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Variation in Endosymbionts of Phosphine Resistant and Susceptible Key Stored Grain Insect Pests

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Stored grain insect pests are one of the major factors for the post harvest grain wastage. Stored grain insect pests posses several primary and secondary endosymbionts. Some of the endosymbionts are involved in the insecticide degrading mechanism that leads to the development of phosphine resistance. In this study, different types of endosymbionts (*Arsenophonus, Rickettsia, Wolbachia, Hamiltonella, Fritschea, Cardinium*) were recorded in phosphine resistant and susceptible population of *Tribolium castaneum, Sitophilus oryzae* and *Rhyzopertha dominica* when analyzed with 16S rRNA gene primer specific selective amplification of respective endosymbionts. Level of variations in the population of endosymbionts was also recorded in phosphine resistance and susceptible populations of all the three stored grain insect pests.

Key words: *Tribolium castaneum, Sitophilus oryzae, Rhyzopertha dominica,* Endosymbiont variations, 16S rRNA gene primer, Phosphine,

India is one of the largest producer and consumer of food grains after China producing an average of 250 million tons annually. Among the total grains produced, more than 50 per cent of the grains are retained by the farmers for their consumption and seed purpose. Significant amount of postharvest grain losses are reported in India. Studies have reported that the annual losses during storage are estimated at 50,000 crore rupees (Singh, 2010). Post-harvest grain losses are mainly caused by several biotic (insects, mold and other bioagents) and abiotic factors (moisture, heat and humidity). Among them, the biotic factors particularly, the insect pests play a major role in deterioration of food grains and can cause the post-harvest grain loses up to 5 – 10 per cent (Cao *et al*., 2002). Worldwide, more than 1500 species of insect pests are reported in stored grain ecosystem. Among the stored grain insect pests, the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera; Tenebrionidae); lesser grain borer, *Rhyzopertha dominica* (Fab.) (Coleoptera; Bostrichidae) and rice weevil, *Sitophilus oryzae* (L.) (Coleoptera; Curculionidae) are reported as the key stored grain insect pests because of their infestation potential and they also deliberated serious threat for the long term storage of the food grains (Bell, 2000).

For the management of these insect pests fumigation with phosphine gas is followed throughout the world (Choudhry, 2000). In India 80% of food grains in storage unit are protected by phosphine fumigation only (Mohankumar, 2017). For residual free treatment, fumigant phosphine is used which is readily available as aluminium phosphide (Celphos) in tablet form and is a cheap source of fumigation (Collins *et al*., 2001). However, lack of ideal airtight condition, increases the frequency of control failure thereby consequently, increasing the frequency of application which leads to the high selection pressure for phosphine resistance in stored grain insect pests in many countries including India (Rafter *et al*., 2017). That also produces heritable resistance in many stored grain insect pests in many countries including India (Choudry, 2000). Two broad level of resistance exists viz., strong (Collins, 1998) and weak resistance (White and Lambkin, 1990) based on the presence of two resistance loci *rph1* and *rph2*. Based on the preliminary survey conducted by Rajendran (1998) in India, five major stored grain insects have developed resistance for phosphine viz., *Tribolium castaneum* (100%), *Rhyzopertha dominica* (95%), *Oryzophilus surinamensis* (92%), *Cryptolestes ferrugineus* (88%) and *Sitophilus oryzae* (72%) developed resistance for phosphine (Mohankumar, 2017). In this study we tried to identify the presence of endosymbionts in stored grain pests particularly in phosphine resistance and susceptible population of *Tribolium castaneum, Sitophilus oryzae* and *Rhyzopertha dominica* plays vital role for the resistance or susceptible for phosphine.

