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High Prevalence of *Pantoea* in *Diaphorina citri* (Hemiptera: Liviidae): Vector of Citrus Huanglongbing Disease

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Abstract

As an important insect vector, Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) transmits the pathogen '*Candidatus* Liberibacter asiaticus' (*C*Las) that is associated with citrus greening also known as Huanglongbing (HLB) disease. The bacterial endosymbionts have a potential role in shaping the host range of insect herbivores and their performance on different host plants, which might affect the endosymbiont distribution in insect populations. Here, we detected and characterized *Pantoea* endosymbiont in nymph and adult ACP specimens collected from *Citrus reticulata* Blanco and *Cordia myxa* L. plants. The phylogenetic tree constructed using endosymbiotic bacteria 16S ribosomal RNA sequences indicated that *Pantoea* sp. was closely related to *Mixta calida*, sharing about 98% identity and was grouped with other *Mixta* and *Pantoea* endosymbionts. Our findings showed 100% and 92.3% infection of *Pantoea* in adults while 61.5% and 90% infection of *Pantoea* in nymphs collected from *C. reticulata* and *C. myxa* plants, respectively. Understanding the interaction of endosymbiotic bacteria with ACP associated with host plants could be useful for developing an effective management strategy for both ACP and HLB disease.

Introduction

The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is an economically important insect pest of citrus crop and vectors the pathogen '*Candidatus* Liberibacter asiaticus' (*C*Las). *C*Las is a gram-negative, phloem-limited, fastidious bacterium, which is associated with the most threatening disease, citrus greening, also known as huanglongbing (HLB) disease. ACP and HLB have been spread in almost all citrus-growing regions worldwide. HLB has resulted in tremendous losses and the death of millions of trees worldwide [1]. There is no cure or effective control method for this disease [2].

The host range of ACP includes more than 25 species in the Rutaceae family [3, 4]. The population of ACP is well established on almost all citrus cultivars in Pakistan [5]. Besides Rutaceae, ACP may feed on a wide range of alternative host plants, which are important in allowing the insects to survive in the absence of suitable hosts. Recently, *Cordia myxa* L. (Family: Boraginaceae) has been reported as a new host for ACP in the south region of the state of Punjab, Pakistan [6]. However, bacterial endosymbionts associated with ACP feeding on new detected host plant have not been reported yet.

An understanding of the interactions between insects and symbionts can shed light on a new perspective for pest prevention and control technology. Symbiotic bacteria play different roles in insect biology which might affect insect growth and reproduction [7], surpass insects' immune defenses [8], affect the tolerance to heat stress [9], alter resistance level in insect against parasitism [10], and amend the attractiveness of insects toward their host plants by changing in phloem composition [11]. Furthermore, the microbial symbionts associated with insect herbivores may allow their insect hosts to feed on different plant species [12, 13].

Generally, ACP harbors various endosymbiotic bacteria including *Wolbachia*, *Arsenophonus* spp., *Candidatus* Carsonella ruddii, mycetocyte symbiont, syncytium symbiont, Liberibacter sp., and *Candidatus* Profftella armatura

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[14, 15]. The genus *Pantoea* comprise of free-living and both pathogenic and non-pathogenic host-associating species [16]. The prevalence of different *Pantoea* isolates has been reported in a wide variety of insect species [17–20]. In most of them, the association has been termed as mutualistic, wherein the bacteria inhabit the insect intercellularly, and in few cases intracellularly within specialized cells of the host [21]. Recent studies related to *Pantoea* showed that while the bacteria contribute insect nutrition and digestion [20] insect gets to benefit from bacteria to the breakdown of toxic substances [22].

In this study, we identified for the first time *Pantoea* sp. associated with ACP feeding on two different host plants by molecular-phylogenetic sequence analysis based on 16S ribosomal RNA sequences. We provided data on the prevalence of endosymbiont, *Pantoea* with natural populations of ACP collected from *C. reticulata* (known host plants) and *C. myxa* (alternative new host plant). Our research objective was to assess whether there is a host-related differentiation in *Pantoea* harbored ACP nymphs and adult populations associated with *C. reticulata* and *C. myxa* plants.

