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Identification of bacterial endosymbionts in rice weevil, *Sitophilus oryzae* Linnaeus (Coleoptera: Curculionidae) populations of North India

P. Kaundal[1], K.G. Padwal[2], S. Premkumari[1], S. Chakravarty[3] and C.P. Srivastava[1]*

[1]*Department of Entomology and Agricultural Zoology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi - 221 005, India.*
[2]*Department of Applied Agriculture, Rajiv Gandhi South Campus, Banaras Hindu University, Mirzapur- 231 001, India*
[3]*Department of Entomology, Dr. Kalam Agricultural College, Bihar Agricultural University, Kishanganj- 855 107, India*

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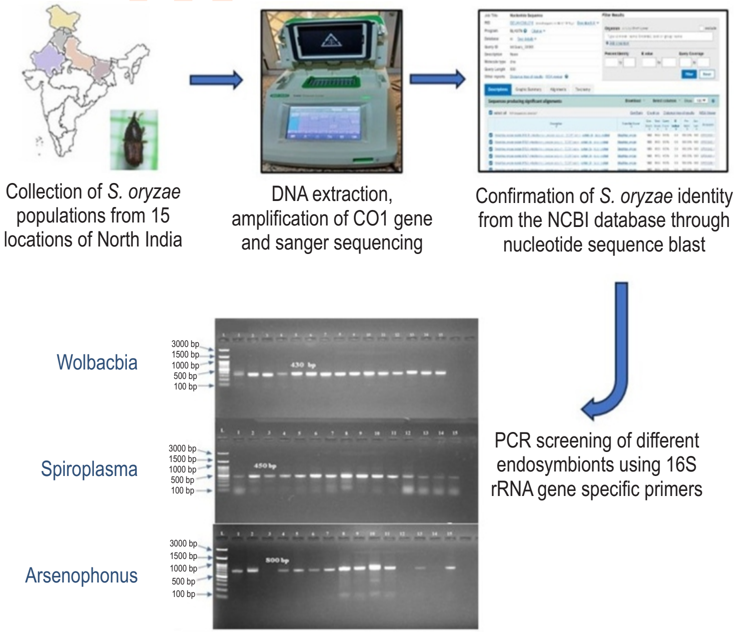
***Corresponding Author Email :** csrivastava63@gmail.com ***ORCiD:** http://orcid.org/0000-0003-3181-7825

Abstract

Aim: The present study was undertaken to screen the most commonly occurring endosymbionts in *S. oryzae* populations from varied geographical regions of North India.

Methodology: The six most common endosymbionts (*Wolbachia, Arsenophonus, Spiroplasma, Rickettsia, Cardinium* and *Hamiltonella*) were screened in 15 different populations of *S. oryzae,* representing five different agro-climatic zones of India. For this, the samples of *S. oryzae* were collected and identified using taxonomic keys and through PCR amplification of the COI gene followed by Sanger sequencing and comparison of obtained sequences with the NCBI database. Thereafter, the screening of endosymbionts was performed using 16S rRNA gene-specific primers for the respective endosymbionts.

Results: The results showed the presence of *Wolbachia* and *Spiroplasma* in all 15 populations with infection rates of 100 and 85 per cent, respectively. *Arsenophonus* was detected in only 12 populations with 60 per cent infection rate. Concurrent infections of *Wolbachia, Arsenophonus* and *Spiroplasma* within the same individuals of *S. oryzae* were also detected. However, no infections of *Cardinium, Rickettsia* and *Hamiltonella* were found.



Interpretation: Thus, it can be inferred that *Wolbachia, Spiroplasma* and *Arsenophonus* are the most common endosymbionts which can be exploited for the development of better management strategies against *S. oryzae.*

Key words: 16S rRNA, Co-infection, Endosymbionts, Rice weevil, *Sitophilus oryzae*

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Introduction

Reducing food loss and waste is recognized globally as an opportunity to combat food and nutritional insecurity and advance towards sustainable development. India, despite being one of the largest producers of food grains in the world (Vignesh *et al.,* 2018), exhibits a relatively low ranking of 107th out of 121 countries on the Global Hunger Index for 2022. One of the primary causes for the same is post-harvest losses of food grains, which amount to 12 to 16 million metric tons each year in the country, inflicting huge monetary losses of over Rs. 50,000 crores (Chakraborty *et al.,* 2021). Damage caused by stored grain insect pests are one of the major factors for such losses. The genus Sitophilus belonging to the order Coleoptera and family Curculionidae, comprises a group of species that are economically significant as stored grain pests all over the world. The rice weevil, *Sitophilus oryzae* Linnaeus is one of the most significant and damaging pest species of this genus. It is highly polyphagous and has been known to damage several stored cereals including rice, wheat, maize, sorghum, barley, and products derived from them (Mehta and Kumar, 2020).

