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Molecular identification of endosymbiotic bacteria associated with *Ferrisia virgata* (Homoptera: Coccoidea: Pseudococcidae) infesting cassava (*Manihot esculenta*)

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Abstract

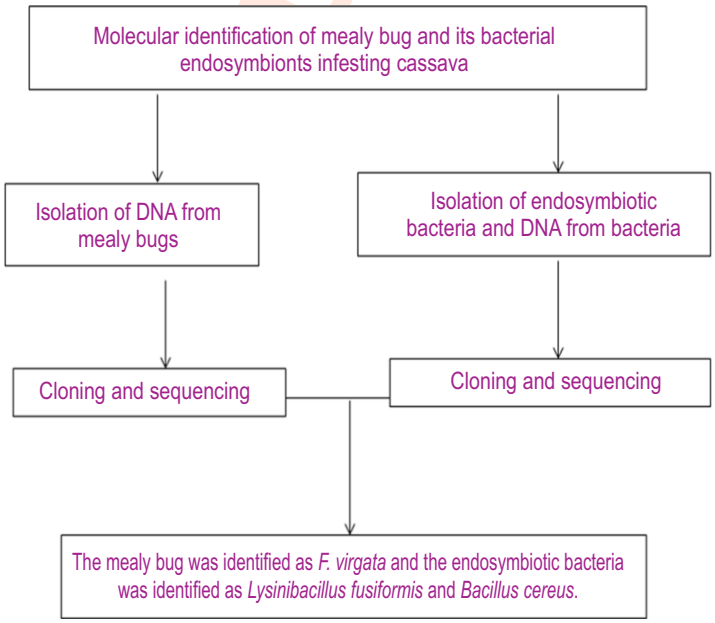
Aim: To identify the mealy bug samples collected from cassava plants and identification of endosymbiotic bacteria associated with the mealy bugs

Methodology: Molecular identification of mealy bugs was done using mitochondrial cytochromeoxidase (COX-1) C1-J-2183 F(CAACATTTATTTTGATTTTTTGG) and CI-N-2568R (GCWACWACRTAATAKGTATCATG) primers. The molecular identification of bacteria was done using 16S rRNA gene universal primers

Results: The mealy bug was identified as *F. virgata* and the endosymbionts associated with mealy bugs were identified as *Lysinibacillus fusiformis* and *Bacillus cereus*.

Interpretation: The endosymbionts associated with the mealy bugs play significant role in completing lifecycle of insects host and for providing nutrients to the host insects.

Key words: Cassava, Endosymbionts, *Ferissia virgata*, Mealybug



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Introduction

Mealybugs (Hemiptera: Pseudococcidae) are small soft bodied insects which feed on more than 200 plant species of fruits, vegetables, grasses and ornamental plants (Miller *et al.,* 2002). Infestation of plants causes wilting, distortion, yellowing of leaves. (Kosztarab and Kozar, 1988; Nagrare *et al.,* 2009). The mitochondrial cytochrome oxidase I (COI) gene is widely used for the identification of insects (De Barro *et al.,* 2011; Rao *et al.,* 2011; Dan *et al.,* 2019). *Candidatus Tremblaya princeps,* is a primary endosymbiont found nearly in all mealybug species (Baumann and Baumann, 2005; Downie and Gullan, 2005). In the subfamily Phenacoccinae, primary endosymbiont *Tremblaya phenacola,* is supplemented with a second bacterial endosymbiont *Moranella endobia* (Husnik *et al.,* 2013; Gruwell *et al.,* 2010). Some of the endosymbionts associated with mealy bugs produce enzyme to detoxify insecticides (Munson *et al.,* 1992; Kantheti *et al.,* 1996).

Previous studies have reported isolation of these endosymbiotic bacteria with the characters for bioremediation of insecticide, polyethylene and waxes (Gatehouse *et al.,* 2012; Kantheti *et al.,* 1996; Munson *et al.,* 1992). Ibrahim *et al.* (2021) reported the degradation of chlorpyriphos and polyethylene by endosymbiotic bacteria *viz Bacillus licheniformis, Pseudomonas cereus, Pseudomonas putida, Bacillus subtilis* from citrus mealybug (*Planococcus citri*). The secondary endosymbionts associated with the insect hosts are not generally required for growth or reproduction, but they are considered as secondary or facultative endosymbionts which can live inside bacteriocytes or in body cavities (Baumann and Baumann 2005; Moran *et al.,* 2008). The endosymbionts inhabiting in the insect gut supply necessary nutrients, help to defense against predators and inter-communication between host and environment. As they play an important role in altering the life cycle of insect hosts, therefore, study regarding the identification of secondary endosymbionts is of great importance to reveal various functions of the bacteria associated with them. In view of the above, this study was conducted to isolate and identify the endosymbionts associated with striped mealy bug *Ferrisia virgata* to understand their diverse roles as insect host.

