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Detection and Characterization of Novel *Wolbachia* Strain in *Spodoptera litura* Collected from Western Ghats, India

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Wolbachia **are maternally inherited endosymbionts found in wide range of arthropod hosts. They alter host sex biology by various phenotypic effects. This is making them as an emerging novel biological tool to control the insect pests. Indian Leaf worm,** ***Spodoptera litura*** **is one of the most devastating pests which cause severe damage to various crops resulting in serious economic loss. Application of chemical insecticides is an extensive practice to control this pest. It develops resistant to various chemical pesticides. Microbial control by a nuclear polyhedrosis virus (SpltMNPV) and entomopathogenic nematodes are available against this pest. However, it has various limitations. The objective of the present study is to explore** ***Wolbachia*** **infection in** ***S. litura*****. In the present study a novel** ***Wolbachia*** **strains from** ***S. litura*** **population has been detected which is further characterized by multigene approach for the first time. Phylogenetic affiliation revealed that this strain is belonging to B supergroup and completely different from those which are already reported for** ***S. litura*** **and** ***S. exempta.*** **This finding further lays a platform for possible use of this** ***Wolbachia*** **strain in biological control of** ***S. litura.***

Key words: *Spodoptera litura*, *Wolbachia*, MLST, *wsp*, biological control.

Wolbachia are intracellular and maternally inherited bacterial symbionts belonging to family Anaplasmataceae and found in many arthropod and filarial nematodes. They were first detected in mosquito host *Culex pipiens*[1] and further described as *Wolbachia pipientis*, the first type species of the genus[2]. These bacteria are known for diverse associations with their hosts ranging from parasitism to mutualism[3]. They induce many phenotypic alterations in their hosts such as male killing (MK), feminization (FI), parthenogenesis (PI), cytoplasmic incompatibility (CI) and speciation through reproductive isolation[3-5]. Hence, they are also called as master manipulator of host sex biology[3]. Along with vertical transmission, they are also known to transfer horizontally from one host to another[6] making them diverse endosymbiont infecting up to 66% of insect species[7]. Gene phylogenies currently divide these bacteria in eleven supergroups (A-K)[8].

The genus *Spodoptera* (Lepidoptera: Noctuidae) comprises more than 25 different species and includes some of the vital pests of agricultural crops in the world. *Spodoptera litura* (Lepidoptera: Noctuidae) is a one of such polyphagous pest species[9], well known as Indian Leaf worm, tobacco cutworm and armyworm[10, 11]. It is one of the most devastating crop pests which cause severe damage to tobacco, cotton,

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groundnuts, maize, rice, soyabeans and vegetables, resulting in serious economic loss. Application of chemical insecticides is an extensive practice to control this pest. However, development of resistance to these chemicals in *S. litura* is cause of concern due to excessive use of insecticides[12, 13]. Microbial control of the *S. litura* by a nuclear polyhedrosis virus (SpltMNPV) has been reported[14, 15]; however, their relatively slow killing speed is a major limitation[16]. Entomopathogenic nematodes were reported to control this cutworm in laboratory conditions[17] however no field trials are taken so far.

Different strains of *Wolbachia* can have different impacts on host biology therefore they are emerging as novel biological tools to control the insect pests[18]. Among the different supergroups of *Wolbachia*, supergroup B is found in arthropods including insects as well as some isopods. Various phenotypic effects are observed for this supergroup *Wolbachia*. Out of which, MK is found in genus *Acraea*, *Ostrinia*, *Hypolimnas* and *Spodoptera* while CI is observed for genus *Chelymorpha*, *Tribolium*, *Gryllus*, *Teleogryllus*, *Drosophila*, *Culex*, *Ephestia*, *Nasonia*, *Colias*, *Eurema*, *Laodelphax*, *Sogatella* and *Leptopilina*. Other phenotypes like FI (genus *Armadillidium* and *Eurema*) and PI (genus *Encarsia*, *Trichogramma*, *Tetrastichus* and *Leptopilina*) were also reported for B supergrroup *Wolbachia*.

