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MOLECULAR CHARACTERIZATION OF ASIAN CITRUS PSYLLID (*Diaphorina citri*) USING MITOCHONDRIAL CYTOCHROME OXIDASE 1 (MTCO1) GENE FROM PUNJAB PAKISTAN

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ABSTRACT

Citrus greening caused by (*Candidatus liberibacter* spp.) is one of the most destructive bacterial disease of citrus all over the world and it is transmitted by grafting and psyllid (*Diaphorina citri)* is responsible for its spread. This study was aimed to detect the prevalence of CLas in different varieties of citrus in Pakistan and molecular characterization of mtco1 gene in *Diaphorina citri*. DNA was extracted from fourteen different citrus cultivars infected with citrus greening from different orchards of Sargodha Punjab, Pakistan. Specific primers were used to amplify (Mtco1 gene) in *Diaphorina citri* and also Las specific primers were also used to detect HLB pathogen. Amplified fragments of 507bp, 1500bp and 2500bp were observed in CLas and Mtco1 gene of Asian isolates. The resulted fragments were TA cloned and sequenced from both strands. The infectious bacterium was found in 3 samples out of 14 (21.5 %) while from psyllid it was found in 5 samples (37.5%). The weather changes effect the prevalence of CLas in citrus but usually it decrease during autumn and found Las positive vector in spring or summer season up to 42°C. So there is a need to research on management of citrus greening disease in Pakistan as it caused major yield loses.

Key word: Mtco1 gene, Clas, liberibacter, haplotypes, HLB, Psyllid.

INTRODUCTION

The *Citrus psyllid* belongs to Psyllidae family is a phloem-inhibited pest of citrus; it's of great importance in all citrus growing areas. Asian citrus psyllid acts as a vector in case of Huanglongbing (HLB) or citrus greening, which is a threat for citrus industry (Bové, 2006). This is a bacterial disease and psyllid transmits bacteria from infected to healthy trees but all the bacteria are not the causal agent of diseases in citrus (Mushtaq *et al.*, 2018) In case of infection, there is no cure at all "treatment is better than cure" (Teixeira *et al.*, 2005). Asian citrus psyllid (*Diaphorina citri*) is a sap sucking insect. It is a widely distributed in south Asian and recorded in all citrus growing areas (Grafton-Cardwell *et al.*, 2006). Bacteria are mostly found in young tender leaves (Michaud, 2004). This disease greatly affects quality, flavor and production of citrus and related species and in this case tree bears small, asymmetrical fruit which were partially green (Graca, 1991).

Citrus greening is a major problem of citrus trees in most parts of Asia and Africa. The diversity of *Diaphorina citri* has been found in Southeast Asia and the Indian subcontinent, Saudi Arabia Brazil (Graca, 1991) and southern Iran (Cermeli *et al.*, 2000), Venezuela and Argentina. *Diaphorina citri* was first reported in Americas in Brazil in 1940s but Huanglongbing was not a problem till 2004. In the 1990s, psyllid made devastating entry in Central and North America, South-eastern and South-central areas of the United States (French *et al.*, 2001), Belize, Eastern and Western Mexico (Halbert *et al.*, 2010), Cuba (Halbert and Manjunath, 2004) and Puerto Rico . The causal agent was transmitted through graft transmission and it was first reported in 1950s in China (Schauff *et al.*, 1998). Vector of Huanglongbing was first reported in 1998 from the continental USA (Florida) and now occurs from Florida to California. The population diversity of CLas has been found in Asia from Japan to South China, South-east Asia and the Indian subcontinent to Pakistan, Brazil and Florida (2005), and Louisiana (June 2008) in the U.S (Rosen, 1978).

The Asian citrus psyllid feeds on all citrus trees, that include lime, orange, mandarin, lemon, kumquat, pomello, tangerine and grapefruit. *Diaphorina citri* has very conserved range of host plants i.e. relatives of citrus, like orange jasmine, curry leaves and related species of *Rutaceae* (Halbert and Manjunath, 2004). Psyllids show increased rate of reproduction on grapefruit than on orange jasmine, sour orange and rough lemon (Tsai et al., 2000). Generally psyllids are narrowly host specific and restricted to perennial dicotyledonous plants (Eastop, 1972).

