Detection of *Wolbachia* Phage (WO) in Indian Lac Insect [*Kerria lacca* (Kerr.)] and Its Implications

S. Kaushik¹ · K. K. Sharma² · R. Ramani² · Suman Lakhanpaul³

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Abstract *Wolbachia*, a maternally inherited bacterium induces reproductive alterations in its hosts such as feminization of males, male killing and parthenogenesis. It is the most diverse endosymbiont infecting more than 70% of the insects ranging from pests to pollinators. *Kerria lacca*—a hemipteran is a sedentary, oriental insect known to produce lac—the only resin of animal origin. The present study was conducted to screen the presence of *Wolbachia* and its associated phages in the two infrasubspecific forms (four insect lines) of *K. lacca* viz. kusmi and rengeeni differing from each other on the basis of host preference. *Wolbachia* and its associated phage were found to be prevalent in all the insect lines analyzed. We, hereby, report the presence of WO-phage (*Wolbachia* phage) for the first time in *K. lacca*. Further, phylogenetic data differentiated the kusmi and rengeeni infrasubspecific forms into two different groups on the basis of WO-phage sequences.

Keywords *Wolbachia* phage · *Wolbachia* · *Kerria lacca* · Lac

Phytophagous insects are well known to harbor various intracellular endosymbionts. Amongst these endosymbionts *Wolbachia* is a rickettsia—like, maternally

inherited, intracellular, ubiquitous bacteria belonging to alpha—Proteobacteria has been reported to infect invertebrates especially insects, mites, filarial nematodes etc. and establish diverse biotic associations with their hosts [1–3]. *Wolbachia* is known to induce various reproductive alterations such as cytoplasmic incompatibility [4], parthenogenesis [5], male-killing [6] and feminization of genetic males [7] in its hosts. It is known to infect and colonize 16–76% of all insect species [2, 8]. Role of *Wolbachia* in inducing speciation in some insect species has also been speculated [4]. However, the molecular mechanisms used by *Wolbachia* to manipulate its host remain uninvestigated and have been speculated due to genes on extra-chromosomal factors such as plasmids or bacteriophages [9–11]. One major factor that slows down the pace of such studies is the lack of artificial transformation techniques and unculturable nature of this bacterium [12]. Presence of phages and plasmids also known as mobile genetic elements are however regarded as potential tools which may enable the genetic manipulation of *Wolbachia* [13]. Out of seventeen identified supergroups of *Wolbachia*, named A–Q, WO phage has been reported to infect *Wolbachia* belonging to supergroup A, B, F and G [14, 15]. In fact, WO phage screening studies have confirmed its presence in invertebrates infected with *Wolbachia* belonging to supergroup A or B (singly infected) or AB (double infected) [4, 10, 16].

The Indian lac insect, *Kerria lacca* (Kerr), is a source of lac, the resinous secretion obtained from unique insect-plant interaction and has immense economic importance. Lac is valued for its three main components namely resin, dye and wax and is also a source of several other chemicals of industrial importance. Presently, the lac is gaining further importance as it is biodegradable and also provides livelihood to landless farmers [17]. In fact, lac is the only

✉ Suman Lakhanpaul
sumanlp2001@yahoo.com

¹ Indira Gandhi National Tribal University, Amarkantak, Madhya Pradesh 484887, India

² Indian Institute of Natural Resins and Gums (IINRG), Namkum, Ranchi 834010, India

³ Department of Botany, University of Delhi, Delhi 110007, India

resinous compound of animal origin. Indian lac insect can be distinguished into two infrasubspecific forms, namely regeeni and kusmi, on the basis of differences in life cycle, host preference and quality of lac produced [18].