CI is a phenomenon, which modulate host incompetent to produce the viable offspring. CI is the most widespread and the most comprehensively studied *Wolbachia*-induced phenotype[4, 5]. This phenomenon was first proven in mosquito *Culex pipiens*[19] and later on reported in many insect orders like Lepidoptera, Coleoptera, Homoptera, Hymenoptera, Orthoptera, etc[18].

Attempts were made to use CI as a way to conquer pest population using the way similar to the sterile insect technique (SIT)[20] and incompatible insect technique (IIT)[21]. Wild populations of agricultural pests such as the European cherry fruit fly, *Rhagoletis cerasi*[22] and the almond moth *Cadra* (*Ephestia*) *cautella*[23] has been controlled using mass released of the incompatible male. It has been also used against disease vectors such as the mosquito *Culex pipiens*[24]. Zabalou and group[25] used this technique by *Wolbachia* mediated CI to control caged medfly *Ceratitis capitata* populations (>99%). Xi *et al.*[26] also used *Wolbachia* to establish strong CI which leads to no egg hatch in an *Aedes aegypti* lines.

Detection and identification of the *Wolbachia* strain types is important to understand their association with hosts. Multi Locus Sequence Typing (MLST) system is a standardized and rigorous framework to study *Wolbachia* strain diversity[27]. It is successfully used to accurately characterize *Wolbachia* strains from various hosts[28]. Similarly, *wsp* typing system is based on the amino acid sequences coding for the four hypervariable regions (HVRs), which is like antigen protein typing and can counterpart with the MLST[27]. The characterization of the strain types using MLST and *wsp* typing systems can also give idea about their phenotypic impacts by searching the information across MLST database (www.http://pubmlst.org/wolbachia/).

Recently, three *Wolbachia* strains have been reported from *S. exempta* collected from Tanzania using MLST system[29]. Out of these, one *Wolbachia* strain showed ability to selectively kill male embryos called male killing (MK) phenotype, while other strains were expected to have same phenomenon. Even though *Wolbachia* infection was reported in Indian Leaf worm, *S. litura*[11], MLST analysis for characterization of this strain was not done yet. In India *Wolbachia* strains from termite[30] and butterfly[28] hosts are successfully characterized so far by MLST approach.

Present report show (I) presence of novel *Wolbachia* in *S. litura* from India; (II) *Wolbachia* strain characterization by using MLST genes and *wsp* typing; (III) phylogenetic affiliation of *S. litura Wolbachia* and (IV) prospective of this *Wolbachia* strain as bio-control agent of *S. litura*.

MATERIALS AND METHODS

Insects and DNA extraction

Larva of *S. litura* (N=5) were collected from surrounding regions of Junnar, Maharashtra, India and stored in absolute ethanol at -20^{0}C till DNA extraction. DNA was extracted from the individual larva by using Blood and tissue DNA Kit (QIAGEN®) following the manufacturer's instructions.

Wolbachia screening and sequencing

All five specimens were subjected to PCR

based *Wolbachia* screening using *wsp* gene as reported by Braig *et al.*[31]. Primer and PCR protocols for amplification of the five *Wolbachia* MLST genes (*ftsZ, coxA, fbpA, hcpA* and *gatB*) and *wsp* gene were used as described[27]. All PCR products were cleaned up using PEG-NaCl method[32]. The successful amplified products so obtained were directly sequenced with both ends using BigDye3.1 Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems). Sequences were obtained using an automatic DNA sequencer (3730xl DNA analyzer, ABI). The sequence data was analyzed using *Wolbachia* MLST database (http://pubmlst.org/*Wolbachia*/) and GenBank database. All the sequences in this study were deposited in the *Wolbachia* MLST (except *fbpA*, since only reverse primer had worked) and GenBank databases with alleles and accession numbers, respectively (Table 1).