The immature stages of this insect develop within grain kernels, which make accurate detection of infestations and the effectiveness of control measures difficult, resulting in pervasive damage (Mehta *et al.,* 2021). The extent of losses due to this pest species in the country ranges between 11-80 per cent, depending upon the infestation level, storage conditions, and period for which the grains are stored (Thakkar and Parikh, 2020). In the preceding two decades, the mitigation of food product losses caused by insect damage has predominantly relied on the utilization of synthetic insecticides. The extensive dependence on a certain set of chemical substances has resulted in many issues such as the development of resistance, the presence of harmful residues, environmental contamination, and failures in control measures (Kumar *et al.,* 2017). These obstacles and the growing awareness of environmental concerns have prompted researchers to investigate on alternatives to chemical pesticide (Nayak *et al.,* 2020). Exploitation of reproductive parasitic endosymbionts as a control strategy, in lieu of pesticides, has emerged as a promising and contemporary technique. These are potentially linked to the fitness of their insect hosts and influence the host's biological and ecological traits in a positive, negative or neutral way (Xu *et al.,* 2023).

Bacterial symbionts namely, *Rickettsia, Wolbachia, Cardinium, Spiroplasma, Arsenophonus* and *Hamiltonella* have been commonly reported in a wide range of insects (Russell *et al.,* 2012). *Wolbachia* has an omnipresent distribution amongst arthropods, known to induce a variety of reproductive manipulations, including parthenogenesis and male-killing (Werren *et al.,* 2008). Rickettsia has been found to increase the host's susceptibility to insecticides (Kontsedalov *et al.,* 2008) whereas Cardinium induces feminization and causes cytoplasmic incompatibilities, but can also provide fitness benefits by enhancing its hosts' fecundity (Zchori-Fein and Perlman, 2004). *Spiroplasma* contributes to the host's defense against both biotic and abiotic stresses (Pina *et al.,* 2020) whilst *Arsenophonus* and *Hamiltonella* play an important role in virus transmission (Morin, 1999). Although these endosymbionts are not crucial for survival, they serve an important role in many metabolic processes related to development, reproduction and insecticide metabolism. Therefore, they possess potential utility in pest management initiatives by leveraging their impact on host insects, such as the implementation of the sterile insect technique, paratrangenesis, symbiont-mediated RNAi or the manipulation of pesticide resistance (Liu and Guo, 2019; Zhang *et al.,* 2023). To effectively plan and evaluate pest management strategies including bacteria, it is important to conduct an initial investigation into the prevalence of these bacteria within natural pest populations (Ipekdal and Kaya, 2020).

However, findings on rice weevil endosymbionts from India are extremely rare. Further, there are reports about rice weevil populations from India exhibiting differential responses to phosphine susceptibility (Vignesh *et al.,* 2018), which can be possibly due to variations in their bacterial flora that are imparting insecticidal resistance. Recent studies have utilized the application of species-specific molecular markers, particularly the 16S rRNA gene, for the detection of various endosymbionts in stored product insect pests (Naik *et al.,* 2016). Thus, the current study was undertaken to screen the adult populations of *S. oryzae* sampled from different geographical regions of India for the incidence of the most common endosymbionts, which will provide valuable information for the development of better management strategies against *S. oryzae.*

Materials and Methods

Insect sampling and identification: The rice weevil infested grain (rice and wheat) samples were collected from the local shops from 15 geographical regions representing five agro-climatic zones of India (Table 2), between February 2021 and March 2022. The collected samples were maintained under controlled laboratory conditions (Biocontrol Laboratory, Department of Entomology and Agricultural Zoology, Banaras Hindu University) at 70-75% relative humidity and 25±1°C temperature, on the sterilized grains of rice variety 'Sonam'. The species identity of collected specimens was confirmed as *S. oryzae* morphologically using taxonomic keys and by dissecting their genitalia (Halstead, 1963).