Previously, we have shown the existence of *Wolbachia* and Yeast like Symbionts (YLS) in the female insects of *K. lacca* [19]. The presence of *Wolbachia* in the lac insect made it imperative to further investigate the (a) screening and molecular subgrouping of the *Wolbachia* infecting *K. lacca* infrasubspecific forms kusmi and regeeni (b) divulging the tripartite association of *Wolbachia*-WO phage (if any) in the infrasubspecific forms of *K. lacca* (c) Phylogenetic position of *Wolbachia*-WO-Phage infecting the two different infrasubspecific forms viz. kusmi and regeeni.

Polymerase chain reaction (PCR) technique was performed for screening of *Wolbachia* using supergroup A and B specific primers (*ftsZ*, a cell cycle gene) and characterization of WO was carried out using specific phage sequences *orf7*, a minor capsid protein gene [16] followed by phylogenetic analyses.

Gravid female insects of four different lac insect accessions, two each belonging to kusmi and regeeni forms were procured from Indian Institute of Natural Gums and Resins (IINRG), Ranchi. Total genomic DNA of individual insect was isolated by using Qiagen kit with minor modifications. Quality of DNA was checked on 0.8% agarose gel. 15–20 ng DNA quantified using Qubit Fluorometer (Invitrogen) was used as DNA template.

A PCR based assay for the detection of *Wolbachia* A and B was carried out using specific sequence primers (for supergroup A and B respectively), in four representative lac insect lines. Primers used were: *ftsZ* A Forward 5'-CTC AAG CAC TAG AAA AGT CG-3'; *ftsZ* A Reverse 5'-TTA GCT CCT TCG CTT ACC TG-3'; and for B supergroup *ftsZ* B Forward 5'-CCG ATG CTC AAG CGT TAG AG-3'; *ftsZ* B Reverse 5'-CCA CTT AAC TCT TTC GTT TG-3'. The PCR reaction was carried out using Eppendorf Thermal Cycler in a 25 µl reaction mixture containing 1 × PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.15 mM MgCl₂), 0.2 mM dNTPs (each of dATP, dGTP, dCTP and dTTP), 2.5 mM MgCl₂, and 0.5 U *Taq* DNA Polymerase (Bangalore Genei), 0.1 µM of each forward and reverse primer (IDT), 20 ng template DNA and final volume of deionised water to make up 25 µl. WO phage was detected using *ORF7* forward-5'-CCC ACA TGA GCC AAT GAC GTC TG-3' and reverse-5'-CGT TCG CTC TGC AAG TAA CTC CAT TAA AC-3' primers under same PCR parameters, except for the annealing temperature (T_H), 57 °C. Amplicon size of appropriate size, 1000 bp and 400 bp were confirmed by running the PCR product on 1% Agarose Gel for *Wolbachia* and WO phage respectively. The PCR-positive clones were regarded as

true positives and sequenced using Sanger sequencing and the sequences were deposited in GenBank (Table 1).

A total of 48 minor capsid gene sequences namely *ORF7* of WO phage comprising four sequences obtained from the present study and 44 gene sequences downloaded from NCBI were aligned using Sequencer 5.1 (Gene Code corporation, USA) followed by manual adjustments in Mesquite v. 2.72. Length conservation and high nucleotide sequence variability of the minor capsid protein gene suggested were readily alignable across closely related taxa, yet suitable to address phylogenetic questions. Bayesian analysis was done using Mr. Bayes 3.1.2. The best model was interpreted using J-model test which was GTR+ G. Three lakh generations were done till the standard deviation fell below 0.01 and stationarity was achieved. Finally, 50% majority consensus tree was taken for interpretation.