Materials and Methods

Insect Samples

The nymphs and adult ACP samples were collected on 15 June 2018 from *C. myxa* plants at Multan (29° 35′ 20.3″ N 71° 10′ 06.5″ E) and additional location Muzaffargarh district (30° 03′ 11.5″ N 71° 10′ 16.1″ E); 472.3 m west of the initial site. The plants were about 3 to 4 years old, and the closest citrus orchards from both locations were 1.04–1.20 km northeast. For comparison, the ACP population was collected from *C. reticulata* orchard in Sahiwal (31° 58′ 48.5″ N 72° 18′ 37.5″ E) and Bhalwal (32° 16′ 34.7″ N 72° 55′ 32.8″ E) tehsils of Sargodha, most citrus growing district in Pakistan. The insect specimens were collected using aspirator and preserved in 96% ethanol. The preserved specimens in 1.5 ml tubes were analyzed in Molecular Entomology Laboratory, Department of Plant Protection, Ankara University, Turkey.

DNA Isolation

A total of 100 ACP individuals from two different host plants were used in this study. Only fifth nymphal instars and adults were used in molecular experiments, which were separated using morphological characters [23]. Genomic DNA was extracted from single nymph and adult of ACP. The whole insect samples were homogenized in lysis buffer (100 mM Tris, 50 mM EDTA, 1.4 M NaCl, 2% CTAB) using a sterile pestle and incubated at 65 °C for 12 h. DNA was extracted using chloroform-isoamyl alcohol (24:1) followed by isopropanol precipitation. The pellet was washed with 70% ethanol, centrifuged for 5 min at 7500 rpm, dried and eluted in sterile water [24]. DNA samples were electrophoresed in 1% agarose gel containing Pronasafe Nucleic Acid Staining Solution (Laboratorios, CONDA, S.A.), and gel images were obtained using GelCapture Software (DNR Bio-Imaging Systems, Jerusalem, Israel). The concentration of DNA samples was analyzed using the NanoDrop2000 spectrophotometer (Thermo Scientific, USA).

Polymerase Chain Reaction (PCR) Amplification and Sequence Analysis

PCRs were carried out in a 50-µl reaction volume using an equal nanogram of DNA template and GoTaq Flexi DNA polymerase (Promega, Madison, WI, USA) according to the manufacturer's instructions. Primers set for identification of *Pantoea* (5′-ACGGAGGGTGCAAGCGTTAAT-3′ as forward primer, 5′-AGGTAAGGTTCTTCGCGTTGCA-3′ as reverse primer) were designed using the sequences of the bacteria identified in ACP given in GenBank including the species *Pantoea ananatis*, *P. cypripedii*, *P. agglomerans* (GenBank accession numbers: KC153128.1; KC153127.1; KC153126.1). *Pantoea* sp. was tested for the presence of the bacteria with the expected product size of approximately 450 bp. A second primer set for mitochondrial *cytochrome oxidase I* (mt*COI*) gene coding region, COI-F (5′-AGGAGG TGGAGACCCAATCT-3′) and COI-R (5′-TCAATTGGG GGAGAGTTTTG-3′) [25], was used to assess the quality of the template DNA with the expected product size of approximately 821 bp. Amplification conditions were as follows for *Pantoea* sp.; 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 90 s and a final step at 72 °C for 10 min. Amplification conditions for *COI* primers consisted of an initial denaturation at 94 °C for 3 min, followed by amplification for 35 cycles at 94 °C for 1 min, 53 °C for 30 s, 72 °C for 2 min, and a final step at 72 °C for 10 min. PCR products were visualized using gel electrophoresis on a 1.5% agarose gel containing Pronasafe Nucleic Acid Staining Solution (Laboratorios, Conda, S.A.), and gel images were obtained using GelCapture Software (DNR Bio-Imaging Systems, Jerusalem, Israel).

Pantoea-specific PCR products were purified with Wizard SV Gel and PCR Clean-up System (Promega) according to the manufacturer's instructions. Sequencing reactions were carried out using CEQ 8800 Genetic Analysis System (Beckman Coulter). The sequenced rRNA sequence was submitted to BLAST against the NCBI 16S ribosomal RNA sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and to the Ribosomal Database Project II server (https://rdp.cme.msu.edu). The amplified ACP *Pantoea* rRNA sequence was submitted to GenBank under accession number MN251864.1.

