

Associated bacterial diversity of insecticide-susceptible and -resistant brown planthopper, *Nilaparvata lugens* (Homoptera: Delphacidae) analyzed by culture-dependent and -independent methods

V. M. Malathi · Sushil Kumar Jalali · Vazhakkala Jose Lyju · R. Gandhi Gracy · Ravi Prabhakar More · Rangasamy Anandham · Apoothy Thulasi · Thiruvengadam Venkatesan

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Abstract *Nilaparvata lugens* (Stål) is one of the major pests of rice throughout tropical and temperate Asia. Indiscriminate use of insecticides for suppressing *N. lugens* has resulted in the development of resistance to multiple insecticide classes, causing frequent control failures in the field. Analysis of gut bacterial diversity within an insect host is the initial step towards understanding the ecological roles of the symbionts. Present study aimed to survey the bacterial diversity associated with laboratory-reared (insecticide-susceptible) and field-collected (insecticide-resistant) populations of *N. lugens* by culture-dependent and PCR-Denaturing Gradient Gel Electrophoresis (DGGE) methods. Seventeen

bacterial isolates were obtained by the culture-dependent method. Molecular characterization using the 16S rRNA gene and phylogenetic analysis revealed that the isolates belonged to *Firmicutes* and *Proteobacteria*. Taxonomic assignment placed these isolates into seven families representing 10 genera. *Enterobacteriaceae* was the most dominant family with its occurrence in four out of the five populations studied. The DGGE profiles indicated a low complex gut bacteria associated with *N. lugens* with limited number of bands. The Shannon-Wiener index ranged from 0.898 in insecticide-susceptible population to 0.946–1.035 in resistant populations. Sequencing and phylogenetic analysis revealed that the DGGE bands belonged to *Firmicutes*, *Proteobacteria* and *Bacteroidetes*. Results of this study illustrated that gut bacterial community associated with *N. lugens* is dominated by *Proteobacteria* and *Firmicutes*. Present findings could provide the basis for future work on the possible role of the bacterial symbionts in insecticide resistance and to formulate potential resistance management strategies.

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V. M. Malathi · S. K. Jalali (✉) · R. G. Gracy · R. P. More · T. Venkatesan

Division of Molecular Entomology, Indian Council of Agricultural Research-National Bureau of Agricultural Insect Resources, Post Bag No. 2491, Hebbal, Bengaluru, Karnataka 560 024, India
e-mail: jalalisk@gmail.com

V. J. Lyju · A. Thulasi
Gut Microbiology Laboratory, Indian Council of Agricultural Research -National Institute of Animal Nutrition and Physiology, Adugodi, Bengaluru, Karnataka 560 030, India

R. Anandham
Department of Agricultural Microbiology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai -625104, India

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Introduction

Insects host large number of microorganisms, the greater part of which inhabits the intestinal tract with

interactions ranging from symbiosis to pathogenesis (Mrazek et al. 2008). Besides contributing to the digestive physiology and nutrition, gut associated symbiotic bacteria are known to perform numerous other functions that are essential for the insect host. These functions comprise vitamin synthesis, pheromone production, methanogenesis, nitrogen fixation, development and maintenance of host immune system (Reeson et al. 2003; Engel and Moran 2013). In insect vectors, manipulation of microbial symbionts is an effective strategy for controlling the spread of pathogens (Engel and Moran 2013). Further, Kikuchi et al. (2012) showed that symbiotic bacteria impart insecticide resistance to their insect hosts. Considering the significance, there is a growing interest in studying microorganisms associated with insects in recent years.

Insect gut microbiota has been studied by both culture-dependent and culture-independent methods. Culture-dependent methods offer the advantage of further functional characterization of the isolated microbe of interest and can be readily engineered for insect control strategies (Hernández et al. 2015). Essentially, culture-dependent methods formed the basis of our present understanding of insect gut microflora, but, they usually produce biased results depending on the selective media used for cultivation (Dillon and Dillon 2004). The advent of various culture-independent methods based on the analysis of 16S rRNA gene dramatically improved our knowledge of bacterial communities associated with insect gut. Denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) and single strand conformation polymorphism (SSCP) are among the methods being widely used to investigate bacterial community structures from various ecological niches which are not readily culturable (Mrazek et al. 2008). In particular, PCR-DGGE has been extensively used to study the diversity of gut associated microflora in many insects including *Plutella xylostella* (Lin et al. 2015), *Oryctes agamemnon* (El-Sayed and Ibrahim 2015) and certain wasp species like *Vespula germanica* (Reeson et al. 2003).