Phylogenetic analysis and construction of similarity matrix

STs and *wsp* alleles currently available in the MLST database for order Lepidoptera along with few others from A and B supergroup were retrieved and aligned using ClustalX (version 2.0.9)[33]. All sequences were manually edited using MEGA5[34] and uninformative sites were removed from further analysis. Bayesian inference and Neighbor joining methods were used to construct unrooted phylogenetic trees for each dataset as described by Salunke *et al.*[28, 30]. The selected model of nucleotide substitution was 'GTR+I+G' for concatenated MLST gene dataset and *wsp* gene. The final alignments consisted of 2079 bp for concatenated MLST gene sequences and 498 bp for *wsp* gene fragments. Three independent runs were performed for each dataset. Bayesian posterior probabilities (BPP) below 0.50 are not shown in phylogenetic trees. Similarity matrix was constructed for each dataset (excluding GenBank sequences) using dnadist from Phylip (version 3.68) package[35].

RESULTS AND DISCUSSION

Detection of *Wolbachia* in *S. litura*

Initially, five larval samples were diagnosed for *Wolbachia* infection based on PCR assay using *Wolbachia* specific *wsp* gene primers that yielded expected amplification product of 650 bp. These results were further confirmed by PCR amplification using primers for *Wolbachia* specific 16S rRNA gene, which gave PCR products of expected size (900 bp). All the five samples were positive for *Wolbachia* infection and showed same *wsp* and 16S rRNA sequences. Therefore, one representative sample was subjected to full MLST sequencing. Super infection of *Wolbachia* can be ruled out since clear chromatograms were obtained in sequencing. Sequences from gene *coxA* and *ftsZ* are new to the MLST database hence new allele numbers were assigned to them (allele 198 and 175, respectively) (Table 1).

Characterization of *Wolbachia* from *S. litura* and its Phylogenetic affiliation

A phylogenetic tree based on the 16S rRNA gene sequences (tree not shown) constructed using the Kimura-2-distances and the Neighbor joining method indicated that *Wolbachia* from *S. litura* was grouped within the clade of B supergroup. Hence, for further analysis, only the members of B supergroup along with some representative of A supergroup *Wolbachia* as outgroup were considered and selected. All the STs and *wsp* alleles for order Lepidoptera along with few others were retrieved from MLST database. Unrooted phylogenetic trees were constructed using Bayesian inference. In both the trees, *S. litura Wolbachia* was clustered together with the supergroup B clade supported by high posterior probability. Phylogenetic trees inferred from the concatenated MLST dataset (Figuer-1A), consistently showed the presence of *S. litura Wolbachia* in B supergroup. It was clustered

Table 1. GenBank accession and MLST allele numbers of *Wolbachia* from *S. litura*

Gene	MLST allele number	GenBank accession number
gatB	9	KC915387
coxA	198	KC915384
hcpA	40	KC915388
fbpA	-*	KC915385
ftsZ	175	KC915386
wsp	664	KC915390
W16S		KC915389

MLST id for *S. litura* from this study is 596
*allele cannot assign since only reverse sequencing primer had worked