Insect pests have evolved symbiotic relationship with several microbes (endosymbionts) and that can significantly alter their physiology and ecology (Douglas, 2016). Secondary endosymbionts are not localized to specific location, rather they are found throughout the host insect. Though these endosymbionts are not essential for survival rather they play important role in different metabolic processes of reproduction, development and viral transmission. The common and widespread group of endosymbiont Wolbachia was observed

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in the reproductive tissues of several arthropods. Wolbachia plays role in reproductive manipulation in arthropods (Werren, 1997), whereas Cardinium plays its role in including cytoplasmic incompatibility, parthenogenesis and feminization (Zchori-Fein and Perlman, 2004). Rickettsia population increases the susceptibility to insecticides and becomes harmful for its host (Kontsedalov *et al*., 2008). Hamiltonella is host-dependent metabolic symbiont which depends on its host to fulfill its nutritional requirements. Arsenophonus belongs to γ- Proteobacteria of phylum Proteobacteria is reported in many arthropods plays role in transmission of virus. Like Wolbachia, Cardinium has been reported that it causes cytoplasmic incompatibilities (Weeks *et al*., 2001).The insecticide degrading bacterial endosymbiont (Burkholderia) was identified in the posterior midgut region of the bean bug Riptortus pedestris and reported that the Burkholderia was associated with the development of resistance against fenitrothion. The better understanding of endosymbionts associated with insecticide / fumigant resistance will not only provide the information on evolution and function of insect microbial symbiosis, but may also lead to the development of effective management strategy through targeting the microbes which contributes to the resistance. Thus, the identification of endosymbionts in the resistant and susceptible population of key stored grain insect pests is crucial for the development of phosphine resistance management strategies.

Material and Methods

Insect cultures

Phosphine resistant and susceptible strains of key stored grain insect pests viz., T. *castaneum*, R. *dominica* and S. *oryzae* were collected from the Toxicology Laboratory, Department of Agricultural Entomology, Centre for Plant Protection Studies (CPPS), Tamil Nadu Agricultural University, Coimbatore for assessing the diversity of endosymbionts. Then screening of endosymbionts was done by following the methodology of FAO (1975).

Insect species	Resistant strains	Susceptible strains
Tribolium castaneum	Madurai and Chrompet	Vellore
Rhyzopertha dominica	Coimbatore North	Kumbakonam and Avadi
Sitophilus oryzae	Kumbakonam and Salem	Vellore and Madurai

Genomic DNA extraction

Genomic DNA was isolated from five insects of *T. castaneum, R. dominica* and *S. oryzae* following the CTAB method (Doyle, 1987). The DNA extraction buffer contained 100mM Tris. HCl (pH 8), 10mM EDTA, 1.4M NaCl, 2.0 per cent CTAB and 5.0 per cent β-mercaptoethanol. Individual insect samples were homogenized with 200 µl of DNA extraction buffer and incubated at 65°C for 1 h. The tubes were removed from the water bath and allowed to cool at room temperature. Chloroform: isoamyl alcohol mixture (24:1, v/v) (0.8 volume) was added and mixed by inversion for 10 min. to form an emulsion. It was centrifuged at 12,000 rpm for 10 min. and the clear aqueous phase was transferred to a new sterile tube. Ice-cold isopropanol (0.7 volume) was added and mixed gently by inversion and it was stored at -20°C for overnight. It was then centrifuged at 12,000 rpm for 10 min. to pellet the DNA and the supernatant was discarded. The DNA pellet was washed with 70 per cent ethanol. After washing, DNA pellet was air dried and dissolved in 20-40 µl of TE buffer depending on size of the pellet and stored at -20°C until use. The isolated DNA was checked for its quality by separating in 0.8 per cent agarose gel electrophoresis and quantified by Spectrophotometer.

Screening of endosymbionts

Polymerase chain reactions were performed in 25 µl volumes in PCR machine (Sure cycler 8800, Agilent Technologies). PCR reaction cycling conditions for the amplification of endosymbionts specific16S rRNA gene primers were furnished in Table 1. The composition of cocktail mixture (for 23 µl reaction mix) contains 15.50 µl sterile water, 2.5 µl of dNTPs (Mixture of dATP,dCTP, dGTP and dTTP); 2.5 µl of 10X PCR Buffer; 1 µl each of 10 µM forward and reverse primer and 0.5 µl of Taq polymerase (1.0 Units). The cocktail mixture (23 µl) was added to each tube containing 2 µl of template DNA. Amplified products were screened using agarose gel electrophoresis (1.5%), 7.5 µl of PCR product along with 2 µl of loading dye loaded on the agarose gel in electrophoresis at 65V for 1 h and products were visualized on UV transilluminator and the gel was documented using gel documentation unit (GELSTAN, 1312).