The brown planthopper (BPH), *Nilaparvata lugens* (Stål) (Homoptera: Delphacidae) is one of the most destructive pests of rice and also is a vector of grassy stunt and ragged stunt virus diseases (Hibino 1996). The annual crop loss due to BPH has been estimated as more than \$300 million (Min et al. 2014) and is often considered as a difficult to control pest. Most studies on the

management of this pest have been focusing on chemical control strategies and development of resistant rice plant varieties. However, very few information is available regarding microbes associated with BPH. Sasaki (1996) reported that BPH harbors eukaryotic, Yeast Like Symbionts (YLS) in their fat bodies and are known to be involved in the recycling of uric acid. Tang et al. (2010) examined bacterial microbes from different biotypes of *N. lugens* and identified 18 Operational Taxonomic Units (OTUs) by sequencing of 16S rRNA clone libraries and Xu et al. (2014) studied the structures of bacteria from different geographic and resistant virulent populations of BPH. However, broader studies regarding bacterial communities harbored by *N. lugens* are still lacking. Furthermore, recent studies have reported the possible role of gut associated microbiota in imparting insecticide resistance to their insect hosts (Kikuchi et al. 2012; Xia et al. 2013). Hence, in the present study, bacterial community associated with laboratory reared, insecticide-susceptible and field collected, insecticide-resistant populations of BPH were surveyed hypothesizing that the gut bacteria would vary between these populations. We employed both culture-dependent and culture-independent (PCR-DGGE) techniques to investigate gut bacteria of BPH. Knowledge of the bacterial community associated with insecticide-susceptible and resistant populations could provide opportunities for developing novel pest management strategies.

Materials and methods

Insect samples

Field populations of *N. lugens* were collected from various rice growing areas of South India, viz., Nellore (Andhra Pradesh) (14°38'N, 80°05'E), Gangavati (Karnataka) (13°01'N, 77°44'E), Warangal (Telangana) (18°12'N, 79°75'E) and Tiruchirappalli (Tamil Nadu) (10°86'N, 78°53'E) between March to December, 2013. The laboratory BPH population (insecticide-susceptible population) was obtained from Rice Entomology Section, Zonal Agricultural Research Station (ZARS), V.C. Farm, Mandya, Karnataka (12°31'N, 76°53'E), where the population has been maintained for more than 10 years on Taichung Native 1 (TN1, a BPH-susceptible rice variety) seedlings, cultured in wooden cages (70 cm × 62 cm × 75 cm), under

laboratory conditions at 28 ± 1 °C and 14: 10 h L: D, without exposure to any insecticides or environmental stresses (Basanth et al. 2013).

Laboratory reared and field collected BPH populations were subjected to toxicological bioassays (rice-stem dipping method) to assess their resistance levels to five commonly insecticides in the sampled areas viz. acephate (organophosphate), imidacloprid, thiamethoxam (neonicotinoids), buprofezin (insect growth regulator) and etofenprox (synthetic pyrethroid) (Malathi et al. 2015). The mortality data from the insecticide bioassays were subjected to probit analysis for the determination of lethal concentration values (LC_{50}). Resistance factors (RF) were estimated at the LC_{50} level as $RF = LC_{50}$ of field population / LC_{50} of laboratory reared susceptible population. The classification of resistance levels was done based on the RF as RF value <10-fold as low resistance, RF = 10–40-folds as moderate resistance, RF = 40–160-folds as high resistance and >160-fold as extremely high resistance (Kim et al. 1999). The field populations were found to exhibit low to moderate levels of resistance to the tested insecticides (Supplementary Table S1).

Culture-dependent method

Bacterial isolation and DNA extraction

The most destructive early fifth instar nymphal stages of *N. lugens* were used for toxicological bioassay and hence were used for isolation of gut bacteria. Ten nymphs were selected from each population and starved for 24 h to allow gut clearing (Broderick et al. 2004; Indiragandhi et al. 2007) in order to reduce transient microbiota derived from food. For the isolation of culturable gut bacteria, the method adopted by Indiragandhi et al. (2007) was followed with some modifications. The nymphs were surface sterilized with 75% ethanol for one min and the abdomen was excised, individual gut contents were filtered out and thoroughly disrupted using a sterilized tissue crusher device in 250 μ l of sterile water (Hernández et al. 2015; Gracy et al. 2016); subsequently diluted (10^{-4}) and 25 μ l of the diluted suspension was aseptically spread onto Lysogeny agar (LA) and Nutrient agar (NA) plates (M/s Himedia Laboratories, Mumbai, India) in duplicates. The plates were then incubated for 72 h at 30 °C and checked every 24 h for the appearance of new colonies.

