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Molecular identification of tortoise beetle and its endosymbiotic bacteria

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Abstract

Tortoise beetles are one of the important defoliator pests of sweet potato, associated with a wide variety of bacterial endosymbionts that confer many ecologically relevant traits to the host insect. Endosymbiotic bacteria (ESB) play a vital role even in the physiology of the host, hence identification of ESB associated with the aphids will help to develop important strategies for the management of this noxious pest. Cassidini is the largest tribe of tortoise beetle in Kerala represented by 18 species in four genera, followed by Aspidimorphinia and notosacanthini. In the present study, molecular characterization of the sweet potato defoliator and endosymbiontic bacteria associated with them, was done. By molecular characterization they were identified as *Chiridopsis* sp. and sequences were deposited at NCBI with accession no OR416859. Morphological characters of the isolated revealed that each isolate has different colony characters. Further, the genomic DNA was isolated from each of the EPB isolates and PCR amplification of 16S rRNA gene was carried out using universal primers. The 16S rDNA gene sequences of endosymbiotic bacterial isolates were generated by sequencing the PCR product and were aligned with each other by using BioEDIT software. The nucleotide sequences were compared with those in the NCBI databases using the Basic Local Alignment Search and were identified as were confirmed as *Kosakonia cowanii* and *Kosakonia* sp. The 16S rRNA gene sequences were also deposited at NCBI database with accession no OR426444, OR418414. From the aligned sequences phylogenetic tree was constructed by the Neighbor- Joining method using MEGA version 11.

Keywords: Sweet potato, tortoise beetle, 16S rRNA, endosymbiotic bacteria

Introduction

The symbiotic microorganisms attached with the herbivorous insects always play major role in lifecycle of insects. In particular, these microorganisms can supplement essential amino acids or vitamins, or enzymes for digestion or detoxification of noxious plant secondary metabolites in their host diet (Douglas, 2009; Douglas, 2015; Feldhaar, 2011). Hence, these endosymbiotic associations with beneficial microbes always provide essential nutrients to the hosts. Many insects such as wood-feeding termites, passalid beetles and the leaf-chewing tortoise beetles are assisted by symbiotic microbes for the break-down of the major components of plant cell walls that are enzymatically challenging sources of carbon and energy (Brune, 2014; Navarro, et al., 2019; Salem, 2017; Cortes et al., 2012). Tortoise beetles (subfamily Cassidinae) are one of the specialized herbivores that feed on sweet potato leaves (McKenna., 2020). These are associated with a broad range of host plants. Many of the species Aspidimorpha, Chiridopsis, Laccoptera and Cassida infest sweet potato plants.

Recent genetic analysis studies revealed that the thistle tortoise beetle lacks the genes responsible for the production of the pectinases. These studies also revealed

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that the pectin present in leafy plant parts eaten by the beetle digested by symbiotic γ-proteobacterial symbiont, *Stammera.* These leaf beetles are able to degrade components of the plant cell wall, such as cellulose and pectin, with the help of digestive enzymes by these bacteria. Moreover, these bacteria posses reduced genome and posses pectinases enabling the host to digest the foliage. The tortoise beetle genome do not encode pectin digesting enzymes but they obtain nutrients with the help of these bacteria. These bacteria reside in sac like organs in the adult and larval tortoise beetle foregut, where they produce pectinases. They also reside in the reproductive tract of adult females, where they play a role in bacterial transmission to beetle oxffspring (Salem et al., 2020). Their genome also lack many genes that code for essential cellular functions that are typically found in free-living bacteria which made them completely dependent on the beetle (Salem et al., 2017). In darkling beetles of the subfamily Lagriinae (Tenebrionidae), specific β-proteobacteria of the genus *Burkholderia* are associated with larvae and adult females extracellularly and provide protection against pathogen by producing antibiotics (Florez et al., 2017; Kaltenpoth and Florez, 2020). Therefore, the knowledge about interaction between insects and symbionts is gaining importance in agriculture due to the potential application for the management of insect pests. Some of the insect gut symbionts are capable of enhancing insecticide resistance in several insectspecies (Kikuchi et al., 2012; Xia et al., 2018). Hence the identification of these endosymbiotic bacteria is gaining more importance for the monitoring and management of chemical insecticide resistance (Cheng et al., 2017). Hence the complete exclusion of these primary endosymbionts from insects may reduce their lifespan and suppress population within a few days or weeks.