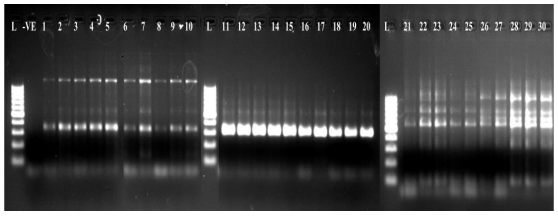
Results and Discussion

Wolbachia (Breeuwer, 1997), *Arsenophonus* (Gherna *et al*., 1991), *Rickettsia* (Werren *et al*., 2008), *Cardinium* (Zchori-Fein *et al*., 2004), *Hamiltonella* (Degnan *et al*., 2009) and *Fritschea* (Everett *et al*., 2005) have been documented in a wide range of insects. Development of phosphine resistance was recorded in key stored grain insect pests like *T. castaneum, S. oryzae* and *R. dominica* (Chaudry, 2000). Many factors like improper handling, frequent use of phosphine are the reasons for development of phosphine resistance (Rafter *et al*., 2017). Symbiotic microbes give impacts on morphology, immunology, physiology and increasing the tolerance against environment stresses. In this study, variation in endosymbionts between phopsphine resistant and susceptible insect populations of *T. castaneum, S. oryzae* and *R. dominica* were documented (Fig 2). Based on 16S rRNA gene primer specific selective amplification of respective endosymbionts, presence of endosymbionts in insects was recorded (Fig 1). They showed variations in the percentage of

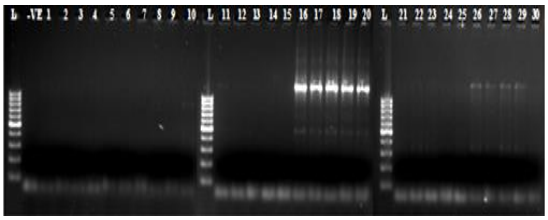
Table 1. Endosymbionts specific 16S *rRNA* gene primers and PCR program for the identification of endosymbionts

Primer	Sequence (5'→3')	Temperature profile
WOL_16S_315F	GCATGAGTGAAGAAGGCC	95°C for 2 min, then 94°C 30 sec, 50°C 45 sec, 72°C 2 min in 30 cycles, 72°C 4 min
WOL_16S_628R	AGATAGACGCCTTCGCCA	
RIC_16S_RBF	GCTCAGAACGAACGCTATC	95°C for 2 min, then 94°C 30 sec, 47.8°C 30 sec, 72°C 2 min in 30 cycles, 72°C 4 min
RIC_16S_RBR	GAAGGAAAGCATCTCTGC	
HAM_16S_92F	TGAGTAAAGTCTGGGAATCTGG	95°C for 2 min, then 94°C 30 sec, 52.6°C 30 sec, 72°C 2 min in 35 cycles, 72°C 4 min
HAM_16S_1343R	CCCGGGAACGTATTCACCGTAG	
CAR_16S_CLOF	GGAACCTTACCTGGGCTAGAATGTATT	95°C for 2 min, then 94°C 30 sec, 53.3°C 30 sec, 72°C 2 min in 30 cycles, 72°C 4 min
CAR_16S_CLOR	GCCACTGTCTTCAAGCTCTACCAAC	
FRI_16S_U23F	GATGCCTTGGCATTGATAGGCGATGAAGGA	95°C for 2 min, then 94°C 30 sec, 50°C 60 sec, 72°C 2 min in 35 cycles, 72°C 4 min
FRI_16S_23SIGR	TGGCTCATCATGCAAAAGGCA	
ARS_16S_F	GGGTTGTAAAGTACTTTCAGTCGT	95°C for 2 min, then 94°C 30 sec, 52°C 30 sec, 72°C 1.30 min in 35 cycles, 72°C 5 min
ARS_16S_R2	GTAGCCCTRCTCGTAAGGGCC	