OBJECTIVES

The main objective of this study was identification and molecular characterization of psyllid (a major cause of Huanglongbing disease) using mitochondrial cytochrome oxidase 1 (mtCO1) gene to assess the genetic relationships between different collections of *D. citri* from Sargodha Punjab, Pakistan.

MATERIALS AND METHODOLOGY

Collection of *Diaphorina citri:* Individuals of *D. citri* were collected from the orchards of citrus located in Sargodha District. Insects were collected in falcon tubes containing 70%

ethanol and labeled properly. Young leaves of symptomatic and non-symptomatic plants were observed, collected, kept in plastic bags, labeled and stored at -80 °C.

DNA extraction from *D. citri:* Insect was taken in a 1.5mL eppendorf and crushed properly with the help of a sterilized pipette tips. After that 600µL pre-heated extraction buffer (100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 500 mM NaCl, 1% beta mercapto ethanol) was added in it and incubated at 65˚C for 15 minutes in a water bath and tubes were inverted 3-4 time during the incubation period (Hung *et al.*, 2004). Tubes were placed at room temperature (RT) for 3 minutes and then 600µL of cold chloroform was added. Samples were then thoroughly vortexed and centrifuged for 3 minutes at 12,000 rpm. Uppermost 500µl of the aqueous phase was transferred to a new 1.5mL eppendorf tubes, 500µL of cold isopropanol was mixed and placed on ice for 10 minutes. Centrifuged at 12,000 rpm for 10 minutes for the precipitate of nucleic acid. Liquid supernatant was poured off. 500µL of 70% cold ethanol was used to wash the pellet and tubes were centrifuged at 12,000 rpm for 1 minute. Ethanol was removed by using pipette and DNA pellets were dried at RT or 37˚C for 10 minutes 30 µL TE buffer was added in tubes.

PCR amplification of *D. citri (mtco1) gene* and ***Liberibacter*** **from DNA:** DNA amplification was conducted through PCR (Polymerase chain reaction) assay by making a reaction mixture of 25µL in a thin walled 0.5mL PCR tube. The reaction mixture was made by mixing 2.5µL 10X *Taq polymerase* buffer (Fermentas), 2.5µL dNTPs, 1.5µL $MgCl_2$, 0.5µL primers PSYCO-F (5´ CAT TTA TTC TGA TTT TTT GGA CAT CC 3´); PSYCO-R (5´ GAA TAT AGA AAT TTG ATT TAG TCG TCC 3´) of each and 0.25µL *Taq polymerase* (Fermentas) and 5µL of DNA. The PCR machine was programmed for the preheat treatment of 94 ˚C for 5 minutes and followed by 35 cycles of 94˚C for 1 minute, 50˚C for 1 minute and 72˚C for 1 minute. It is followed by final incubation for 10 minutes at 72˚C. Specific primers L10-F (5´ CATC GGG AGA TGA AAG TTG AAT A 3´); L10-R (5´ TTC CCC TGC CGC AGA CGC AAC A 3´); O11/O12c-R (5´ GCC TCG CGA CTT CGC AAC CCA T 3´); O11/O12c-F (5´ GCG CGT ATG CAA TAC GAG CGG CA 3´) were used for amplification of DNA. The PCR machine was programmed for the preheat treatment of 94˚C for 10 minutes and followed by 30 cycles of 94˚C for 30 seconds, 60˚C for 30 seconds and 72˚C for 1 minute. It was followed by final incubation of 10 minutes at 72˚C. Agarose gel electrophoresis was performed to check amplifications under UV trans-illuminator and fragment length was estimated by comparison with a co-electrophoresed 1kbp DNA ladder (Fermentas).

Cloning and sequencing of the desired PCR products were performed by using standard protocols of INSA Clone TA Cloning kit. Confirmed clone plasmids were sent to Macrogen Korea for Sanger sequencing with specific primers. The sequenced files were analyzed through software (DNA Star Inc., Madison, WI, USA). The sequences were blast at the NCBI website to search the similarity of clone with other reported ones. Phylogenetic analysis was constructed through MEGA 7.0 software using Clustal W (neighbor-joining method) compared with other reported sequences (Tamura et al., 2011).