Negative controls with sterile water spread onto the plates were routinely employed. After 72 h, the colonies were classified based on their size, shape, color, margin, opacity, elevation and consistency; single distinct colonies were picked and streaked five to six times in their respective growth medium to obtain pure cultures. The pure cultures were maintained in 20% glycerol at -80 °C.

For experimental purpose, the bacterial isolates were grown in respective liquid medium for 24 h and preceded for DNA isolation. DNA was extracted using HiPurA™ Bacterial Genomic DNA purification kit (M/s HiMedia Laboratories, India) following manufacturer's instructions. Extracted DNA was checked by electrophoresis on 0.8% agarose gels stained with ethidium bromide and stored at -20 °C until used for polymerase chain reaction (PCR).

Identification of bacterial isolates

The DNA of each bacterial isolate was partially amplified with 16S rRNA gene target primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1401R (5'CGGTGTGTACAAGACCC-3') (Lindh et al. 2005; Sánchez et al. 2013). PCR was performed using Biorad C1000 thermal cycler (M/s Biorad Laboratories, California). The reaction mixture contained 1 U of Platinum Taq DNA polymerase (M/s Invitrogen, California), 10 X PCR buffer (2.5 mM $MgCl_2$ included), 10 pmol of each primer, 200 mM dNTPs and 70–80 ng extracted DNA in a total volume of 30 μ l. The thermal cycling conditions were as described (Lin et al. 2015). The partial 16S rRNA nucleotide sequences (with an expected product size of ~1300 bp) were identified by PCR direct sequencing using the fluorescent dye terminator method with ABI Prism equipment and a Big dye terminator cycle. The products were then purified using a Millipore-montage kit, and finally run in an automatic ABI3730XL capillary DNA sequencer (50 cm capillary). The partial 16S rRNA gene sequences were aligned using the CLUSTALW program (Larkin et al. 2007) and the bacterial identities deduced using BLAST analysis (<http://www.ncbi.nlm.nih.gov/blast/>). Neighbor-joining phylogenetic tree was constructed using MEGA version 7.0 (Kumar et al. 2016) with the bootstrap values of 1000.

Culture-independent method

DNA extraction, PCR amplification and DGGE

Ten early 5th instar nymphs were randomly sampled from insecticide-resistant and -susceptible populations and starved for 24 h. The nymphs were surface sterilized with 75% ethanol for one min. The abdomen was excised and gut contents were aseptically placed in sterile 1.5 ml microcentrifuge tubes. DNA was extracted with Qiagen DNeasy blood and tissue kit (M/s Qiagen, Hilden, Germany) according to the manufacturer's instructions. A nested PCR was carried out wherein, the isolated DNA was initially amplified using universal eubacterial primers 27F/1492R (Lane 1991) and the products were further used as template for subsequent amplification targeting V₃ region of the 16S rRNA gene, using the primers 338F and 518R (Kim et al. 2013). In the latter amplification, the forward primer was extended at the 5' end with a 40 bp GC-rich clamp for further use of amplicons in Denaturing Gradient Gel Electrophoresis (DGGE). The PCR conditions for the initial amplification were same as described above. For the amplification of DGGE primers, a touch down PCR was performed. The reaction mixture contained 1.25 units of iTaq DNA polymerase (M/s Biorad, California), 10X PCR buffer, 25 mM MgCl₂, 5 pmol of each primer, 200 mM each dNTP and 160–175 ng of template in a total volume of 50 µl. Reactions were performed in an Applied Biosystems 9700 thermal cycler (Applied Biosystems, France) with an initial denaturation at 95 °C for five min followed by 20 cycles, each consisted: denaturation at 95 °C for 30 s, annealing at 65 °C for 30 s which was decreased by 0.5 °C every second cycle, and extension at 72 °C for 30 s; then additional 5 cycles with annealing at 55 °C for 30 s followed by a final extension step at 72 °C for 7 min. Amplified products (approximately 200 bp) were checked by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining. The PCR products were purified by QIA Quick Gel extraction kit and were stored at –20 °C before performing DGGE.

DGGE was performed with TV- 400 DGGE system (SciePlas, UK) as described previously (Lin et al. 2015). Gel was stained with silver staining as described by Sanguinetti et al. (1994) and photographed. The digitized DGGE image was analyzed with Bionumerics software 7.0 (M/s Applied Maths, Germany). Automated band calling was performed with a 5% minimum

profiling. Cluster analysis was performed with Dice similarity coefficient and a dendrogram was constructed by unweighted pair group method using arithmetic averages (UPGMA). The DGGE banding data was used to estimate the Shannon-Wiener index (H'), which describes the diversity of the bacterial communities (Shannon and Weaver 1963) and Simpson index (Simpson 1949). Also, DGGE bands of interest were excised as described by Dillon et al. (2008) and reamplified as described above with primer pair 338F and 534R without a GC clamp. The purified products (QIA Quick gel extraction kit, Qiagen, Germany) were cloned using pGEM-T easy vector (M/s Promega, Madison, Wisconsin). The recombinant plasmids were transformed into *E. coli* JM109 competent cells. The positive clones were verified and two clones per band were sequenced (M/s Eurofins Genomics, Bengaluru, India) to confirm band identity.