Materials and Methods

Identification of insects and endosymbiotic bacteria: Mealybugs samples were collected and maintained in cassava plants inside insect proof cage at the Entomology Laboratory, ICAR-Central Tuber Crops Research Institute, Thiruvananthapuram (Fig. 4). The genomic DNA was isolated using the modified method of Gawel and Jarret (1991). The polymerase chain reaction (PCR) was carried out in a thermal cycler (BioRad, Veriti 96 wells). The primers used were specific to mitochondrial cytochrome oxidase (COX-1) C1-J-2183F (CAACATTTATTTTGATTTTTTGG) and CI-N-2568R (GCWACWACRTAATAKGTATCATG) (Brady *et al.,* 1990; Simon *et al.,* 1994). PCR amplified fragments were eluted using Nucleospin® Extract II (Thermoscientific, USA) and ligated to T/A cloning vector, (PTZ57R/T) InsT/Aclone kit (Thermoscientific, USA). Plasmids were isolated from the overnight culture of white colonies using GeneJET™ Plasmid Miniprep Kit (Thermoscientific, USA). The plasmids isolated from white colonies were sent for sequencing. The homologies of sequences were analyzed using BLAST (http://www.ncbi.nlm.nih.gov). The phylogenetic tree was constructed using MEGA 11 software (Tamura, 2021). Adult mealy bugs were collected and the endosymbiotic bacterias' (ESB) were isolated following the protocol of Sreerag *et al.* (2014). The shape, margin, elevation and Gram stained images of each isolates were observed under a stereomicroscope (Leica DMLB compound microscope) at 100x magnification. PCR amplification of 16S rDNA gene was carried out using universal primers (Weisburg *et al.,* 1991). The reaction was carried out in a Biorad thermal cycler and the PCR amplified fragments (1500bp) were purified using Nucleospin® Extract II (Thermoscientific, USA) and ligated using (PTZ57R/T) InsT/Aclone kit. Plasmids were isolated from the overnight culture of white colonies using Gene JET™ Plasmid Miniprep Kit (Thermoscientific, USA). The plasmids were sent for sequencing. The nucleotide sequences were compared with those in the NCBI databases using (http://www.ncbi.nlm.nih.gov/ BLAST). The phylogenetic tree was constructed by the Neighbor-Joining method using MEGA11 software (Tamura, 2021).

Results and Discussion

The amplification of COX-1 gene of CAMB isolate was carried out and the amplicon product size was 384 bp. All the sequences obtained were aligned using BioEdit. BLAST analysis of mealybug isolates CAMB showed 100% similarity to Coccoidea: Pseudococcidae *F. virgata* (Homoptera) 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 phylogenetic tree of mealybug *Ferrisia virgata* based on Mt (COX1) gene sequences is shown in Fig. 1. The mealy bug isolate associated with cassava in this study showed 100% similarity with the *Ferrisia virgata* isolate Kn28bMT deposited in NCBI database with accession no MK090646.1. The complexity in morphological identification can be overcome by the molecular identification of mealy bugs which is quicker, cheaper and more reliable method. With the aid of DNA marker regions viz mitochondrial cytochrome oxidase subunit I (COI) gene, leuA-16S region of primary endosymbionts and internal transcribed spacer 2 (ITS2) the molecular identification of mealy bugs can be done accurately (Silva *et al.,* 2014).