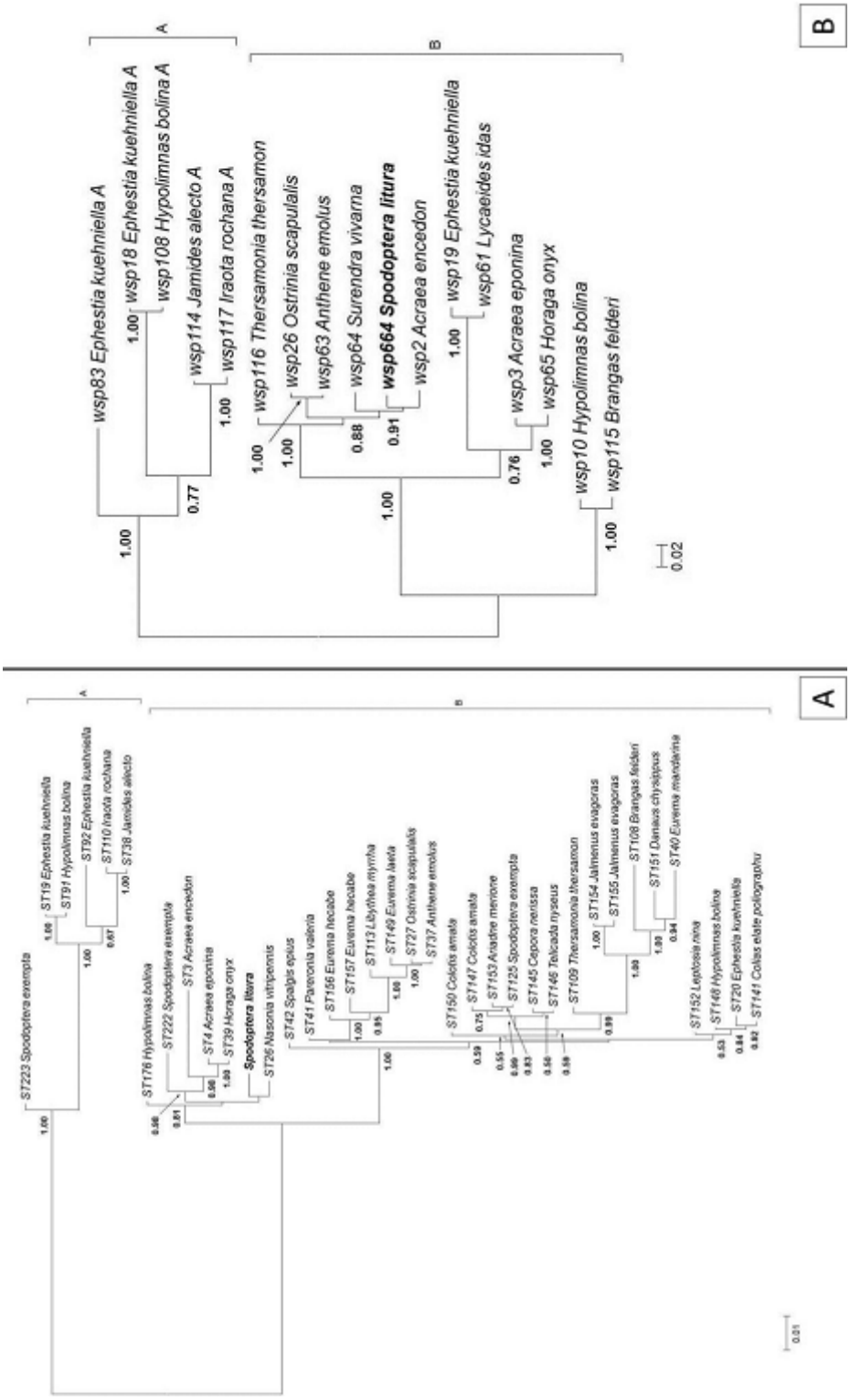

Fig. 1. Unrooted phylogenetic relationships between *S. litura Wolbachia* (bold) and those infecting other organisms, based on *concatenated* gene dataset (A) and *wsp* gene (B). Names are those of the host species

together with the wasp *Nasonia vitripennis* (ST26). The ST26 represented the *Wolbachia* strain that is responsible for cytoplasmic incompatibility (CI). A completely new WSP profile to the database has been characterized for *S. litura Wolbachia* and was assigned the new number (*wsp* 664). This is the first report for *wsp* typing of *Wolbachia* from the genus *Spodoptera*. Phylogenetic tree obtained using this *wsp* gene sequence (Figuer-1B) also showed the presence of the *S. litura Wolbachia* (*wsp* 664) in supergroup B clade. In this tree, *Wolbachia* from *S. litura* showed strong clustering with *Acraea encedon* (*wsp* 2) which was characterized for male killing (MK).