The sequences were subjected to search analyses in GenBank using the nucleotide Basic Local Alignment Search Tool (BLASTn) to identify sequences of the highest similarity.

Phylogenetic analysis

Neighbor-joining phylogenetic tree of the sequences obtained in the present study and its closest phylogenetic relatives was constructed using MEGA version 7.0 (Kumar et al. 2016) with the bootstrap values of 1000.

Data deposition

All the sequences obtained in the present study were deposited in GenBank database under the accession numbers KF359494-KF359495, KF448523, KF448525-KF448526, KF512549, KF901493-KF901494, KF901496-KF901497, KJ655531-KJ655537 for the cultured bacterial sequences and KR024811-KR024821 for DGGE derived sequences.

Results

Culturable bacterial diversity of laboratory-reared (insecticide-susceptible) and field-collected (insecticide-resistant) *N. lugens*

The gut bacterial isolates and their closest neighbor, along with their accession numbers were presented

(Table 1). Molecular characterization using the 16S rRNA gene revealed that the 17 isolates belonged to 9 genera distributed in 7 families of the phyla *Firmicutes* and *Proteobacteria*. *Enterobacteriaceae* was found to be associated with 4 (insecticide-susceptible, Warangal, Tiruchirappalli and Gangavati) out of the 5 populations followed by *Bacillaceae* and *Moraxellaceae* in 3 of the studied populations (insecticide-susceptible, Warangal, Tiruchirappalli and insecticide-susceptible, Nellore, Tiruchirappalli populations, respectively). The family *Staphylococcaceae* was found to be associated with the insecticide-resistant populations collected from Gangavati and Tiruchirappalli, but not associated with the insecticide-susceptible population. Isolates belonging to *Comamonadaceae*, *Planococcaceae* and *Bacillales* family 12 were less predominant and were found to be associated with only one field population; the former 2 families being associated with Nellore and latter, associated with Warangal field populations (Table 1).

Neighbor-joining phylogenetic tree of the culturable bacteria obtained in the present study and its closest phylogenetic relatives were presented (Supplementary Fig. S1).

Bacterial diversity based on DGGE

PCR-DGGE profile of gut associated bacteria from different populations of *N. lugens* is presented (Fig. 1a) (Supplementary Table S2). Overall, the DGGE profiles showed a low complexity with limited number of bands. At least 7 bands were common to more than 80% of the populations with 5 bands being present in all the populations. Most bands present in the laboratory population were also found in at least two field populations while three bands were found to be exclusively associated with field population.

Cluster analysis based on the Dice similarity coefficient revealed that all the field populations formed a coherent cluster with a similarity level of 64% (Fig. 1b). Within this cluster, Nellore and Gangavati populations were grouped together with 84% similarity and Warangal and Tiruchirappalli populations were grouped together with 72% similarity. The slightly low similarity of laboratory population with field populations was 57.38% and the laboratory population formed a separate clade. The Shannon-Wiener index (H'), ranged from 0.8983 in the laboratory population to 0.9461–1.0354 in the field populations. Among the field populations, *N.*

lugens from Warangal and Tiruchirappalli showed the highest H' (1.030 and 1.035 respectively) followed by Nellore (0.9920) and Gangavati (0.946). The Simpson index (1-D) for the laboratory population was 0.873; while the field populations exhibited slightly higher values with Warangal, Tiruchirappalli, Nellore and Gangavati showing 0.905, 0.907, 0.897 and 0.885 respectively.

Identification of bacteria by sequencing of DGGE bands and phylogenetic analysis

Prominent DGGE bands were sequenced to identify representative members of the gut bacterial community. A total of eleven sequences (200 bp corresponding to the V_3 region) were obtained (corresponding bands are marked in Fig. 1a) and all of them matched to reported sequences of GenBank database with identity $\geq 98\%$ (Supplementary Table S2). A Neighbor-joining tree (Fig. 2) was constructed to better visualize the phylogenetic relationship of the sequences obtained in the present study and its closest relatives of the GenBank. Phylogenetic analysis revealed that sequences belonged to *Proteobacteria* (γ -*Proteobacteria*), *Firmicutes* (*Bacilli*) and *Bacteroidetes* (*Cytophagia*) (Fig. 2). Band numbers 1 and 2 belonged to *Bacillaceae*; and band number 4 and 7 belonged to *Staphylococcaceae*. Band numbers 5, 6, 8 and 12 belonged to *Enterobacteriaceae*. Band number 6 showed similarity with *Serratia* and bacterium symbiont of *Heliothrips haemorrhoidalis* and band number 9 showed similarity with *Cardinium* endosymbiont belonging to *Amebophilaceae*. Band numbers 10 and 11 belonged to *Vibrionaceae* were exclusively present in the field populations (Warangal and Tiruchirappalli) but not in the laboratory population.