Molecular identification: Prior to extraction, test insects were sterilized with absolute ethanol for 30 seconds and then cleaned using distilled water to remove any external microbial contaminants. The genomic DNA of individual insect (including bacterial DNA) was extracted using Qiagen's DNeasy Blood and Tissue kit by following the provided user's protocol. In order to confirm the identity of rice weevil, the amplification of the mitochondrial cytochrome c oxidase subunit I (COI) gene was done by using species-specific primers (forward primer 5'-AGTTTGCTAATTCGGGCAGA-3' and reverse primer 5'-

ACTCCGGTTAATCCTCCAAT-3') given by Correa *et al.* (2013). The PCR master mix consisted of 25 ng genomic DNA, 10 pmol forward and reverse primers each, 10X PCR buffer, 0.3 unit of Taq polymerase (Kapa Biosystematics TM), 2mM dNTP and 2mM $MgCl_2$ collectively. By introducing the required quantity of deionized double distilled water, the final reaction mixture was made up to 50µl. Thermocycling consisted of initial denaturation at 94 °C for 5 min, followed by 35 cycles of 95 °C for 30 sec, 55 °C for 45 sec, and 72 °C for 1 min, with a final elongation step of 72 °C for 10 min. The amplification products were electrophoresed on 1.5% agarose gel having 0.05 µg ml^{-1} ethidium bromide and sequenced by Sanger's method using ABI3730xl DNA sequencer (Applied Biosystems, USA) and Big Dye Terminator v3.1 kit (Applied Biosystems, USA) at M/s Eurofins Analytical Services India Pvt. Ltd. (Bengaluru, India).

Screening of endosymbionts: The screening of the endosymbionts was done by following the methodology of İpekdal and Kaya (2020). PCR amplifications were performed with the aforementioned procedure, using the 16S rRNA gene-specific primers as described in Table 1. The amplicons were separated by gel electrophoresis on 2% agarose gel, and visualized in BIO-RAD Gel Doc^{TM} System.

Data analysis: The COI sequences were imported into FASTA format for alignment and trimming using the ClustalW algorithm (Thompson *et al.*, 1994) with default parameters in the MEGA 6.0 software package (Tamura *et al.*, 2013). The sequences were analyzed for gene homology using nBLAST from National Center for Biotechnology Information (NCBI), and subsequently submitted to get GenBank accessions. The presence of endosymbionts in *S. oryzae* populations was detected by the standard band length of the PCR products as mentioned in Table 1, using Image Lab Software Version 6.0.1 (Trofimova and Jaschke, 2021). The infection percentage of these endosymbionts for each zone was worked out by dividing the number of endosymbiont positive individuals/ total number of individuals ×100.

Results and Discussion

The adults of rice weevil were identified by using the taxonomic keys as well by dissecting their genitalia. Sixty adults of *S. oryzae* sampled from 15 locations were analysed in the present study for endosymbionts using species-specific primers. PCR amplification of the COI gene resulted in the amplicon size of 950 bp for all the populations of *S. oryzae.* The PCR products were sequenced, and the obtained sequences of the COI gene were compared with that of *S. oryzae* database on NCBI. Sequence identities were found to be 99% or more, thereby confirming the identity of insect species. After verification, all the sequences were trimmed in Bioedit software and submitted to GenBank for accession numbers (Table 2).

The results of PCR screening of *S. oryzae* individuals for infections with six most common endosymbionts are presented in Table 2 and Fig. 1. Of the six endosymbionts tested, only three species of facultative endosymbionts, *Wolbachia, Spiroplasma* and *Arsenophonus* were detected. The *Wolbachia*-specific primers detected the presence of *Wolbachia* in all the individuals, while the *Spiroplasma*-specific PCR detected only 51 individuals out of 60 tested samples in which every location recorded at least two positive individuals for *Spiroplasma* infection. *Arsenophonus* showed very low infection as it was encountered in only 36 individuals belonging to 12 locations. It showed negative results for the Kinnaur, Samastipur and Kota populations of *S. oryzae*. Whereas, the PCR screening of Rickettsia, *Cardinium* and *Hamiltonella* did not detect the presence of these bacteria in any of the individuals tested. Infection percentages of these facultative endosymbionts in *S. oryzae* individuals belonging to different agro-climatic zones were also worked out (Fig. 2). For *Wolbachia,* the infection was 100 per cent as it was prevalent in all the locations.