Phylogenetic Analysis

16S rRNA nucleotide sequences of *Pantoea* and different bacteria genus from ACP were collected from the NCBI database and aligned with MEGAX [26] suit using ClustalW [27]. The evolutionary history was inferred using the Neighbor-Joining method [28]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [29] and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Evolutionary analyses were conducted in MEGA X [26].

Results

In this study, an endosymbiotic bacterium belonging to ACP was identified as *Pantoea* sp. for the first time by sequencing and phylogenetic analysis based on 16S ribosomal RNA sequences. 16S rRNA BLAST analysis revealed that sequence of *Pantoea* 16S rRNA from ACP displayed a high sequence similarity among *Pantoea* and *Mixta* species with the highest possible scores including *Mixta calida* (homotypic synonym *Pantoea calida*) and *M. gaviniae* (homotypic synonym *Pantoea gaviniae*) (Table 1).

The *Pantoea* 16S rRNA gene sequence obtained from ACP was used for phylogenetic analyses. The phylogenetic tree was constructed using endosymbiotic bacteria showing highest identity to *Pantoea* 16S rRNA from ACP, wherein *Syncytium* placed in ingroup. Additionally, *Wolbachia* and *C.* Liberibacter asiaticus from *D. citri* were used as an outgroup. Figure 1 depicts the tree constructed by Neighbor-joining analysis. According to the phylogenetic tree, *Pantoea* sp. was closely related to *M. calida*, sharing an identity of 98% (on average). The phylogenetic tree grouped *Pantoea* sp. according to its expected classification within the Erwiniaceae family clade, and separated this node from the other endosymbionts of ACP. Three major clades were produced that included taxa belonging to the γ-, β-, and α-Proteobacteria, respectively, and the separation of each major eubacterial division was strongly supported by the bootstrap method. *Pantoea* and *Mixta* species were grouped in the γ-Proteobacteria in a clade. While Syncytium endosymbiont of ACP was grouped in β-Proteobacteria; *Wolbachia* endosymbiont of ACP and *C.* Liberibacter asiaticus were grouped in α-Proteobacteria.

Pantoea primer set was used to detect the presence and prevalence of *Pantoea* infection in ACP by observing an approximately 450 bp PCR fragment. Among 100 adult and nymph ACP samples, 91 were found positive for *Pantoea* (91%). Out of 67 adult specimens, 65 were positive for *Pantoea* (97.01%), while 26 specimens out of 33 nymphs were found to be positive (78.8%) from four locations including Sahiwal, Bhalwal, Muzaffargarh, and Multan locations. As presented in Table 2, the numbers of adult ACP samples infected with *Pantoea* (95.3%) were higher than nymphal stages (78.8%) collected from three different locations.

Discussion

Pantoea species has been characterized by molecular phylogenetic approaches in a variety of insects such as bark beetles [30], fruit fly [31], flesh flies [32], wild mosquito [33], and termites [34]. The most abundant bacterial community genus across the different life stages of ACP samples collected from a navel orange orchard in China was detected as *Profftella*, *Wolbachia,* and *Pantoea* through Illumina MiSeq sequencing [35]. As far as we know, this is the first molecular phylogenetic analysis report of ACP harboring *Pantoea* associated with two different host plants in Pakistan. Proteobacteria was attributed to the most prevalent bacterial phylum across all many insects [33, 36, 37] due to insects actively recruiting Proteobacteria or due to proteobacterial taxa being more effective than other bacterial

Table 1 Highest 16S rRNA BLAST similarities of *Pantoea* sp. from ACP

GenBank accession number	Gene	Species	Max score	Identity (%)
NR_117304	16S ribosomal RNA	*Mixta calida* 1400/07	647	98.13
NR_117305	16S ribosomal RNA	*Mixta gaviniae* A18/07	636	97.60
NR_116246	16S ribosomal RNA	*Pantoea eucrina* LMG 2781	630	97.33
NR_104928	16S ribosomal RNA	*Pantoea stewartii* subsp. *indologenes* CIP 104006	630	97.33
NR_116750	16S ribosomal RNA	*Pantoea ananatis* LMG 2665	630	97.33
NR_116247	16S ribosomal RNA	*Pantoea conspicua* LMG 2434	630	97.33
NR_116114	16S ribosomal RNA	*Pantoea deleyi* LMG 24200	630	97.33
NR_115258	16S ribosomal RNA	*Pantoea allii* BD 390	630	97.33

