It was observed that islets showed 92 ± 2.5% viability and remained unstained with trypan blue, whereas the acinar cells present in the preparation showed almost zero viability, turning blue in colour. The viability of frozen–thawed islets was comparable to that of freshly isolated islets (94 ± 3.1) The viable islets stained positive (brick-red in colour) with dithizone indicating their specificity and identity, whereas acinar cells remained unstained with dithizone.

These results clearly indicate that the protocol used for cryopreservation of islets at –70°C leads to selective killing of acinar cells, permitting survival of islets only. We repeated these experiments 8 times and obtained similar results on all the occasions, indicating susceptibility of acinar cells and resistance of islets to cold storage at –70°C under similar set of conditions.

Islet purity has been considered to be of major importance in islet transplantation programme as it determines the long-term survival of islets on transplantation. Several methodologies have been used to get pure islet preparations free of acinar-cell contaminants[7,8]; however they are laborious and affect islet yield adversely.

Our results indicate that impure islet preparations containing acinar cells could be safely subjected to cryopreservation at –70°C leading to selective killing of acinar cells, without complicating the purification procedure. The method described in this communication provides an alternative approach to purification of islets without affecting islet yield and viability. These cryopreserved and revived islets have been shown to retain their insulin secretory activity in response to glucose challenge[5], indicating their functional state. Cryopreserved pancreatic microfrag-