Table 1: Details of the species-specific primers and their PCR conditions for screening of endosymbionts

Target genus and gene region	Names	Sequence (5'-3')	PCR Product (bp)/ Annealing Temperature (°C)	References
Wolbachia	WOL_16S_315F	GCATGAGTGAAGAAGGCC	430/53	Vignesh *et al.* (2018)
16S rRNA	WOL_16S_628R	AGATAGACGCCTTCGCCA		
Arsenophonus	Ars-F	GGGTTGTAAAGTACTTTCAGTCGT	800/52	Duron *et al.* (2008)
16S rRNA	Ars-R2	GTAGCCCTRCTCGTAAGGGCC		
Rickettsia	Rb-F	GCTCAGAACGAACGCTATC	900/58	Gottlieb *et al.* (2006)
23S rRNA	Rb-R	GAAGGAAAGCATCTCTGC		
Cardinium	Clo-F	GCGGTGTAAAATGAGCGTG	466/54	Weeks *et al.* (2003)
16S rRNA	Clo-R	ACCTMTTCTTAACTCAAGCCT		
Spiroplasma	63-F	GCCTAATACATGCAAGTCGAAC	450/55	Fukatsu and Nikoh (2000);
16S rRNA	TK55-R	TAGCCGTGGCTTTCTGGTAA		Mateos *et al.* (2006)
Hamiltonella	Ham-F	TGAGTAAAGTCTGGAATCTGG	730/54	Zchori-Fein and Brown (2002)
16S rRNA	Ham-R	AGTTCAAGACCGCAACCTC		

Table 2: Rice weevil samples for various bacterial endosymbionts detection

Agro-climatic zones	Locations	Geographical Co-ordinates	GenBank Accession Number(s) COX1 gene	Endosymbionts (Infection) W	R	C	A	S	H
WHR	Baramulla	34˚12' N, 74˚20' E	OP618395, OP618396, OP618397, OP618398	+ (4:4)	-	-	+ (4:4)	+ (4:4)	-
	Mandi	31˚73' N, 76˚93' E	OP618405, OP618406, OP618407, OP618408	+ (4:4)	-	-	+ (3:4)	+ (4:4)	-
	Kinnaur	31˚52' N, 78˚24' E	OP618415, OP618416, OP618417, OP618418	+ (4:4)	-	-	-	+ (3:4)	-
TGPR	Ludhiana	30˚54' N, 75˚48' E	OP618425, OP618426, OP618427, OP618428	+ (4:4)	-	-	+ (1:4)	+ (4:4)	-
	Sirsa	29˚53' N, 75˚03' E	OP618435, OP618436, OP618437, OP618438	+ (4:4)	-	-	+ (4:4)	+ (2:4)	-
	Chandigarh	30˚73' N, 76˚78' E	OP618445, OP618446, OP618447, OP618448	+ (4:4)	-	-	+ (4:4)	+ (3:4)	-
UGPR	New Delhi	28˚61' N, 77˚21' E	OP618455, OP618456, OP618457, OP618458	+ (4:4)	-	-	+ (1:4)	+ (4:4)	-
	Pantnagar	29˚02' N, 79˚49' E	OP618465, OP618466, OP618467, OP618468	+ (4:4)	-	-	+ (3:4)	+ (3:4)	-
	Lucknow	26˚85' N, 80˚95' E	OP618475, OP618476, OP618477, OP618478	+ (4:4)	-	-	+ (3:4)	+ (4:4)	-
	Agra	27˚18' N, 78˚01' E	OP618485, OP618486, OP618487, OP618488	+ (4:4)	-	-	+ (4:4)	+ (4:4)	-
MGPR	Varanasi	25˚15' N, 82˚59' E	OP618495, OP618496, OP618497, OP618498	+ (4:4)	-	-	+ (4:4)	+ (3:4)	-
	Samastipur	25˚86' N, 85˚79' E	OP618505, OP618506, OP618507, OP618508	+ (4:4)	-	-	-	+ (4:4)	-
	Gaya	24˚79' N, 85˚00' E	OP618515, OP618516, OP618517, OP618518	+ (4:4)	-	-	+ (3:4)	+ (2:4)	-
WDR	Kota	25˚07' N, 75˚56' E	OP618525, OP618526, OP618527, OP618528	+ (4:4)	-	-	-	+ (4:4)	-
	Udaipur	24˚34' N, 73˚42' E	OP618535, OP618536, OP618537, OP618538	+ (4:4)	-	-	+ (2:4)	+ (3:4)	-

+ Presence of endosymbiont, - absence of endosymbiont, W: Wolbachia, R: Rickettsia, C:Cardinium, A: Arsenophonus, S:Spiroplasma, H:Hamiltonella; WHR: Western Himalayan Region, TGPR: Trans-Gangetic Plains Region, UGPR: Upper Gangetic Plains Region, MGPR: Middle Gangetic Plain Region, WDR: Western Dry Region