Recently, Choi and Lee (2022) also reported the identification of mealybugs using nuclear ribosomal RNA, mitochondrial (COI) and nuclear protein-encoding genes and the molecular identification of *Dysmicoccus neobrevipes* was also done by using mtCOI gene (Nurbaya *et al.,* 2022). *Crisicoccus matsumotoi* and *Planococcus kraunhiae* was identified using molecular methods by using ITS1 and 2 (Park *et al.,* 2010). A total of two bacterial strains were successfully isolated from the *Ferrisia virgata* and

Table 1: Identification of mealy bug and endosymbiotic bacterial isolates

Isolates	Identification	Accession No	Similarity (%)
CAMB	*Ferrisia virgata* isolate Kn28bMT cytochrome oxidase subunit I gene, partial cds, mitochondrial (MK090646.1)	OR392288	100
CAM2	*Lysinibacillus fusiformis* strain CSR-A-11 16S ribosomal RNA gene, partial sequence (KU745624.1)	OR388567	99
CAB2	*Bacillus cereus* 16S ribosomal RNA gene, partial sequence (KY777711.1)	OR388567	98

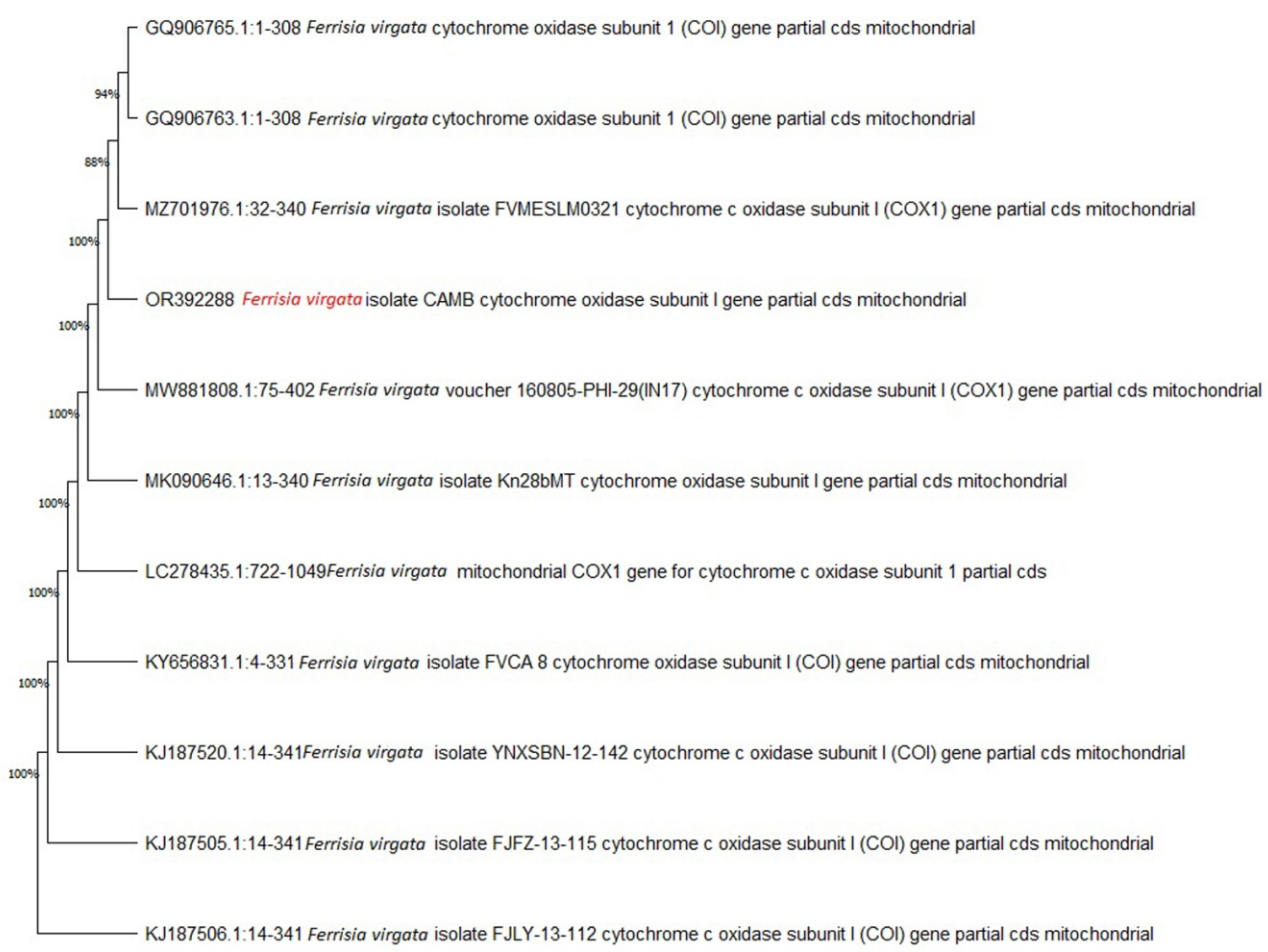

Fig. 1: Phylogenetic tree of mealy bug isolate CAMB from mitochondrial cytochrome oxidase (COX-1) sequences.