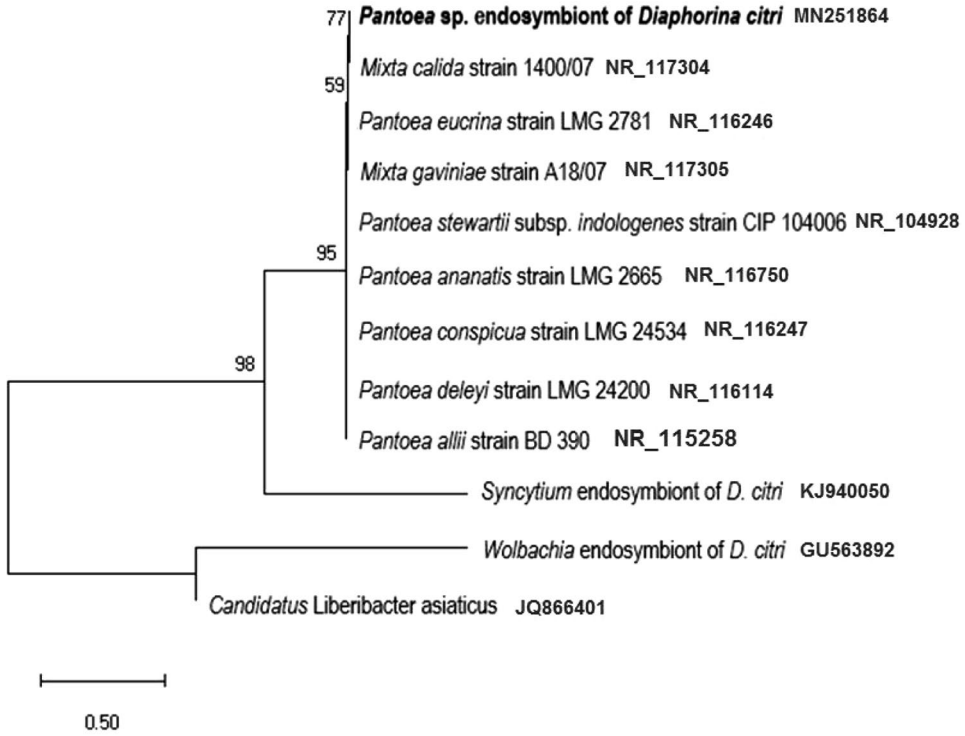

Fig. 1 Phylogenetic analysis of endosymbiotic bacteria belonging to ACP. The species and GenBank accession numbers of the sequences used to construct the phylogenetic tree are as follows

Table 2 Infection status by *Pantoea* sp. in adult and nymphal stage of ACP population collected from two different host plants at various sites

Location	Coordinates	Host plants	*Pantoea* infection status			
			Insect stage	+ve	−ve	% Infected
Sahiwal	31° 58′ 48.5″ N 72° 18′ 37.5″ E	*Citrus reticulata*	Adult	24	0	100
Bhalwal	32° 16′ 34.7″ N 72° 55′ 32.8″ E	*Citrus reticulata*	Adult	17	0	100
			Nymph	8	5	61.5
Muzafargarh	30° 03′ 11.5″ N 71° 10′ 16.1″ E	*Cordia myxa*	Adult	5	0	100
			Nymph	7	0	100
Multan	29° 35′ 20.3″ N 71° 10′ 06.5″ E	*Cordia myxa*	Adult	19	2	90.5
			Nymph	11	2	84.6

groups at invading and proliferating within new insect hosts [38]. As in many other insects, ACP microbiota was mainly dominated by species of the Proteobacteria phylum [35], our result also confirms the classification of ***Pantoea*** sp occurring in the ACP in γ-Proteobacteria class. It has been recently reported that *Pantoea calida*, *Pantoea gaviniae*, *Pantoea theicola*, and *Pantoea intestinalis* were moved to *Mixta* genus [39]. Placing of both *Pantoea* and *Mixta* genus belonging to *Erwiniaceae* family in the same clade underlined very high similarity of these two genera in the phylogenetic tree.