Discussion

In-depth analysis of the diversity of microflora within an invertebrate host is essential to characterize the ecological roles of insect symbionts and their interactions with the host. Here, we have analyzed the diversity of gut bacterial communities of insecticide-susceptible and insecticide-resistant *N. lugens* using culture-dependent and PCR-DGGE based culture-independent methods.

Previous reports on bacterial symbionts of *N. lugens* from China (Tang et al. 2010; Xu et al. 2014) suggested that BPH typically harbors a low complex gut bacterial

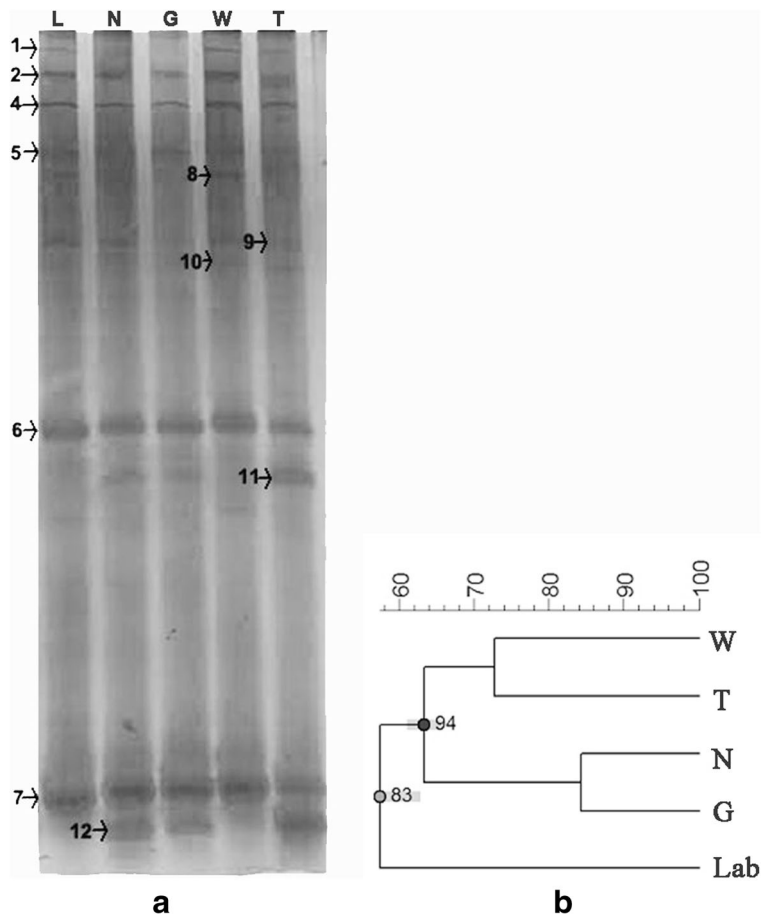
Table 1 Culturable bacteria from different populations of *Nilaparvata lugens*