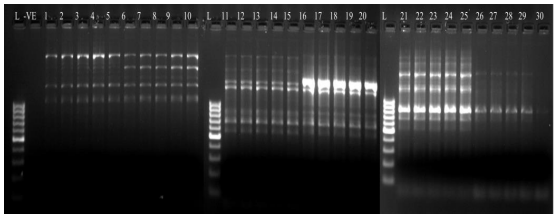
presence between different insects and also between phosphine resistant and susceptible population in the same insect. The possibility of *Wolbachia* infections influencing pesticide resistance was raised by the observation that there was a higher *Wolbachia* load in insecticide resistant *Culex*



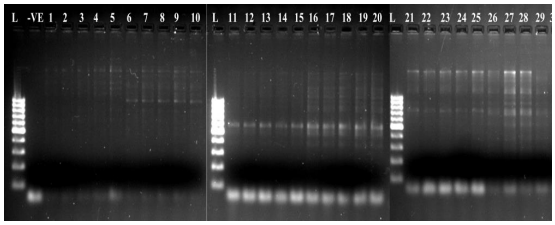
1.1 *Wolbachia* 16S rRNA amplification



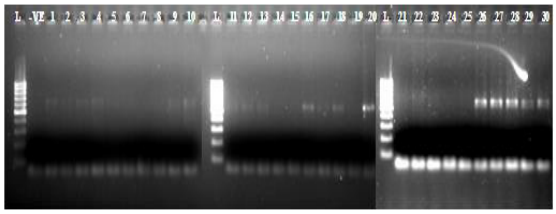
1.4 *Hamiltonella* 16S rRNA amplification



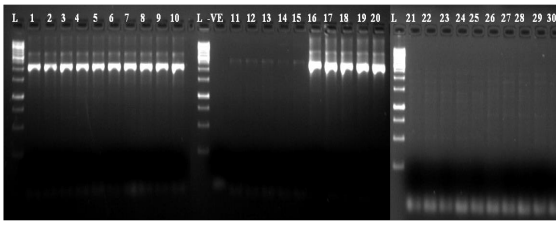
1.2 *Rickettsia* 16S rRNA amplification



1.5 *Cardinium* 16S rRNA amplification



1.3 *Fritschea* 16S rRNA amplification



1.6 *Arsenophonus* 16S rRNA amplification

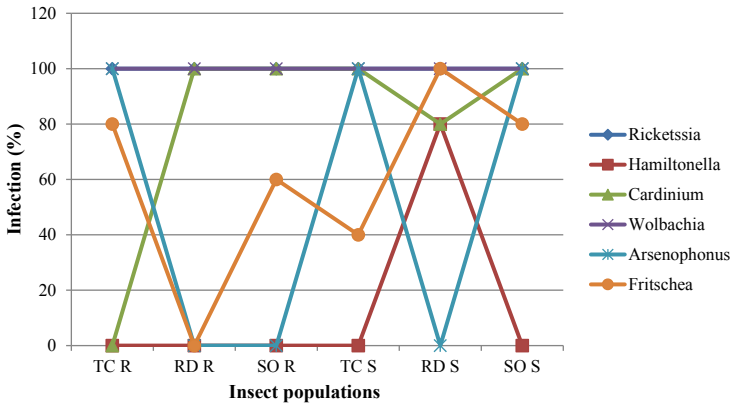
L- 100bp Ladder -ve- Negative
1 to 5 *T. castaneum* resistant population 6 to 10 *T. castaneum* susceptible population
11 to 15 *S. oryzae* resistant population 16 to 20 *S. oryzae* susceptible population
21 to 25 *R. dominica* resistant population 26 to 30 *R. dominica* susceptible population