The present study concerns the presence of *Pantoea* endosymbiotic bacteria associated with the natural populations of ACP collected from *C. reticulata* and *C. myxa* plants. An investigation from both host plants revealed uniformity in the distribution frequency of *Pantoea*. We detected high prevalence with 91% of *Pantoea* infection rate among 100 adult and nymph ACP samples, which are consistent with 93.0% infection rate by *P. agglomerans* in blueberry maggot fly [20]. Furthermore, high-throughput sequencing of bacterial DNAs of gut microbiota of an invasive pest, *Agrilus mali* Matsumara (Coleoptera: Buprestidae) resulted in 98.8% *Pantoea* infection [40].

In this study, there was a significant difference detected in the infection rate of *Pantoea* in the nymphal and adult populations of ACP feeding on *C. reticulata* plants. However, no difference was found in terms of infection rate among different life stages collected from *C. myxa* plants that could be closely associated with the development and insect feeding habits, e.g., nymphs and adults feeding exclusively on the phloem from leaves of their host plants. A recent study reported that *Pantoea* was found to be more abundant during the nymph 2–5 stages among ACP samples collected from a navel orange orchard in China [35]. This study suggests that

there may be a difference associated with the bacterial abundance depending on hosts and life stages. However, infection frequency by *Pantoea* between different instars of ACP required detailed investigation with more samples to make clear that it is more closely related to developmental stages.

Previously, a broad host plant range within the Rutaceae family has been reported for ACP [41, 42]. Development and oviposition of ACP are similar on almost all citrus cultivars and additionally on orange jasmine [43]. ACP also lays eggs on fig *Ficus carica* L. (Moraceae) and feeds on potatoes *Solanum tuberosum* L.; hackberry [44] and *C. myxa*, as well [6]. In comparison with two different host plants, 100% adults and 61.5% nymphs collected from *C. reticulata* were found to be infected with *Pantoea*. While the rate of *Pantoea* infection was 92.3% adults and 90% nymphs collected from *C. myxa* plants. Differences in leaf surface, wax composition, availability of sugars, and interactions with other bacterial species are notable parameters that can alter the bacterial community [45]. Furthermore, the concentrations of plant secondary compounds can also affect the bacterial composition in insect guts [46]. Further genome sequencing analysis will provide data on *Pantoea* metabolism and putative role in ACP physiology. Also understanding the interaction of ACP with *Pantoea* might be an important aspect of the integrated management of HLB disease.

The prevalence of *Pantoea* with symbiotic nature in insect hosts or inside the plants affected by arthropods pests originates the promising possibilities to counter the arthropods pests or pathogens by using the paratransgenesis technique. The genetically modified strains of *Pantoea* might have a greater chance to develop in the arthropod's body compared to exogenous strains commonly used to kill the pest. Therefore, paratransgenesis might be also a promising tool for the management of ACP and HLB disease, as it has been identified as an antagonist of various plant pathogens including bacteria and fungi, which is related to the production of antibiotics or any other mechanisms. This technique could be used as a biocontrol agent, and being an environment-friendly procedure, which might be helpful in minimizing chemical control [47].

Conclusion

This study contributes to our understanding of the prevalence of *Pantoea* endosymbionts in a natural population of ACP from two host plants, *C. reticulata* and *C. myxa*. By using metagenomics libraries and genome sequencing, it is possible to identify reluctant microbes and mechanisms in order to develop evolutionary models explaining the changes undergone by endosymbiotic bacteria in their adaptation to the intracellular host environment. Vectoring of beneficial bacteria is an important concern that could replace the other methods for ACP and HLB control. Further study should be conducted to investigate whether this endosymbiotic bacterium offers any kind of physiological advantage to ACP and the fitness costs for ACP harboring this bacterium on different host plants.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with Ethical Standards

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

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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