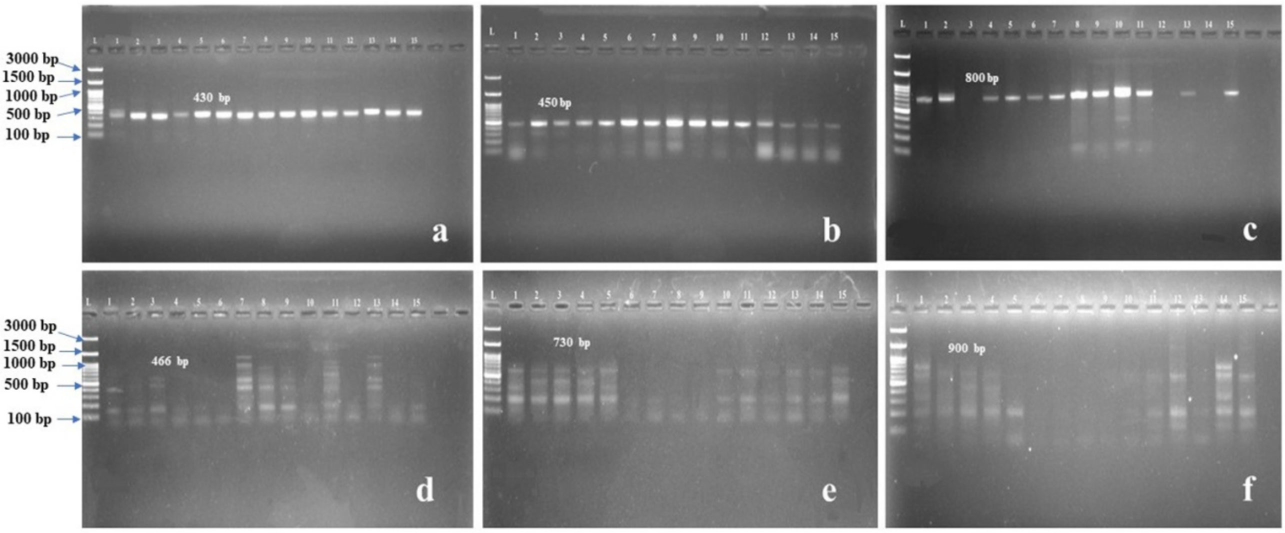

Fig. 1: Agarose gel electrophoresis of a) Wolbachia, b) Spiroplasma, c) Arsenophonus, d) Cardinium, e) Hamiltonella, and Rickettsia specific primer PCR products (L: 100bp ladder, 1) Baramulla, 2) Mandi, 3) Kinnaur, 4) Ludhiana, 5) Sirsa, 6) Chandigarh, 7) New Delhi, 8) Pantnagar, 9) Lucknow, 10) Agra, 11) Varanasi, 12) Samastipur, 13) Gaya, 14) Kota, 15) Udaipur.

For *Spiroplasma*, the highest value was observed for Western Himalayan region individuals (96.67 %), followed by Upper Gangetic Plain region (93.75 %), Western Dry Region (87.5 %) and Trans Gangetic Plain Region and Middle Gangetic Plain Region (75 %). For *Arsenophonus*, the infection percentage varied from 75 per cent for the Trans Gangetic Plain Region to 25 per cent for the Western Dry Region. However, the overall infection percentages of *Wolbachia, Spiroplasma* and *Arsenophonus* in *S. oryzae* adults were 100 %, 85 % and 60 %, respectively. The co-infections of different endosymbionts in a single individual were also observed The present findings revealed that the *S. oryzae* populations in northern states of India hosted three species of facultative endosymbionts, *Wolbachia, Spiroplasma and Arsenophonus* while *Rickettsia, Cardinium* and

