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Molecular identification of *Araecerus fasciculatus* and its endosymbiotic bacteria

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Abstract

Araecerus fasciculatus are one of the important storage pests of cassava, 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 storage pests will help to develop important strategies for the management of the noxious pest. In the present study molecular characterization of the *Araecerus fasciculatus* and endosymbiotic bacteria associated with them, was done. By molecular characterization they were identified as *Araecerus fasciculatus* and sequences were deposited at NCBI with accession no OR415335. 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 *Bacillus megaterium.* The taxonomy had been recently changed and was renamed *Priestia megaterium*, which is a new separate genus from *Bacillus*. The 16S rRNA gene sequences were also deposited at NCBI database with accession no OR418413. From the aligned sequences phylogenetic tree was constructed by the Neighbor-Joining method using MEGA version 11.

Keywords: *Araecerus* sp., Endosymbiotic bacteria, 16S rRNA, Pests, Symbiosis

Introduction

Endosymbiotic bacteria and fungi associated with the insect host play important role in digestion of food, protection against pathogens, parasites, predators, modulating the interaction of phytophagous insects with host plants and in inter and intraspecific communication (Alvarado et al., 2021). These endosymbionts of insects are classified into primary and secondary endosymbionts. The primary endosymbionts have an obligatory relationship with the insect host, providing essential amino acids and showing phylogenetic congruence with their host. Primary endosymbionts are morphologically similar to each other and are restricted to bacteriocytes (Baumann et al., 2005). The secondary endosymbionts play many functional roles on their hosts such as providing fitness benefits, increasing tolerance to heat stress, increasing resistance to parasitic wasps, causing host plant specialization, conferring invasiveness (Fadden, 2001). The knowledge about interaction between insects and their gut-associated symbionts has importance in agriculture due to their potential application in pest management strategies. Moreover, the insect gut symbionts were capable of enhancing insecticide resistance in several insect species (Kikuchi

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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). With the increasing number of agriculturally relevant pests, recent studies focus on microbiome-insect mutualism with the help of culture-independent techniques such as shot gun metagenomic studies (Bharti and Grimm, 2019; Gurung et al., 2019). The coffee bean weevil (CBW), *Araecerus fasciculatus* De Geer, (Coleoptera: Anthribidae) is an important pest of stored products such as grains, coffee beans, cassava, and traditional Chinese medicine materials (Yang et al., 2017). A yield loss of up to 20% was caused by the pest in stored and processed cassava products as reported by Chijindu and Boateng, (2008). The weevils were associated with an ancient γ-proteobacterial lineage *Nardonella* which are localized to bacteriomes (Lefevre et al., 2004; Conord et al., 2008; Hosokawa and Fukatsu, 2020; Hosokawa et al., 2015; Anbutsu et al., 2017). In some of the *Sitophilus* grain weevils *Nardonella* was replaced by γ-proteobacterial *Sodalis* endosymbionts and in Curculionini *Acorn* weevils was occupied by γ-proteobacterial *Curculioniphilus* endosymbionts (Heddi et al., 1999; Heddi and Nardon, 2005; Vigneron et al., 2014, Toju et al., 2010, Toju et al., 2013; Toju and Fukatsu, 2011). Hence the identification of the functional role of these primary as well as secondary endosymbionts from insects is important as they play vital role in the lifecycle of the insects. The exclusion of these bacteria may reduce their lifespan and suppress population within few days or weeks.

Materials and Methods

Collection of insects

Adult insects were collected and maintained at the Insect Microbiology Laboratory, ICAR-Central Tuber Crops Research Institute, Thiruvananthapuram, Kerala, India. Earlier studies used morphological keys for organism identification. However, in most of the recent studies, identification and confirmation of insects pests are usually done by molecular techniques.

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 with 500 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 hr 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.

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 45 sec 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). The PCR analysis 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 µg ml^{-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 storage pests were collected from the stock culture maintained at the Insect Microbiology Laboratory, ICAR-CTCRI, Thiruvananthapuram 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

The 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.

Phenotypic characterization of ESB

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 100X 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

The PCR amplification of 16S rDNA gene by universal primers: forward primer fD1 5'AGAGTTTGATCCTG GCTCAG3' and reverse primer RP2 5'CGGCTACCTTGTTACGA CTT3' (Weisburg et al., 1991) were used. The PCR was performed in a 25 μl reaction mixture having 2.5 μl of 10X Taq buffer A (containing 15 mM $MgCl_2$, 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 visualized under UV transilluminator and the purified PCR products of 1500 bp were sequence d at Eurofins Genomics India Pvt. Ltd., Bengaluru, Karnataka, India.

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 storage pest

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).