assigned code numbers as CAM2, CAB2 (Fig 5a,b). BLAST analysis of isolates CAM2 and CAB2 showed 100% similarity to *Lysinibacillus fusuformis* and *Bacillus cereus* available in the GenBank. The sequence data was deposited in the GenBank nucleotide database (NCBI) and the accession numbers assigned are given in Table 1. The phylogenetic tree of the endosymbiotic bacteria based on 16S rRNA gene sequences is shown in Fig. 2-3. The bacterial isolates identified in this study showed 99% similarity to *Lysinibacillus fusiformis* strain CSR-A-11 16S with accession no KU745624.1 and 98% similarity to KY777711.1. Previous studies have also reported the degradation of polythene/paraffin/hydrocarbons by *Bacillus* species (Yang *et al.*, 2014), and also the association of *Bacillus cereus*, from citrus mealybug *Planococcus citri* with paraffin wax degrading properties (Ibrahim *et al.,* 2020).

According to a study conducted by Jose *et al.* (2020), endosymbiont communities vary between *P. marginatus* and *F. virgata*. *Bacillus* spp. is reported as one of the most predominant

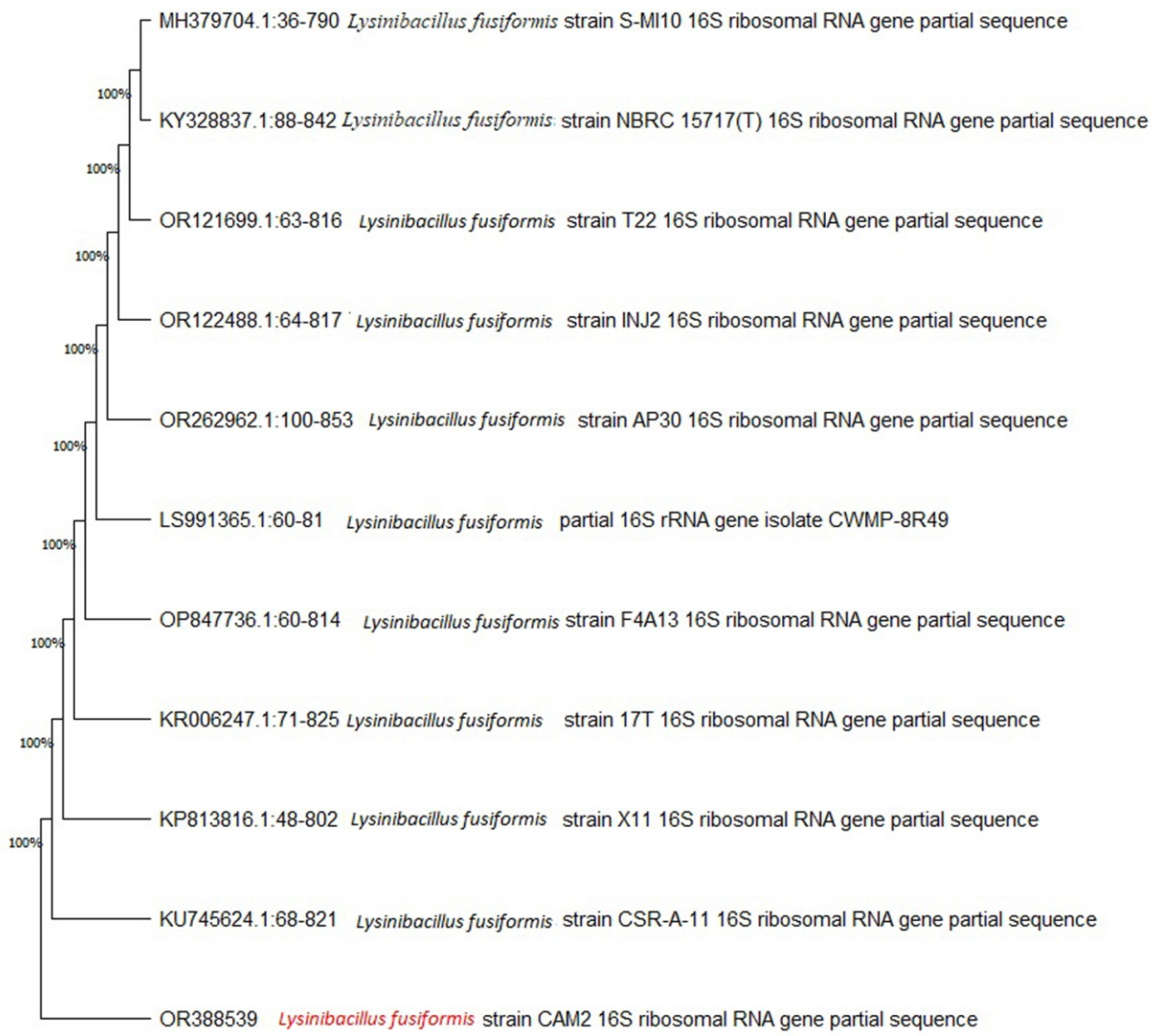

Fig. 2: Phylogenetic tree of endosymbiotic isolate CAM2 from 16S rDNA sequences.