Bacterial isolate	Phylogenetic affiliation ^b					
	Strain name	Accession number	Nearest phylogenetic neighbor	Accession number	Similarity ^{a*} (%)	Phyla; Family; Genera
Laboratory population	<i>Acinetobacter</i> sp. MB2	KF359494	<i>Acinetobacter</i> sp. S79	KT025922	99 (977/979)	<i>Proteobacteria</i> ; <i>Moraxellaceae</i> ; <i>Acinetobacter</i>
	<i>Bacillus</i> sp. MB3	KF359495	<i>Bacillus</i> sp.R01S-5	KJ413087	99 (899/901)	<i>Firmicutes</i> ; <i>Bacillaceae</i> ; <i>Bacillus</i>
	<i>Pantoea</i> sp.MB4	KF512549	<i>Pantoea</i> sp. S71	KT025920	99 (897/901)	<i>Proteobacteria</i> ; <i>Enterobacteriaceae</i> ; <i>Pantoea</i>
Nellore	<i>Acinetobacter</i> sp. Nellore FB2	KF448525	<i>Acinetobacter</i> <i>lwoffi</i> AG-37	KF817651	97 (920/949)	<i>Proteobacteria</i> ; <i>Moraxellaceae</i> ; <i>Acinetobacter</i>
	<i>Chryseomicrobium</i> sp. Nellore FB3	KF448526	<i>Chryseomicrobium</i> sp.AK52	HG529985	97 (1052/1088)	<i>Firmicutes</i> ; <i>Planococcaceae</i> ; <i>Chryseomicrobium</i>
Warangal	<i>Delftia</i> sp. Nellore FB1	KF448523	<i>Delftia acidovorans</i> B208	KJ781879	96 (817/851)	<i>Proteobacteria</i> ; <i>Comamonadaceae</i> ; <i>Delftia</i>
	<i>Enterobacter asburiae</i> WMB1	KF901493	<i>Enterobacter asburiae</i> RB2	KC431780	99 (844/846)	<i>Proteobacteria</i> ; <i>Enterobacteriaceae</i> ; <i>Enterobacter</i>
	<i>Exiguobacterium</i> sp.WMB2	KF901494	<i>Exiguobacterium aquaticum</i> F-50	KT027701	99 (693/700)	<i>Firmicutes</i> ; <i>Bacillales</i> family 12; <i>Exiguobacterium</i>
Tiruchirappalli	<i>Lysinibacillus</i> sp. WMB4	KF901496	<i>Lysinibacillus</i> sp. MD5	HQ257252	99 (791/792)	<i>Firmicutes</i> ; <i>Bacillaceae</i> ; <i>Lysinibacillus</i>
	<i>Bacillus circulans</i> WMB5	KF901497	<i>Bacillus circulans</i> KAR81	KR055041	100 (858/858)	<i>Firmicutes</i> ; <i>Bacillaceae</i> ; <i>Bacillus</i>
	<i>Acinetobacter soli</i> Trichy FB1	KJ655531	<i>Acinetobacter soli</i> HD51A	JN644600	100 (940/940)	<i>Proteobacteria</i> ; <i>Moraxellaceae</i> ; <i>Acinetobacter</i>
	<i>Bacillus</i> sp. Trichy FB2	KJ655532	<i>Bacillus</i> sp. AZ6	KT221633	99 (917/918)	<i>Firmicutes</i> ; <i>Bacillaceae</i> ; <i>Bacillus</i>
	<i>Staphylococcus sciuri</i> Trichy FB3	KJ655533	<i>Staphylococcus sciuri</i> DHAN01	KT270573	100 (972/972)	<i>Firmicutes</i> ; <i>Staphylococcaceae</i> ; <i>Staphylococcus</i>
Gangavati	<i>Pantoea</i> sp. Trichy FB4	KJ655534	<i>Pantoea</i> sp. UJWRF1322	KR189345	100 (1020/1020)	<i>Proteobacteria</i> ; <i>Enterobacteriaceae</i> ; <i>Pantoea</i>
	<i>Staphylococcus sciuri</i> GFB1	KJ655535	<i>Staphylococcus sciuri</i> DHAN01	KT270573	100 (974/974)	<i>Firmicutes</i> ; <i>Staphylococcaceae</i> ; <i>Staphylococcus</i>
	<i>Staphylococcus xylosum</i> GFB2	KJ655536	<i>Staphylococcus xylosum</i> LN29	KC790245	100 (896/896)	<i>Firmicutes</i> ; <i>Staphylococcaceae</i> ; <i>Staphylococcus</i>
	<i>Pantoea ananatis</i> GFB3	KJ655537	<i>Pantoea ananatis</i> XFZ19	KR296701	100 (942/942)	<i>Proteobacteria</i> ; <i>Enterobacteriaceae</i> ; <i>Pantoea</i>

*represents the exact alignment length in bp

^a Similarity represents % identity shared with non-redundant sequences of GenBank database^b Phylogenetic affiliation (Phyla; family; genus) was made based on BLASTn results

Fig. 1 **a** DGGE profile of PCR amplified V₃ region of bacterial 16S rRNA gene in the guts of different populations of *Nilaparvata lugens*. Numbers indicate sequenced bands; (See Supplementary Table S2). Band 3 was not cloned; hence not included in the Supp. Table S2. **(b)** Dendrogram of DGGE profiles of PCR amplified V₃ region of bacterial 16S rRNA gene in the guts of different populations of *Nilaparvata lugens*. The values on the top line represent the similarity level. Letters represent the following populations: L- Laboratory-reared, N - Nellore, G - Gangavati, W- Warangal and T- Tiruchirappalli