Therefore, screening and distribution of *Wolbachia* and WO phage sequences were studied based on PCR detection followed by sequencing of the partial *ftsZ*, a cell cycle gene involved in cell division and a putative minor capsid protein, *orf 7* respectively. We report the first molecular detection of *Wolbachia* and WO-phage infecting kusmi and regeeni infrasubspecific forms of *K. lacca*. The representative infrasubspecific forms of kusmi and regeeni were found to be singly infected by *Wolbachia* belonging to Supergroup B (Table 1). The single *Wolbachia* infection status (based on *ftsZ*) in all the insect lines studied irrespective of their infrasubspecific forms corroborates the earlier phylogenetic position of *Wolbachia* (based on *wsp*) infecting *K. lacca* to supergroup B [17]. WO phage was found to be present in all the four insect lines. The results obtained are comparable with earlier studies done on *Drosophila* where 70–90% infection of WO-phage was observed [16]. It is hereby noteworthy that PCR detection technique does not unravel whether the bacteriophage infecting is in a lytic or lysogenic state. Interestingly, changes in the titer of *Wolbachia* due to the lytic and lysogenic nature of WO phage are speculated to induce reproductive alterations such as CI, male killing, feminization or parthenogenesis [6, 9]. Considering the above manifestations and diversity of WO phage obtained in the kusmi and regeeni forms, we speculate that presence of WO phage can be a switch in divulging the evolutionary regime of the insect in terms of infraspecific divergence.

Due to sequence similarity in the *ftsZ* sequences obtained from the kusmi and regeeni infrasubspecific forms further phylogenetic analysis was not carried out. However, the phage sequences belonging to kusmi and regeeni infrasubspecific forms were very distinct thus requiring further phylogenetic analysis. On the basis of *orf 7* sequences, the phylogenetic tree differentiates the three strongly supported groups of bacteriophage WO-B, as

Table 1 Occurrence of *Wolbachia* and *WO* Phage along with their NCBI sequence identities respectively, in four different lines belonging to Kusmi and Rengeeni strains in Indian Lac insect (*Kerria lacca*)

S. no.	Insect lines ID	Strain	<i>Wolbachia</i> A	<i>Wolbachia</i> B (NCBI accession no.)	<i>WO</i> phage (NCBI accession no.)
1	LIK0001	Kusmi	–	+	(JQ820158)
2	LIK0003	Kusmi	–	+	(JQ710840)
3	LIK0016	Rengeeni	–	+	(JQ820157)
4	LIK0028	Rengeeni	–	+	(JQ820159)

–, not detected; +, present

group I, II, and III (Fig. 1). Interestingly, regeeni accessions (LIKBL0016 and LIKBL0028) clustered with group III and the kusmi accessions (LIKBL0001 and LIKBL0003) clustered with group I of the phylogenetic

tree. Thus, phylogenetic findings on the basis of *orf* 7 sequences suggest the correspondence between differentiation of kusmi and regeeni infrasubspecific forms into two different groups with that of *Wolbachia* phage. Most

Fig. 1 Phylogenetic tree of *Wolbachia* phage (WO) ORF 7 nucleotide sequences from kusmi and regeeni strains of *Kerria lacca* along with those of other Arthropod species constructed using likelihood analysis and Bayesian mc-mc approach (50% majority consensus tree)

importantly, kusmi and reneeni forms of Indian lac insect are getting differentiated from each other on the basis *orf7* sequences. It is of utmost importance to point out that the two forms of Indian lac insect differ from each other on the basis of host preference and the lac quality aspects, yet they are indistinguishable morphologically. Further studies are required to understand the tripartite association of *K. lacca*-*Wolbachia*-WO phage and the implications of this on their role in its infraspecific divergence and reproductive behavior of the insect and in turn, on the lac production.

In summary, this is the first report to divulge the presence of *Wolbachia* in the kusmi and reneeni infrasub-specific forms of *K. lacca* and in particular of WO phage in this commercially important insect. Both the infrasub-specific forms were found to be singly infected with *Wolbachia* belonging to supergroup B. However, WO-phage infecting the two infrasubspecific forms was found to be distinct on the basis of sequence analysis and phylogenetic data. The study provides an important platform to further investigate the role of *Wolbachia* and WO phage infecting *K. lacca* particularly for their potential role in reproductive alterations and also divergence ultimately for the improvement of lac production.

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