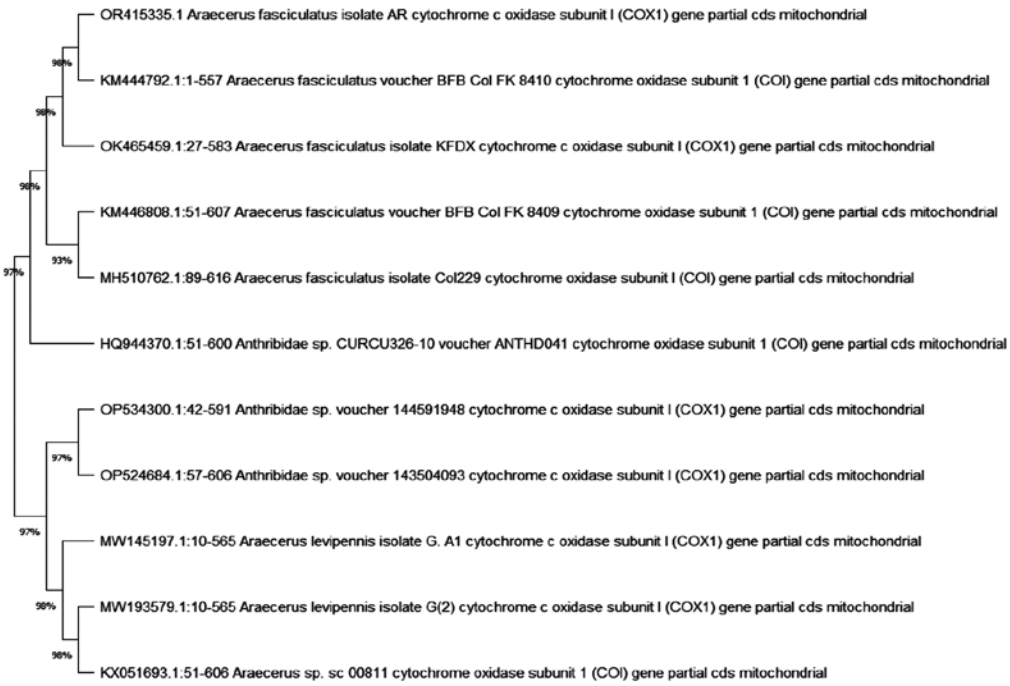

Fig. 1. Phylogenetic tree inferred from mitochondrial cytochrome oxidase (COX-1) sequences analysis of Araecerus fasciculatus isolate AR

Phenotypic and molecular characterization of bacterial isolates

Bacterial symbionts were isolated from the *Chiridopsis* sp. and were assigned code number as isolate A1. Morphological variations were observed for each endosymbiotic bacterial strains, but no pigmentation was observed. Colonies formed on nutrient agar were circular, raised, convex, flat, entire white in colour, gram positive, rod-shaped with no pigmentation. 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 amplified products were sequenced. BLAST analysis of the sequences of the isolate showed 98% similarity to *Bacillus megaterium* 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).

The taxonomy of *Bacillus* had been recently changed and *Bacillus megaterium* was renamed as *Priestia megaterium*, which is a new separate genus from *Bacillus* (Gupta et al., 2020). Earlier studies have reported *P. megaterium* as a potential biological control agent, with antimicrobial activities and against plant pathogens (Alvarez et al., 2012; Yang, 2019; Nair and Radhakrishnan, 2021). It is also reported that P. *megaterium* can be isolated from various plants, such as alfalfa, black pepper, carrot, clover, cotton, cucumber, potato, wheat, ginseng, dendrobium, and *polygonatum sibiricum* (Afzal et al., 2019; Kłosowski