community and our results too demonstrate the same. The bacterial community composition of BPH guts obtained in the present study was comparable to earlier works related to bacterial flora of BPH and other insects. Among the 9 genera identified through molecular characterization of the bacterial isolates, 3 (*Acinetobacter*, *Enterobacter* and *Exiguobacterium*) have been previously reported from Chinese population of *N. lugens* (Tang et al. 2010). Other genera such as *Staphylococcus*, *Pantoea*, *Lysinibacillus*, *Delftia*, *Enterobacter*, and *Bacillus* have been found consistently in many insect species including *Lymantria dispar* and *Plutella xylostella* (Broderick et al. 2004; Lin et al. 2015) and are known to be involved in multiple functions thereby contributing to the overall fitness of the host. *Enterobacter asburiae* isolated from the gut of *P. xylostella* has been known to degrade acephate (Ramya et al. 2016); in the present study, *Enterobacter asburiae* was isolated from Warangal field population. Further, *Bacillus* sp. has been isolated from different *N. lugens* populations in the present study and strains belonging to this genus are capable of

degrading various environmental pollutants including organophosphorus compounds (Singh and Walker 2006). Conversely, the genus *Chryseomicrobium* (*Planococcaceae*) recorded in the present study has not been reported in any insects before.

Likewise, the bacterial sequences obtained by culture-independent method were similar to gut-associated microbes reported in insects. Phylogenetic analysis enabled the establishment of their taxonomic position with known bacteria. Band number 9 shares 99% nucleotide identity (192/194 bp) (corresponding to ~200 bp V₃ hypervariable region) with *Cardinium* endosymbiont of *Sogatella furcifera*, *Euides speciosa* and *Harmalia sirokata* all belonging to Delphacidae. Interestingly, *Cardinium* bacteria was not detected in *N. lugens* in the previous studies by Tang et al. (2010) from China and Nakamura et al. (2009) from Japan, however it was detected in our populations. Bands 5, 8 and 12 shares 99% identity with bacteria belonging *Enterobacteriaceae* previously reported from *N. lugens* and 99% nucleotide identity with *Arsenophonus* symbiont of