RESULTS

Molecular characterization of Asian citrus psyllid (ACP): Psyllids were collected from citrus groves near citrus research station, Sargodha, Pakistan during the survey in 2013 (Figure 1). Universal primers (C1-J-2195 and TL2-N-3014) were used to amplify mtCO1 gene, but no amplification of PCR was observed. Another set of specific primers PSYCO-F/R for *D. citri* mtCO1 gene was used. These primers amplified a fragment of 1500bp (Figure 2). Amplified products were cloned in pTZ57R/T vector and confirmed by restriction analysis (Figure 3 & 4) and selected clones (S11 and S3) were sequenced. Full length sequence (RF5) was trimmed and 507bp sequence was used for further analysis.

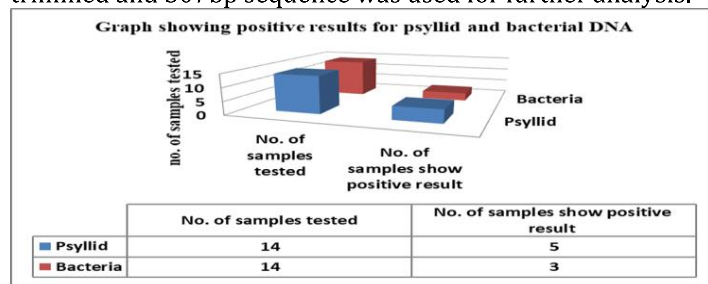

Figure 1: Graph exhibiting number of samples which were positive in case of *Candidatus liberibacter* and *Diaphorina citri.*

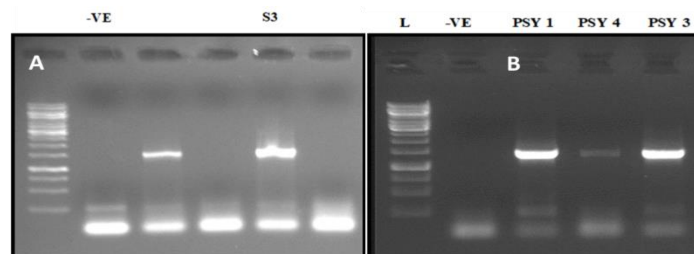

Figure 2: PCR products from genomic DNA of *Diaphorina citri* (psyllid) samples were amplified as 1500bp band (A). In each case the fragments were compared with 1Kb ladder. (B) PCR products from genomic DNA of *Diaphorina citri* (psyllid) samples were amplified as 1500bp band. In each case the fragments were compared with 1Kb ladder.

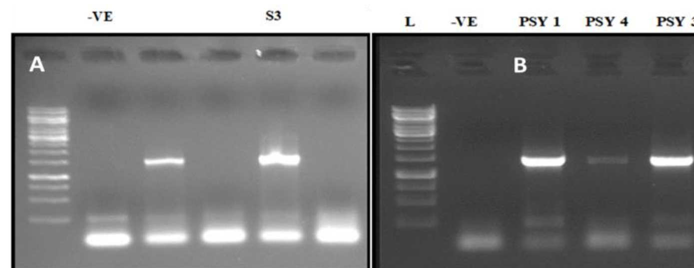

Figure 3: PCR products were digested with EcoR1 and Pst1 enzymes to obtain distinct 1500bp DNA fragments. These DNA fragments were cloned in PTz57R/T vector and screened by restriction analysis.