Previous studies on tortoise beetles of Kerala reported that Aspidimorpha furcata, Aspidimorpha miliaris, Aspidimorpha sanctaecrucis, Cassida circumdata, Chiridopsis bipunctata, Laccoptera nepalensis, Aspidimorpha fuscopunctata infest sweet potato palnts. It is also reported that the tortoise beetles infest about 25 species of plants belonging to the family Convolvulaceae. (Takizawa, 1980; Ghate et al., 2003; Borowiec and Swietojanaska, 2020; Amritha Hari 2020).

Materials and Methods

Collection of insects

Adult tortoise beetles were collected and maintained at the Entomology Laboratory, Division of Crop Protection, ICAR-Central Tuber Crops Research Institute, Thiruvananthapuram.

DNA isolation from insect

The genomic DNA was isolated using the modified cetyl trimethyl ammonium bromide buffer (CTAB) method Gawel and Jarrett (1991). The individual insect samples was homogenized with500 μl of lysis buffer (CTAB 2%, 100 mM Tris-HCI (pH 8.0), 1.4 M sodium chloride, 20 mM EDTA, 0.1% of 2-mercaptoethanol) and suspended in the same buffer. The suspension was incubated at 65°C for 1 h and centrifuged at 10,000 rpm for 10 min. Then an equal volume of chloroform: isoamylalcohol (24:1) was added and the suspension was centrifuged at 6000 rpm for 15 min at room temperature. The upper aqueous layer was transferred to a fresh micro centrifuge tube and DNA was precipitated by adding 40 μl of sodium acetate, 600 μl of 95% ethyl alcohol. The tubes were kept at -20ºC for 20 min and centrifuged at 8000 rpm for 10 min. The supernatant was discarded and the resultant pellet was washed with 70% ethanol, dissolved in 50 μL DNase-, RNase- and Protease-free molecular biology water. The intact genomic DNA was further quantified using Nanodrop ND-1000 (Thermo Scientific, Belgium). The DNA samples were diluted with sterile water to get a working solution of 50-100 ng μL^{-1}.

Polymerase Chain Reaction and DNA sequencing

The polymerase chain reaction (PCR) was carried out in a thermal cycler (BioRad, Veriti 96 wells) with the following cycles; initial denaturation 94°C for 5 min as followed by 35 cycles of denaturation 94°C for 45 sec, annealing 47°C for 45 sec, extension 72°C for 45sec and final extension 72°C for 10min, hold at 4°C. The primers used were specific to mitochondrial cytochrome oxidase (COX-1) F- LCO (GGT CAA CAA ATC ATA AAG ATA TTG G), R- HCO(TAA ACT TCA GGG TGA CCAAAA AAT CA). PCR was performed in 25 μL total reaction volume containing 20 Pico moles of each primer, 1.0 μL of 20 mM dNTP, 2.5 μL of 10X buffer and 1.0 μL of 1.0 U Taq DNA polymerase (Fermentas Life Sciences, Maryland, USA). The amplified products were resolved in 1.0% agarose gel, stained with ethidium bromide (10 ng μL^{-1}) and visualized in a gel documentation system (UVP). The PCR amplified fragments were eluted using Nucleospin® Extract II (Thermo Scientific, USA). The purified PCR products were sent for sequencing. Sequencing was carried out in an automated sequencer both in forward and reverse directions at Eurofins Genomics India Pvt. Ltd., Bengaluru, Karnataka. Homology search was carried out using BLAST (http://www.ncbi.nlm.nih.gov). From the aligned sequences phylogenetic tree was constructed by the Neighbor - Joining method using MEGA 11 software (Tamura, 2021).

Isolation of ESB

Adult beetles were collected and were surface sterilized with absolute ethanol. These were homogenized in sterile 0.9% saline and plated directly on to the nutrient agar media and kept for incubation at 30°C overnight under aerobic condition.

Identification of ESB

Pure culture of each ESB was obtained by streaking the individual colony on a fresh nutrient agar plate and incubated for 24 h at 30°C. The colony characters were observed from each separated colony (Sreerag et al., 2014)

Phenotypic characterization of ESB strains

Cultural characteristics of each bacterium, which include shape, margin and elevation of the isolates of each colony type were observed using stereomicroscope (Carl Zeiss, Stemi 2000C) under 40× magnification, by using research microscope (Leica DMLB) under 100× magnification. Gram staining was done using the Hi-Media kit (Hi-Media Laboratories Pvt. Ltd., India) according to the manufacture's protocol for the identification of unknown bacterial strains collected from the nutrient broth of 24 h culture and were observed under a compound microscope (Leica DMLB) with 100× magnification.