L- 100bp Ladder -ve- Negative
1 to 5 *T. castaneum* resistant population 6 to 10 *T. castaneum* susceptible population
11 to 15 *S. oryzae* resistant population 16 to 20 *S. oryzae* susceptible population
21 to 25 *R. dominica* resistant population 26 to 30 *R. dominica* susceptible population

Fig. 1. Endosymbionts presence in stored grain insect pests detected by endosymbionts specific 16S rRNA gene primer amplification

pipiens compared with insecticide susceptible individuals, possibly due to decreased control of *Wolbachia* load as a physiological cost of insecticide resistance (Berticat *et al.,* 2002). Kontsedalov *et al*. (2008) reported that *Rickettsia* infected whiteflies showed susceptibility to acetamiprid, thiomethoxan, spiramesifen, imidacloprid, diofenthiurom and pyriproxyfen and *Rickettsia* uninfected strains showed resistance to acetamiprid, thiomethoxan, spiramesifen. Similar results have been shown with *Wolbachia* and *Rickettsia,* both singly and as a double infection which increased susceptibility of *Bemisia tabaci* to insecticides (Kontsedalov *et al.,* 2008). Here the presence of *Wolbachia* and *Rickettsia* were documented (100%) in all populations of three insects like this multiple infection of group of endosymbiont

species in a single type of insect is common (Weeks *et al*., 2001). *Hamiltonella*, *Arsenophonus*, *Cardinium* and *Fritschea* showed variations between resistant and susceptible populations of *T. castaneum, S. oryzae* and *R. dominica* (Fig 2). So, the presence of these endosymbionts may be the reason for development of phosphine resistance. Naik *et al.* (2016) studied the endosymbionts in *R. dominica* and *T. castaneum* collected from different geographical regions of southern India using endosymbionts specific 16S rRNA gene primers and identified the digestion related endosymbionts *viz*., Non-diaspididae



TC- *Tribolium castaneum,* RD- *Rhyzopertha dominica*, SO- *Sitophilus oryzae*

R- Resistant population, S- Susceptible population

Fig. 2. Diversity of endosymbionts observed in resistant and populations of key stored grain insect pests through PCR

and gammaproteobacteria in *R. dominica* and *T. castaneum*, respectively. *Fritschea* was documented in all the populations (*T. castaneum* resistant - 80%, *T. castaneum* susceptible – 40%, *R. dominica* susceptible - 100%, *S. oryzae* resistant – 60% and *S. oryzae* susceptible – 80%) except resistant populations of *R. dominica*. *Cardinium* infection was observed in all the populations (*T. castaneum* susceptible – 100%, *R. dominica* susceptible - 80%, *R. dominica* resistant – 100%, *S. oryzae* resistant – 100% and *S. oryzae* susceptible – 100%) except resistant populations of *T. castaneum*. *Hamiltonella* was observed only in the susceptible populations of *R. dominica* (80%) and *S. oryzae* (100%). *Arsenophonus* was not observed in both resistant and susceptible populations of *R. dominica* and resistant populations of *S. oryzae*. Endosymbionts other than *Wolbachia* and *Rickettesia* might have influenced the susceptibility which needs further investigation (Fig 2).

Conclusion

The present study revealed that bacterial symbionts of *T. castaneum*, *R. dominica* and *S. oryzae* differed between resistant and susceptible populations collected from different parts of Tamil Nadu. The endosymbiont *Hamiltonella* was present in susceptible population of *R. dominica* and *S. oryzae,* while *Cardinium* was present in both resistant and susceptible populations of *R. dominica* and *S. oryzae* and only in susceptible population of *T. castaneum.* Another endosymbiont *Arsenophonus* was found only in susceptible *S. oryzae;* susceptible and resistant populations of *T. castaneum. Fritschea* was found in both resistant and susceptible populations of *T. castaneum* and *S. oryzae* and only in susceptible population of *R. dominica*. This endosymbiont diversity discrimination among the stored grain insect pests may be related to their insecticide resistance, which needs further investigation to develop sustainable eco friendly prophylactic measures for stored grain insect pests.

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