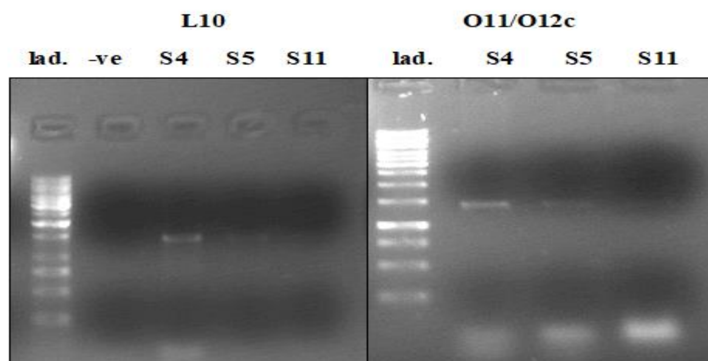

Figure 4: Agarose gel analysis of reaction products for detection of Huanglongbing (HLB) pathogen '*Candidatus liberibacter*' in citrus psyllid samples using standard polymerase chain reaction with primer sets O11/O12c and L10. In case of L10 primer set, S4 and S5 show band of 2500bp. Primer set O11/O12c show distinct band of 1500bp in case of S4 and S5 while S11 show a very minimal band. In each case the fragments were compared with 1 Kb ladder.

Table 1: List of haplotypes and their base locations.

Haplotypes	Base locations				
	130	138	217	447	500
RF5	A	A	G	T	T
Dict-1-1	T	A	A	C	T
Dict-1-2	T	A	A	C	T
Dict-1-3	T	A	A	C	T
Dict-1-4	A	A	G	T	T
Dict-1-5	A	A	G	T	T
Dict-1-6	A	A	G	T	T
Dict-1-7	A	A	G	T	T
Dict-1-8	A	A	G	T	T
Dict-2-1	A	A	G	T	T
Dict-2-2	T	A	A	C	T
Dict-3-1	T	A	A	C	T
Dict-3-2	T	A	A	C	T
Dict-4-1	A	A	G	T	T
Dict-4-2	A	A	G	T	T
Dict-5-1	A	A	G	T	T
Dict-5-2	A	A	G	T	T
Dict-6-1	T	A	A	C	T
Dict-6-2	T	A	A	C	T
Dict-6-3	-	A	A	C	T
Dict-6-4	T	A	A	C	T
Dict-6-5	T	A	A	C	T
Dict-7-1	T	A	A	C	T
Dict-8-1	T	A	A	C	T

Phylogenetic analysis: Cloned sequence was tested for homology and similarity at NCBI (BLAST) web page and analyses showed that RF5 clone match with sequences of cytochrome oxidase subunit 1 gene of psyllid (*Diaphorina citri*). Closely related sequences were extracted from NCBI databank and aligned with RF5. Multiple sequences were aligned using Clustal W, in Megalign software (DNA star Madison) and sequence distance homology was measured. RF5 showed highest sequence homology (96.6%) with two Indian isolates (Dcit-1-5, sequence ID: FJ190342 and Dcit-1-6, sequence ID: FJ190343). It can be clearly seen from the (table 1), that RF5 shows identical bases at location sites 130, 138, 217, 447 and 500 with two Indian strains.

Phylogenetic tree: Sequencing result was subjected to BLAST at NCBI web page and analyses showed that full length 507bp clones of cytochrome oxidase subunit 1 gene of psyllid (*Diaphorina citri*). Results showed that the new isolated clone showed 96.6% homology with two Indian isolates (Dcit-1-5, sequence ID: FJ190342 and Dcit-1-6, sequence ID: FJ190343). It can be clearly seen from (table 1), that RF5 shows identical bases at location sites 130, 138, 217, 447 and 500 with two Indian strains. It's a distinct kind of strain and forms a separate clade in phylogenetic tree (Figure 5).