PCR amplification of 16S rDNA of ESB

PCR amplification of 16S rDNA gene by universal primers: forward primer fD1 5'AGAGTTTGATCCTG GCTCAG3' and reverse primer RP2 5'CGGCTACCTT GTTACGACTT3' (Weisburg et al., 1991) were used. The PCR wasperformed in a 25 μl reaction mixture having 2.5 μl of 10X Taq buffer A (containing 15 mM MgCl2, mM each), 1.0 μl of each primer (20 ng), 2 μl of template DNA and 0.25 μl of (1U) Taq DNA polymerase and 17.75 μl of sterile distilled water. The reaction was carried out in a Biorad thermal cycler with the thermal cycle programme of 92°C for 2min 10 s (initial denaturation), 30 cycles at 94°C for 1min 10 s (denaturation), at 49°C for 30 s (annealing), at 72°C for 2 min (extension) and final extension at 72°C for 10 min. The amplified products were resolved on a 1.2% agarose gel. DNA ladder of 500 bp (Bangalore GeNei, India) was used for determining the size of the amplicon. The DNA bands were visualised under UV transilluminator and the purified PCR products of 1500 bp weresequenced at Eurofins Genomics India Pvt. Ltd., Bengaluru, Karnataka.

Phylogenetic analysis

The sequences obtained for the EPB isolates were aligned with each other by using Clustal alignment programme of MEGA 11 software (Tamura et al., 2021). The nucleotide sequences were compared with those in the NCBI databases using the Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/BLAST). From the aligned sequences phylogenetic tree was constructed by the Neighbor - Joining method using MEGA 11 software.

Results and Discussion

Molecular identification of sweet potato defoliator

The tortoise beetles collected from one month old sweet potato plants maintained at ICAR-CTCRI. The molecular identification of insects was done. Genomic DNA was extracted from the insect samples and the amplification of COX-1 gene was done and product size of amplicon was 658bp.The PCR amplified products were purified and sequenced. The sequences obtained in automated DNA sequencing were aligned and compared by using BioEdit. BLAST analysis of isolates showed 100% similarity to *Chiridopsis* sp. available in the Genbank. The sequence data generated were deposited in the Genbank nucleotide database (NCBI) and the accession numbers assigned are given in Table 1. The phylogentic tree of the isolate based on Mt (COX1) gene sequences is shown in (Fig. 1).

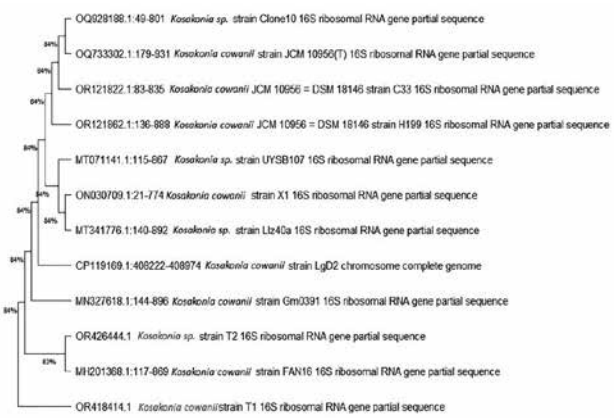

Fig. 1. Phylogenetic tree inferred from mitochondrial cytochrome oxidase (COX-1) sequencesanalysis of *Chiridopsis* sp. isolate TB

Phenotypic and molecular characterization of bacterial isolates

A total of two isolates were isolated from the *Chiridopsis* sp. and were assigned code numbers asisolates T1 and T2. Morphological variations were observed for each endosymbiotic bacterial strain, but no pigmentation was observed. Colonies formed on nutrient agar were circular, raised, convex, flat, entire white in colour with no pigmentation. A total of two bacterial strains were successfully isolated from the *Chiridopsis* sp. and were assigned code numbers as isolates T1 and T2 for laboratory purposes. They were gram positive, rod-shaped bacteria. Genomic DNA was extracted from bacterial samples. The PCR amplification of the 16S rDNA of the with the primers 16SF and 16SR at an annealing temperature of 49°C yielded a fragment of approximately 1500 bp. The PCR ampliflied products were sequenced. BLAST analysis of the sequences of the isolates T1 and T2 showed 98 % similarity to *Kosakonia*

cowanii and *Kosakonia* sp. available in the Genbank. The sequence data generated were deposited in the Genbank nucleotide database (NCBI) and the accession numbers assigned are given in Table 1. The phylogentic tree of the endosymbiotic bacteria based on 16S rRNA gene sequences is shown in (Fig. 2).