group associated with the insects. They frequently inhabit in the gut of several mosquito species from different environments (Takahiro *et al.,* 2006). This bacteria play an important role on the growth, reproduction and immune response of the host insect. Earlier studies have also reported *Bacillus cereus* as mutualistic organism associated with various insects (Margulis *et al.,* 1998). These bacteria are naturally found in the gut of several insects and the intestines of isopods are colonized by bacteria (Jenson *et al*., 2003). They are supported by an inbuilt genetic mechanism for their establishment within the insect hosts. Jenson *et al.* (2003) reported the distinct lifecycles of these bacteria *viz* symbiotic, pathogenic and saphrophytic. Studies have elucidated presence of *B. cereus* for utilizing carbon from insecticides (Ramya *et al.,* 2016). The presence of *B. cereus* in the gut of *E. grisescens* and their involvement in the host lipid metabolism was first reported by Li *et al.* (2022). The population of *Bacillus* in the gut increased significantly and with starvation of the insect host. *Bacillus cereus* isolated from the mid gut of *Anopheles subpictus* play a significant role on the larval development and survival (Mukhopadhyay and Chatterjee, 2016). The endosymbiotic bacteria associated with the midgut of *Drosophila ananassae* was reported to have significant role in their lifecycle.

Moreover, Plearnpis *et al.* (2001) reported the presence of *B. cereus* isolates in the mosquito larval gut. Mortality of larvae