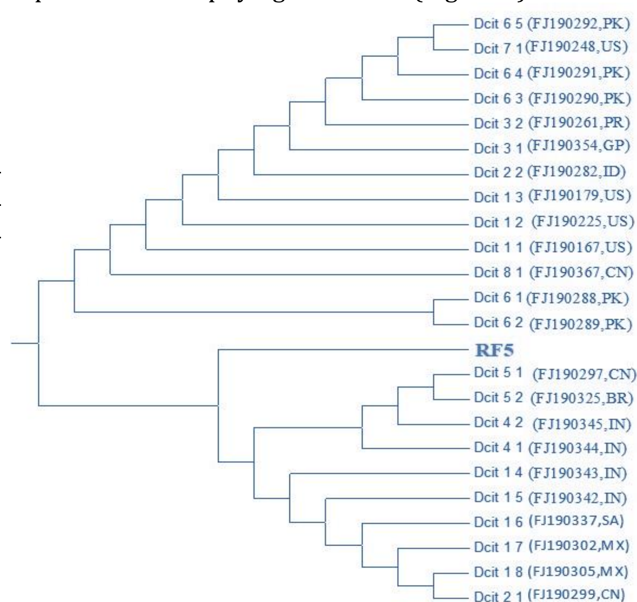

Figure 5: Phylogenetic analysis on the basis of full-length sequence of cytochrome oxidase gene obtained in this study (RF5) and extracted sequences from NCBI databank. Each node contains sequence ID, haplotype and country. RF5 showed a different clade.

Discussion

Asian citrus psyllid is a phloem-inhibited pest of citrus; it's of great importance in all citrus growing areas. Asian citrus psyllid acts as a vector for *Candidatus liberibacter* in case of Huanglongbing (HLB) or citrus greening, which is a threat for citrus industry (McClean, 1970). Vector of Huanglongbing was first reported in 1998 from the continental USA (Bové, 2006) and spread from Florida to California. CLas is found in Asia from Japan to South China, South-east Asia and the Indian subcontinent to Pakistan.

In this study prevalence CLas and *D. citri* were found in Pakistan. Studies showed that CLas was detected from those places where average temperature remains 42°C. In Pakistan warm weather interfere with acquisition and replication of

CLas in *D. citri* and do not transmit CLAs into citrus trees. Population of Psyllid was also found to be reduced in extreme high and low temperature (Razi *et al.*, 2014).

Eight haplotypes (Dict-1, Dict-2, Dict-3, Dict-4, Dict-5, Dict-6, Dict-7 and Dict-8) were highly similar to each other. Two (Dict-1 and Dict-2) of them were most abundantly found while the other two (Dict-7 and Dict-8) were very rare. Dict-7 and Dict-8 haplotypes were recovered from a single individual of Florida, USA and China respectively. Each of them is different from each other in one; there is a change or substitution of bases (Boykin *et al.*, 2012).

The Asian citrus psyllid is most serious pests of citrus in the world, and become the cause of pathogen spread in different areas and cause citrus greening disease. To determine genetic variations among geographic populations of *D. citri*, mitochondrial gene cytochrome oxidase I (mt-COI) was used to characterize Pakistani populations. Mt-CO1 is a mitochondrial encoded cytochrome c oxidase 1 which is the component of the respiratory chain of psyllid that catalyzes the reduction of oxygen to H_2O. mtco1 gene is located in mitochondrial DNA (Lashkari *et al.*, 2014).

Second important reason for this study was detection of CLas from citrus orchards of Pakistan. It is a gram-negative phloem restricted bacteria, belongs to the family Rhizobiaceae. This genus has three known species that causes Huanglongbing i.e. *Candidatus liberibacter asiaticus* (Asian countries), *Candidatus liberibacter africanus* (African countries), and *Candidatus liberibacter americanus* (Brazil and Florida). *Candidatus liberibacter asiaticus* gets transmitted through psyllids (Garnier *et al.*, 2000). Molecular characterization of alpha subgroup of proteobacteria causing Huanglongbing disease of citrus from three different ecological zones of Kenya i.e. lower highlands, upper midlands and lower midlands was done and a DNA fragment of 536bp was amplified and sequenced (Magomere *et al.*, 2009).

The presence of CLas in citrus orchards in the North-West Frontier Province of Pakistan has been confirmed. Samples of DNA Extracted from leaves of infected citrus plants and from the vector of the disease, *D. citri* were amplified using PCR based technique. Characterization of the bacterium was confirmed through sequence which showed 100% identity with reported sequences of the CLas (Chohan *et al.*, 2007). Results were found positive for both D*. citri* and CLas with respect to primers used. Two Indian isolates (FJ190342 and FJ190343) showed maximum (96.6%) similarity with the RF5 clone. Control of HLB disease has not been found anywhere in the world still researchers are doing efforts to control this disease. It is most destructive disease that is known to spread rapidly in new areas and cause huge crop losses. So there is a need to work on citrus greening as it's a really important problem in Pakistan and around the world.

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