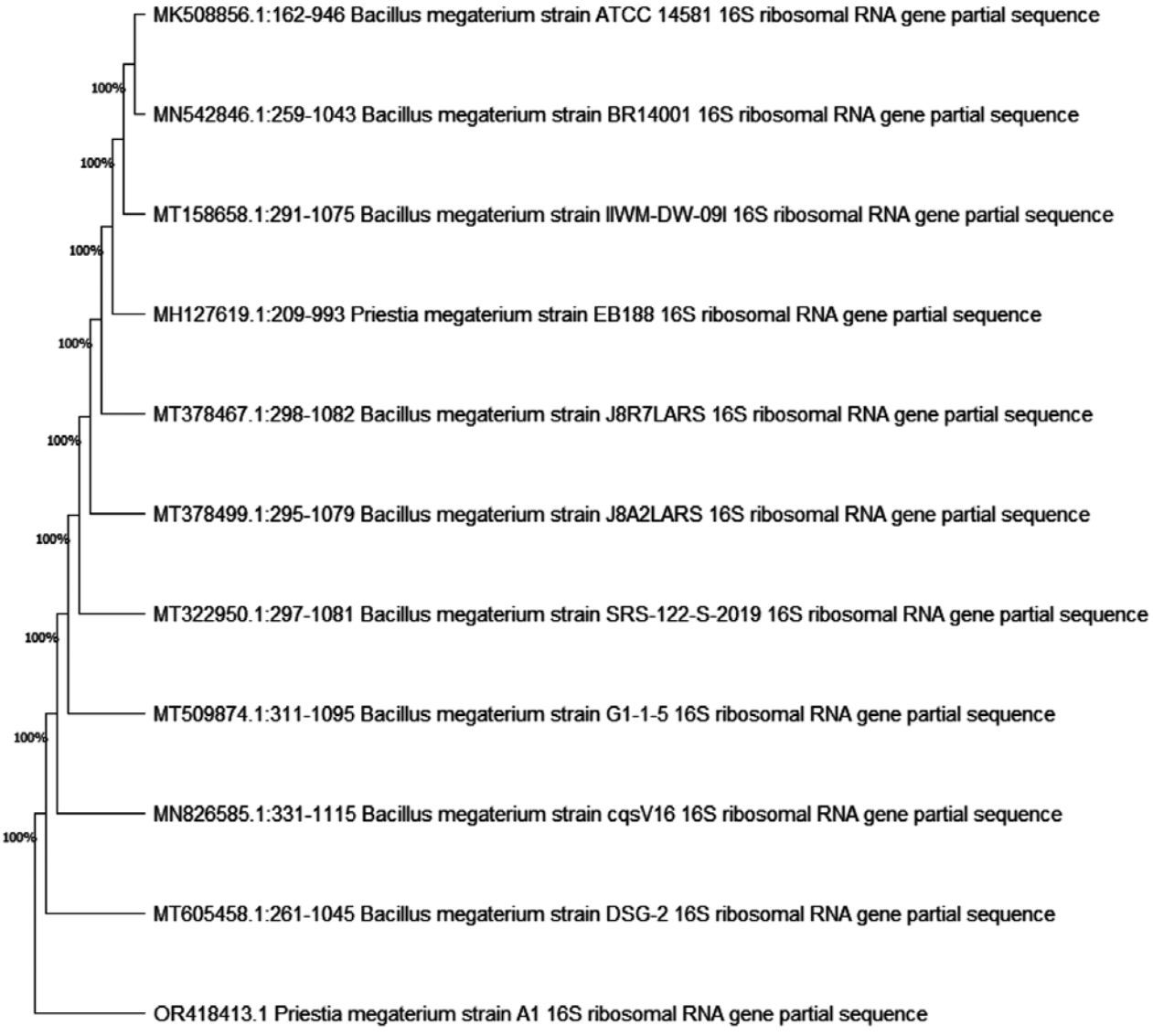

Fig. 2. Phylogenetic tree inferred from 16S rRNA gene sequences analysis of bacterial isolate A1 associated with Araecerus fasciculatus

et al., 2021; Rajan et al., 2021). In our study also, *P. megaterium* was found to be the endosymbiotic bacterium which was associated with the *A. fasiculatus.*

Table 1. Molecular identification of storage pest and endosymbiotic bacteria

Isolate	Identification	Accession No	Similarity (%)
A1	16S ribosomal RNA gene sequence, partial *Priestia megaterium* isolate A1	OR418413	100
AR	Mitochondrial cytochrome c oxidase subunit I (COX1) gene sequence partial *Araecerus fasciculatus* isolate AR	OR415335	98

Salama et al., (2004) have reported the different bacterial isolates *viz.*, *Bacillus sphaericus*, *B. megaterium* and *B. laterosporus*, from infected red palm weevil, *Rhynchophorus ferrugineus. B. megaterium* is also marketed as a crude biofertilizer and studies revealed the ability of the bacteria to promote growth and reduction of diseases in different Indian cultivable plants (Chakraborty et al., 2006). It has also been reported that the bacteria have a probiotic effect on many animals (Jones et al., 2006; Otero et al., 2006). Previous studies also showed the presence of *B. megaterium* from larval tissues of *Ostrinia nubilalis* (Hübner) (Secil et al., 2012). Similar studies also showed the association of *B. megaterium* with lac insects *Kerria lacca* (Kerr) (Gulsaz et al., 2017). *B. megaterium* and organisms belonging to the *B. alvei-B. thiaminolyticus* spectrum were one of the most frequent isolates associated with frass from feral honey bee colonies (Gilliam, 1985). Prvious studies also reported the association of *B. megaterium* with different species of sand flies viz *Phlebotomus papatasi, Lutzomyia evansi, Phlebotomus argentipes* (Vivero et al., 2016; Hillesland et al., 2008; Gunathilaka et al., 2019; Dillon et al., 1996; Mukhopadhyay et al., 2012).

Conclusion

The endosymbiotic bacteria can serve as targets for future functional analysis of diverse genes which are responsible for the various functions within each life stage across different developmental stages of insects. The identification of these bacteria will help to detect the pathway of insect-host interactions and thereby it will help to develop novel pest control strategies.

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