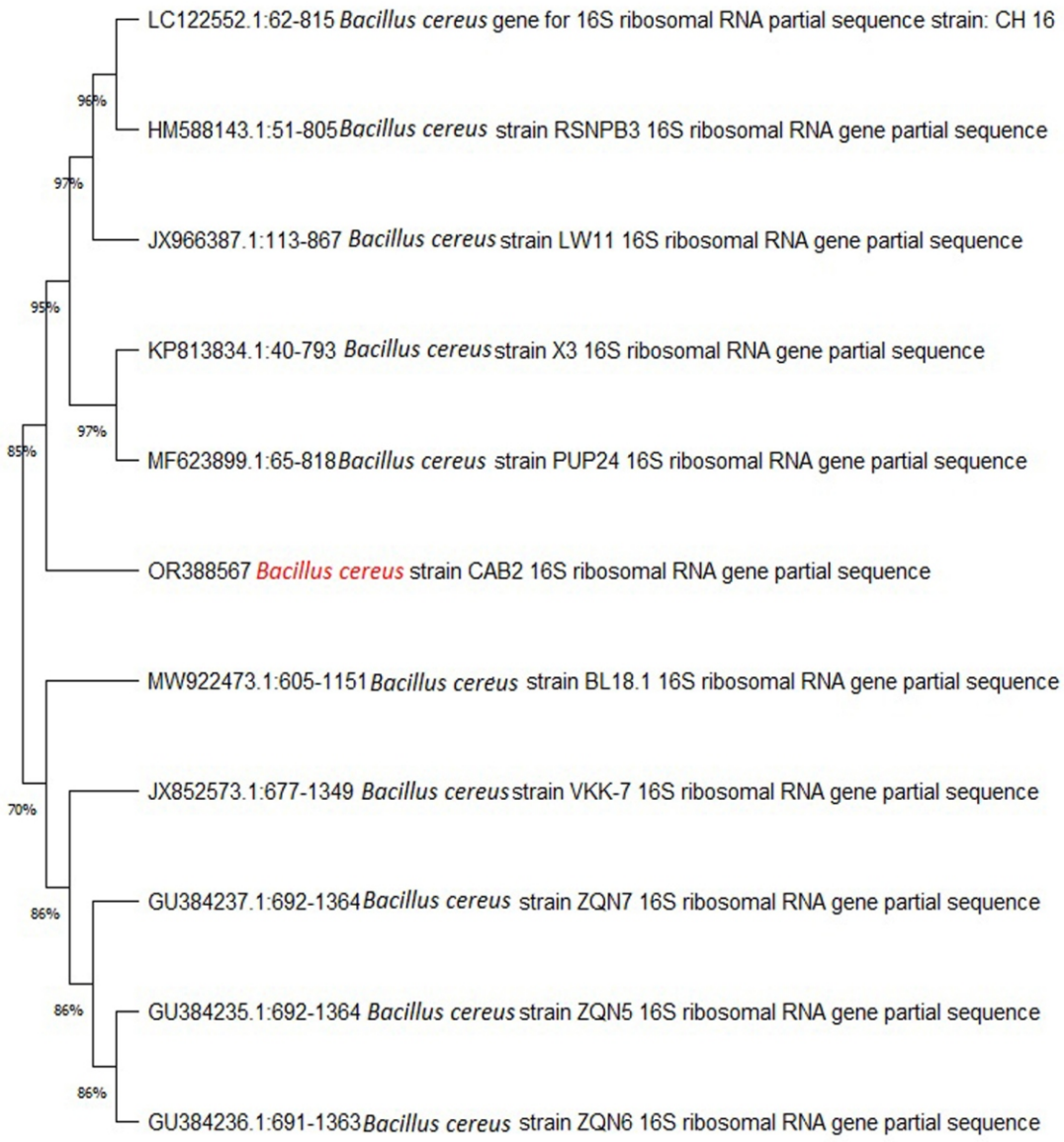

Fig. 3: Phylogenetic tree of endosymbiotic isolate CAB2 from 16S rDNA sequences.

gypsy moth was reported by Broderick *et al.* (2000) with coinfection of *Bacillus thurigiensis* and *B. cereus* due to the production of an antibiotic, zwittermicin A. *B. cereus* usually inhabit digestive tract of sow bug (Jensen *et al.,* 2003) and few studies have shown the symbiotic colonization of these bacteria in the digestive tract of arthropods (Margulis *et al*., 1998). In few arthropods, long filamentous chain named (Arthromitus) were formed in the digestive tract (Margulis *et al.,* 1998). In the present study also, the association of bacterial isolate CAB2 with *F. virgata* showed the symbiotic association of these bacteria within the insect body.

Tago *et al.* (2014) reported the biodegradation of organophosphorus pesticides by the endosymbiotic bacteria *Burkholderia* associated with stinkbugs. The studies on the degradation of paraffin wax, chlorpyriphos and polyethylene by endosymbiotic bacteria has been reported by (Blanton and Peterson, 2020, Ibrahim *et al*., 2021), and in our study also *B. cereus* was identified as one of the endosymbionts from *F. virgata*. Recently, Sangeetha *et al.* (2023) reported the metagenomic DNA analysis of bacterial communities associated with striped mealybugs, *Ferrisia virgata* (Cockerell) (Homoptera: Pseudococcidae) infesting cassava. Molecular identification

Fig. 4: Mealy bug (*Ferrisia virgata*) infesting cassava leaves.