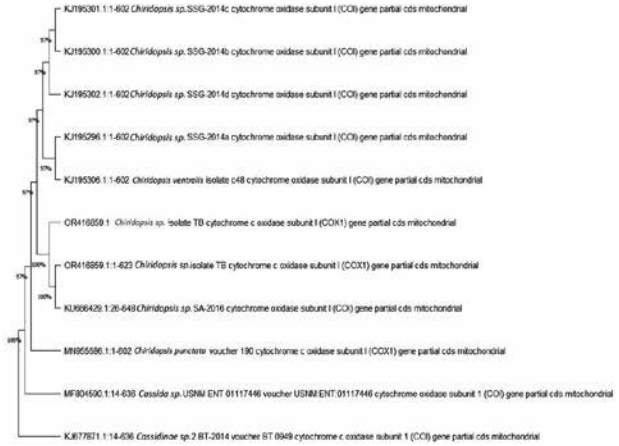

Fig. 2. Phylogenetic tree inferred from 16S rRNA gene sequences analysis of bacterial isolate T1and T2 associated with *Chiridopsis* sp.

Table 1. Molecular identification of tortoise beetle and endosymbiotic bacteria

Isolate	Identification	Accession No.	Similarity (%)
T1	16S ribosomal RNA gene sequence partial *Kosakonia cowanii* isolate T1	OR418414	84
T2	16S ribosomal RNA gene sequence partial *Kosakonia* sp. isolate T2	OR426444	84
TB	Mitochondrial cytochrome c oxidase subunit I (COX1) gene sequence partial *Chiridopsis* sp. isolate TB	OR416859	97

Previous studies have reported specific gut-inhabiting yeast-like symbiotic fungi from cigarette and drugstore beetles which provide sterols to their hosts (Pant and Fraenkel, 1954; Noda and Koizumi, 2003). Similarly in leaf beetles belonging to the subfamilies Donaciinae (Chrysomelidae), specific gut-inhabiting γ-proteobacteria, *Macropleicola* supply enzymes for plant digestion and essential nutrients for their hosts (Reis et al., 2020). Kosakonia has been reported to be associated with plant growth promoting bacteria (Kampfer et al., 2005; Peng et al., 2009). In our study *Kosakonia cowanii* was found to be reported with the *Chiridopsis* sp. and *K. cowanii* was identified as the causal agent of bacterial wilt on tomatoes (Sarkar and Chaudhuri, 2015). The genus *Kosakonia* is recently derived from reclassification of genus *Enterobacter*, and several species, including *Enterobacter arachidis, Enterobacter cowanii, Enterobacter oryzae, Enterobacter radicincitans* previously included in genus *Enterobacter* which have been transferred to the novel genus *Kosakonia* (Brady et al., 2013). *K. cowanii* is reported as phytopathogen causing a variety of plant diseases viz pathogen of eucalyptus and woody plants (Furtado et al., 2012; Wu et al., 2016; Krawczyk and Borodynko-Filas, 2020). Some strains of this species are also opportunistic human pathogens (Grimont and Grimont, 2006; Mardaneh and Soltan-Dallal, 2014; Peleg et al., 2008; Yang et al., 2018). The ability of the species to colonize different ecological conditions revealed its easy adaptable metabolism to new host conditions. Earlier report also showed the ability to cause necrotic spots on leaves of *Mabea fistulifera* Mart. (Euphorbiaceae) (Furtado et al., 2012). Some of the reports also showed *K. cowanii* colonizing the gut of *Anopheles gambiae* displayed direct anti-Plasmodium properties (Dennison et al., 2014). Previous studies conducted using *K. cowanii* B-6-1 isolated from tomato showed biocontrol activity against *F. verticillioides, A. tenuissima*, and *B. cinerea* (Shi and Sun, 2017). *K. cowanii* was found in microbiome of wasp and as an endophytic growth-promoting bacteria in some plants (Meng et al., 2015, Fall and Holley, 2016).

Conclusion

The endosymbiotic bacteria exist within the insect host and play major role in the metabolic process of the insects. They serve as unexploited sources of enzymes, bioactive molecules which helps the insect host to survive in extreme environmental conditions. Currently the research in insect–symbiosis using various genomic tools had led to the identification of the genes which are responsible for the production of these enzymes as well as various bioactive molecules which will be useful for various insect pest management strategies.

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comb. nov. and *Kosakonia arachidis* comb. nov., respectively, and *E. turicensis*, *E. helveticus* and *E. pulveris* into *Cronobacter* as *Cronobacter zurichensis* nom. nov., *Cronobacter helveticus* comb. nov. and *Cronobacter pulveris* comb. nov., respectively, and emended description of the genera *Enterobacter* and *Cronobacter. Syst. Appl. Microbiol.*, **36**:309–319.

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