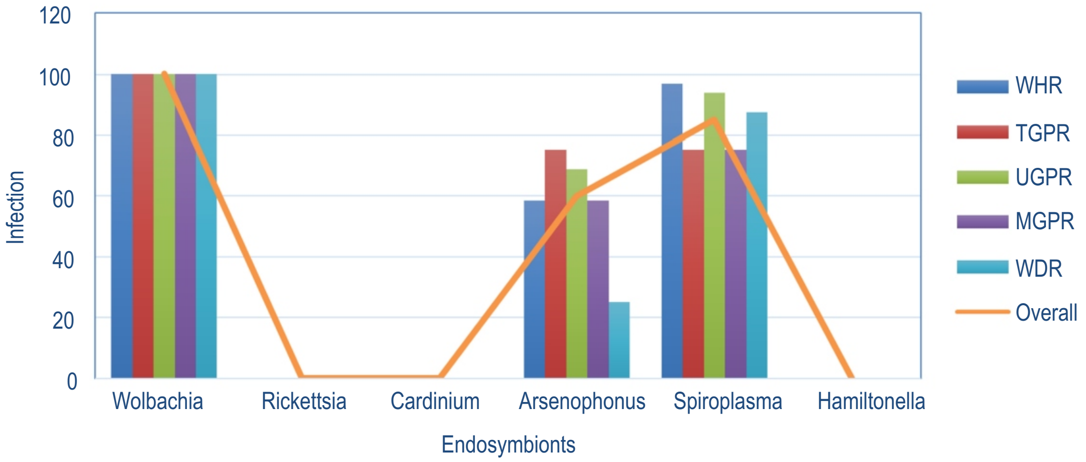

Fig. 2: Infection percentage of endosymbionts present in *S. oryzae* populations from different agro-climatic zones of India.

Hamiltonella were absent. Previous studies have demonstrated the presence of these endosymbionts in different stored-product insects, including rice weevil around the world.

The detection of *Wolbachia* in *S. oryzae* was also observed by Guruprasad *et al.* (2017) from Bangalore, India. Contrary to our findings, Ipekdal and Kaya (2020) did not find any of the above-mentioned symbionts in *S. oryzae* populations from Central Turkey. Similarly, Kageyama *et al.* (2010) from Japan demonstrated the absence of *Wolbachia* in laboratory cultures of rice weevil. Kocak and Yaman (2019) recorded an infection percentage of 55.5% for *Rickettsia,* 22.2% for *Wolbachia* and 8.3% for Spiroplasma in *S. oryzae* populations collected from different provinces of Turkey. It was observed that the diversity of endosymbiont species in insect populations varies from region to region. The varied infection rates of endosymbionts observed in *S. oryzae* populations might be due to the interaction of endosymbionts with different biotic and abiotic factors such as temperature, climate, vegetation, availability of food, and the existence of natural enemies or potential competitors that might affect the population dynamics of insects and the infection patterns of endosymbionts (Liu *et al.,* 2019). Co-infections of these endosymbionts in a single individual of rice weevil were also recorded in the present study. Previous studies conducted by Kondo *et al.* (2002), Zhang *et al.* (2016) and Guo *et al.* (2021) also demonstrated the coexistence of more than one endosymbiont in the same host population.

The absence of *Rickettsia, Cardinium* and *Hamiltonella* in *S. oryzae* populations may reflect their absence in the natural populations or it might be possible that these exist in the populations, but at frequencies too low to be detected in this study of very few individuals (Guo *et al.,* 2014). Myuzer *et al.* (1996) also mentioned that the bacterial endosymbiont population less than 1% in the host insect cannot be detected by PCR. Manipulating the gut symbionts using techniques such as CRISPR/Cas9, RNAi, antimicrobials, and combining IIT and SIT can change the growth and population of the host insects. This manipulation can be a possible focus for developing more effective tactics for pest control (Rupawate *et al.,* 2023). The technique of paratransgenesis like the use of symbiont mediated RNAi to target specific genes in host insects has been demonstrated in numerous insect pests, such as *Rhodococcus rhodnii*, to decrease host's fitness, and in *Frankliniella occidentalis,* to minimise crop damage (Whitten *et al.,* 2016). Similarly, antibiotics like tetracycline and penicillin have been administered orally to the tsetse fly in order to eradicate the symbiotic microbiota. The administration of these antibiotics has been demonstrated to induce sterility in the fly by disrupting the symbiotic relationship with *Wiggles worthia* during the larval stages, resulting in impaired reproductive capacity (Zhong *et al.,* 2007). Thus, the present findings will be helpful in understanding the diversity and ecology of these endosymbionts as well as their utility as biocontrol agents, which can guide future studies in formulating environment-friendly strategies for the management of rice weevil as the technologies based on these endosymbionts are environment friendly and cost-effective.

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Authors' contribution: P. Kaundal: Contribution: Collected the insect samples, performed the experiments, data analysis, and prepared the first draft of the manuscript; **K.G. Padwal:** Helped in data analysis and interpretation, and edited the manuscript draft; **S. Premkumari:** Collection of insect samples and manuscript preparation; **S. Chakravarty:** Performed interpretation of results, and edited the manuscript draft; **C.P. Srivastava:** Designed the study, supervised the experiments, and evaluated and finalized the manuscript draft.

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