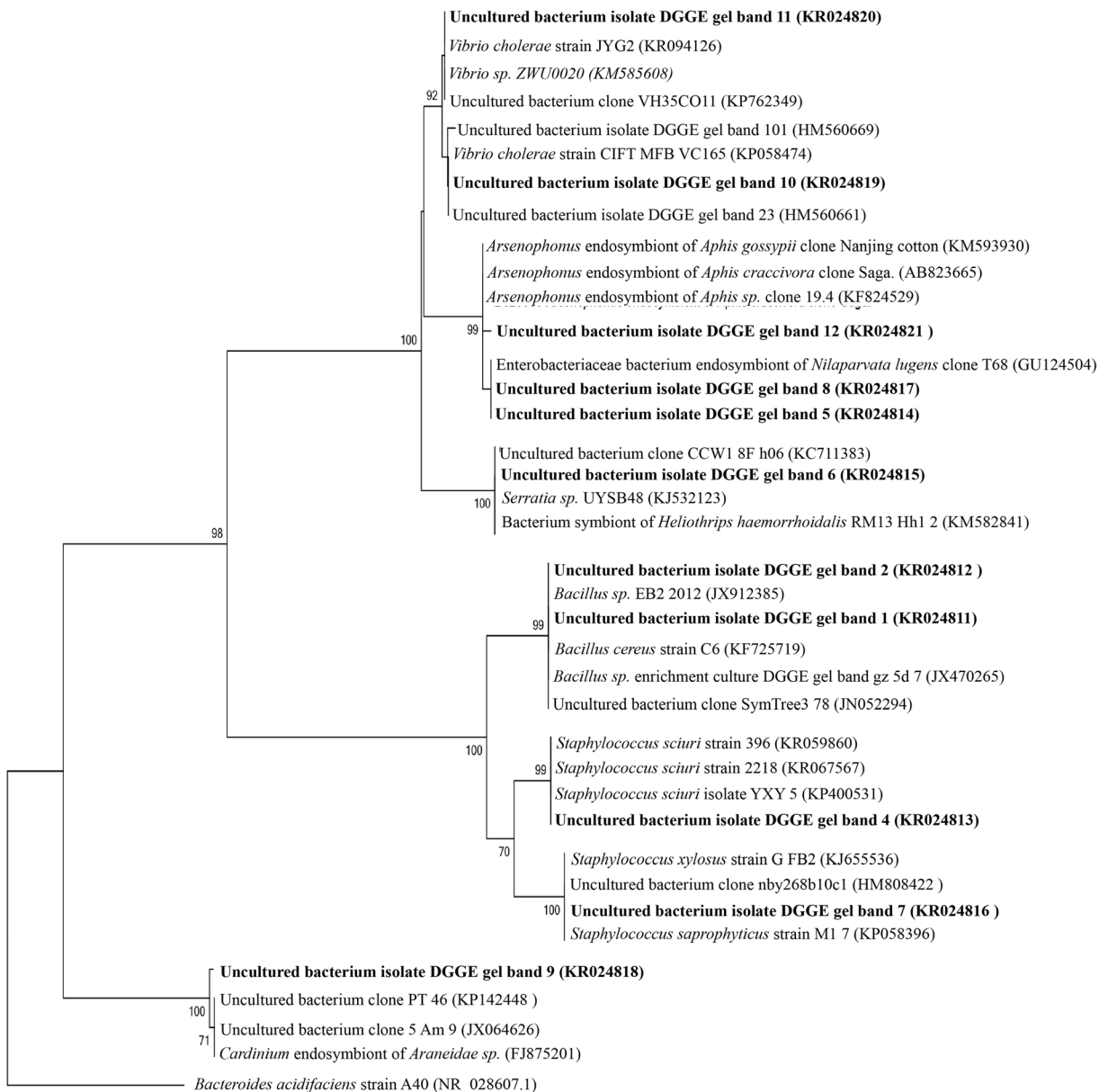


Fig. 2 Neighbor-joining phylogenetic tree based on PCR-DGGE amplified V₃ region of 16S rRNA gene in the guts of different populations of *N. lugens*. Maximum Composite Likelihood

method was used and the numbers at the nodes indicates the bootstrap values out of 1000 replicates

Aphids. Members of *Enterobacteriaceae* are among the common gut-associated microflora of various insect species (Xia et al. 2013; El-Sayed and Ibrahim 2015). Bacteria belonging to *Enterobacteriaceae* reportedly degrade carbohydrates and may play role in the digestive physiology of host insects (Anand et al. 2010). Also, Xue et al. (2014) reported that BPH harbors

facultative bacteria that are phylogenetically close to the genus *Arsenophomus*; further, they observed that the *Arsenophomus* symbiont genome contained the complete functional gene set for the synthesis of B vitamins, which BPH genome lacks. Therefore, it was speculated that *Arsenophomus* symbiont might be involved in the supply of B vitamins to BPH. OTU 6 shares 100%

identity with the genus *Serratia*, which is a Gram negative, facultative anaerobic bacterium and frequently isolated from several insect guts, e.g. Acridid (Dillon et al. 2008), Gypsy moth (Broderick et al. 2004) and Diamond back moth (Indiragandhi et al. 2007). While some of the bacterial species belonging to this genus viz. *Serratia marcescens* are regarded an opportunistic insect pathogen, its consistent occurrence in the gut of many insect species may imply symbiosis (Broderick et al. 2004; Dillon et al. 2008).

In the present study, by culture-dependent method, it was observed that field populations carried some unique bacterial flora which was not detected in the susceptible population. Further, cluster analysis showed that the field collected, resistant populations formed a coherent cluster while the insecticide-susceptible population formed a separate clade. Also, sequencing of DGGE bands revealed presence of bacterial taxa exclusively associated with the field populations (Band numbers 10 and 11). The observed variation in the gut bacterial flora between the laboratory-reared and field-collected BPH populations may largely reflect differences in the bacteria that they might have acquired from the environment. The laboratory-reared *N. lugens* are not exposed to any insecticides and are insecticide-susceptible. Field populations are exposed to xenobiotics such as insecticides, which may play a selective role in shaping the gut bacteria that can degrade these toxins. The resistance level of the studied BPH populations to various insecticides has been documented (Supplementary Table S1) (Malathi et al. 2015). Field populations including Tiruchirappalli, Warangal and Gangavati were found to exhibit low to moderate levels of resistance to the tested insecticides. The intensive use of insecticides is likely to have contributed to the development of *N. lugens* populations with insecticide resistance (Malathi et al. 2015). Apart from the physiological resistance mechanisms which involve elevation of detoxifying enzymes such as esterases, the gut associated bacteria may degrade these insecticides thereby imparting insecticide resistance to their hosts. Recent studies (Kikuchi et al. 2012; Xia et al. 2013; Ramya et al. 2016) demonstrate the role of gut bacteria in imparting insecticide resistance to the host. van den Bosch and Welte (2016) suggested the term ‘detoxifying symbiosis’, where, the insect-

associated microorganisms effect the detoxification of plant allelochemicals and insecticides thereby providing protection to the insect host.

Our basic study revealed the bacterial diversity of the gut of insecticide-susceptible and -resistant *N. lugens* and opens an avenue for research on symbiont-mediated insecticide resistance in *N. lugens*. Bacterial isolates obtained in the present study may be further investigated for their possible role in conferring insecticide resistance to the host and could lead to development of novel, sustainable approaches in the management of this pest.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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