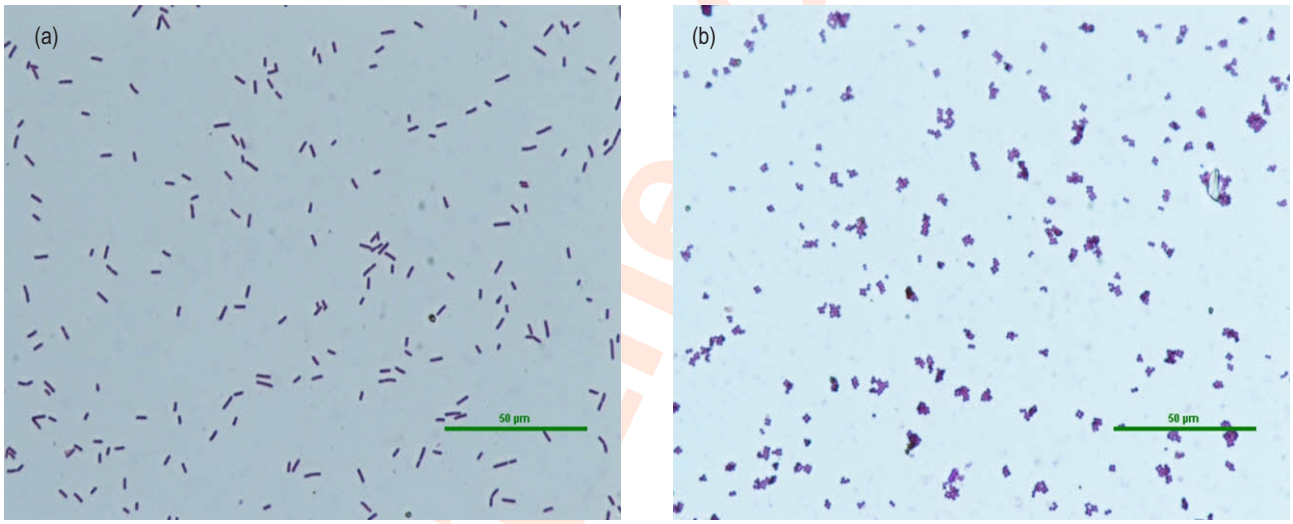

Fig. 5: (a) Gram stain of ESB isolate CAM2 and (b) ESB isolate CAB2.

showed that both the bacterial isolates were identified as predominant bacteria associated with the striped mealy bug. Previous studies on the endosymbiotic bacteria of *Phenococcus solenopsis* reported the presence of uncultured bacteria Firmicutes, Bacteriodetes and Actinobacteria (Padmanabhan *et al*., 2019). this study also revealed the association of *B. cereus* with *F. virgata.*

The beneficial role of Firmicutes in degradation of cellulose and hemicellulose in insects has been reported by Brown *et al.* (2012). Likewise antibiotic susceptibility of bacterial endosymbionts viz *Bacillus cluasii, B. altitudinis, B. siamensis,* Gram-negative bacteria, Serratia marcescens and *Stenotrophomonas maltophilia* of papaya mealybug *Paracoccus marginatus* has been documented by Megaladevi and Kennedy (2021). Whereas *B. pumilus* was found associated with the gut of mealybug, which produce high amount of physiologically active gibberellins and cellulose enzyme (Manero *et al.,* 2001; Ariffin *et al.,* 2006). The association of *B. subtilis, Staphylococcus gallinarum* and *Staphylococcus saprophyticus* with the mealy bug *Rhizoecus amorphophalli* has been eluciated by Sreerag *et al.* (2014). *Lysinibacillus* was earlier included within the genus *Bacillus* and reclassified to the new genus based on unique morphological characters and 16S rRNA sequences (Ahmed *et al.*, 2007). Few *L. fusiformis* were reported as biocontrol agents against wide range of plant pathogens (Ahmed *et al.,* 2014).

Zhang *et al.* (2022) reported that *L. fusiformis* found in the the gut of housefly larvae enhanced the development of growth stages of larvae. Recently, *Lysinibacillus fusiformis* was isolated for the first time from red soil and was found effective in controlling mosquito vectors (Vijayakumar *et al.,* 2023). Similarly, in this study also the isolate CAM2 identified as *L. fusiformis* might have plyaed a role to promote different growth stages in mealy bugs. *L. fusiformis* bacteria was also isolated from the soil nematodes (Loulou *et al.,* 2023). Recent studies of Loulou *et al*., 2023 have also reported the asscociation of Lysinibacillus fusiformis with nematodes. Recently the plant growth promoting activity of *Lysinibacillus* pp. was reported by Guerra *et al.* (2023). *Lysinibacillus* spp. has been reported to possess bioremediation potential, also the beneficial effects of these isolates in enhancing plant growth (Ahsan and Shimizu, 2021) has been demonstrated.

It can be concluded from this study that the association of secondary endosymbionts are always beneficial to the mealy bugs, which provide protection from invading pathogens, predators, pesticide detoxification and supply essential nutrients. The endosymbiotic bacteria have a significant influence in different growth stages of the insect host and can be used as one of the factor for studying the lifecycle of the same. The application of this study will lead to efficient use of these endosymbionts as biocontrol agents against mealy bugs.

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