Comparison between *S. exempta* and *S. litura* strains

Wolbachia MLST data suggests the characterization of three *Wolbachia* STs for *S. exempta*. Out of these, two STs belong to B supergroup (ST125, ST222) while the other is from A supergroup (ST223). Similarity matrix constructed using these three STs from *S. exempta* and our typed ST for *S. litura* suggests that the *Wolbachia* strain from *S. litura* in the present study has similarity with ST222, ST125 and ST223 of 98.4%, 97.2%, and 90.7%, respectively. *Wolbachia* infection was also reported in *S. litura* from Bangalore, India using *ftsZ* gene. When the sequence from this report compare with the sequence obtained in this study, it showed only 98.36% similarity. Therefore, the *Wolbachia* characterized from Indian *S. litura* is very diverse from those which are already characterized for *S. litura* and *S. exempta*.

Wolbachia* as a promising biological control agent of *S. litura

S. litura is one of the most destructive crop pests causing serious economic damage. It has also developed resistance to many chemical pesticides[12, 13]. Biological control by viruses (nuclear polyhedrosis virus - SpltMNPV)[14, 15] and some nematodes[17] has been reported, however, their success in the field is contentious. *Wolbachia* is very diverse endosymbiont and it is emerging as an important bio-control agent against various insects using its different phenotypic effects. Brelsfoard and Dobson[36] reviewed the different *Wolbachia* based strategies with use of both naturally occurring infections and genetically modified *Wolbachia* strains.

Phylogenetic rearrangement using concatenated dataset of the *Wolbachia* in this study acclaimed clustering with the *Wolbachia* from *Nasonia vitripennis* (ST26) which was reported for cytoplasmic incompatibility (CI). While phylogeny using *wsp* gene dataset showed clustering with *Acraea encedon* (*wsp*2) which was characterized for male killing (MK) earlier. If both the effects are considered, the CI causing *Wolbachia* is now well established as a bio-control tool[36]. However, MK *Wolbachia* is also developing as a bio-control agent since it is instead responsible for varying sex ratio leading to competition for mating.

Use of Baculoviruses is currently developed as integrated biocontrol strategy against important lepidopteran pests of economic plants like cotton bollworm (*Helicoverpa armigera*), diamond-back moth (*Plutella xylostella*), Egyptian cotton leafworm (*S. littoralis*) and beet armyworm (*S. exigua*)[37]. Graham *et al*.[37] proposed that susceptibility of lepidopteran host to baculoviruses SpexNPV increases by 6–14 times in the presence of *Wolbachia*. Though *Wolbachia* is naturally present in some of the lepidopteran pest (e.g. *S. littoralis* and *P. xylostella*), they further suggested the need of transinfection of suitable *Wolbachia* strains in naturally uninfected pest. Successful transfer and establishment of the *Wolbachia* strain from *Aedes albopictus* to *Aedes aegypti* have been done[26] which shows same phenotype as present in native host. It is more readily possible because both the hosts were very close to each other. Hence the *Wolbachia* from this study can be used for transinfection in naturally uninfected *Spodoptera* species to increase the susceptibility towards Baculoviruses subsequently leading to their control.

Wolbachia is well known for providing fitness benefits to their hosts. It has been proven to increase the resistance in *Drosophila melanogaster* for RNA viruses like Drosophila C virus[38], Nora virus[39] and West Nile virus[40]. Mosquito *Culex quinquefasciatus* also showed increase resistance to West Nile virus in presence of *Wolbachia*[40]. *Wolbachia* is currently well-known as an obligatory endosymbiont for filarial nematodes. It provides the hosts with some vital chemical which are essential for their survival[41]. *Wolbachia* was also proven to give more

insecticide resistance to their host mosquito *Culex pipiens*[42]. Hence antibiotic treatment against these bacteria is used to overcome the filariasis caused by nematodes.

By considering all these facts, *Wolbachia* from *S. litura* can be a novel finding, since elimination of *Wolbachia* infection might be exploited as an effective pest management programme against this pest. However, it deserves more research attention towards further characterization of the phenotypic effects caused by this *Wolbachia* strain in *S. litura*.

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