Detection of specific endosymbionts of mealybugs infesting cassava (*Manihot esculenta* Crantz) using diagnostic PCR and Sanger sequencing

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Abstract

Cassava is one of the most produced food materials and constitutes the major energy source for about 800 million people worldwide. Mealybugs have been reported to be the main concern in cassava production for the past few years in all the major tuber cultivated ecosystems of India. Endosymbiotic bacteria found in mealybugs have been observed to interact and provide specific advantages to the host and they can be one of the reasons for the pests' insecticidal resistance. In the present study, endosymbiotic bacteria were isolated from cassava mealybugs, allowed to grow in media after insecticidal treatments, and after observing colony morphology, distinct bacterial colonies were sub-cultured and selected for further molecular identification procedures. PCR analysis was carried out for bacteria using 16S rRNA primer, with an annealing temperature of 49^{o}C, and yielded fragments at 1500 bp. Based on the sequencing report, the observations were that the culturable endosymbionts *Pseudomonas oryzihabitans, Staphylococcus* sp. which are resistant to the insecticide thiamethoxam are present in *Paracoccus marginatus,* and malathion resistant *Paenibacillus alvei, Ralstonia* sp., and thiamethoxam resistant *Clostridium lundense* are present in *Ferrisia virgata*. The sequences were submitted to NCBI, and the accession numbers obtained were OP572218, OP572193, OP572215, OP572194, and OP572097, respectively. Also, through diagnostic PCR using specific bacterial primers, the presence of *Wolbachia* is confirmed in mealybug *F. virgata* at a fragment length of 650 bp.

Keywords: Cassava mealybug, Diagnostic PCR, *Ferrisia virgata*, Insecticide resistant endosymbiotic bacteria, *Paracoccus marginatus.*

Introduction

Cassava (*Manihot esculenta* Crantz), the perennial crop belonging to the family Euphorbiaceae, together with maize and rice constitutes the major source of energy and carbohydrates for the people in tropics and subtropics (Ceballos et al., 2012). In Africa, the Indian sub-continent, Latin America, and several Southeast Asian countries, cassava is one of the major root crops and acts as a tropical food crop (Karthikeyan et al., 2016). Various biotic factors (Abaca et al., 2014) considerably reduce the crop's yield potential; among them, mealybugs are one of the most important (Harish et al., 2022).

Cassava mealy bugs (Family: Pseudococcidae) sucks sap from cassava stems, petioles, and leaves and inject a toxin that causes leaf curling, slow shoot growth, and eventual leaf withering (FAO, 2013). They are small, soft-bodied, sap sucking insects that constitute the second-largest family of scale insects (Mamoon-ur-Rasheed et al., 2014). As of now, *Paracoccus marginatus* Williams and Granara de Willink, and *Ferrisia virgata* Cockerell are the most important mealy bugs found attacking cassava in India, especially Kerala. *P. marginatus* is native of

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Central America/ Mexico and infests more than 60 species of plants spread over 50 countries. In 2002, it was reported from the Pacific Islands (Meyerdirk et al., 2004, Muniappan et al., 2006); subsequently from Indonesia, India, and Sri Lanka (Muniappan et al., 2008). *F. virgata* is one of the most highly polyphagous mealybugs, attacking plant species belonging to some 203 genera in 77 families (García et al., 2016). The striped mealybug has achieved economic significance as a pest of several agricultural crops, including cassava (Oliveira et al., 2014, Hodges, 2017).

Insects commonly exhibit a symbiotic relationship with bacteria, and they provide several benefits to their host. These are called endosymbionts. Endosymbionts are organisms that live within the body or cell of another organism in a mutualistic relationship (Mergaert, 2018). Many sap-sucking insects live in close association with bacterial endosymbionts (Baumann, 2005). Mealy bugs have both primary endosymbionts (P-endosymbionts) and secondary endosymbionts (S-endosymbionts). The P-endosymbionts are proteobacterial β subdivision members and S-endosymbionts are α - subdivision (Von Dohlen et al., 2001). A beta proteobacterial endosymbiont *Tremblaya princeps* and an additional gammaproteobacterial endosymbiont were present in many species of sub family Pseudococcinae (Fukatsu and Nikoh, 2000). The primary endosymbiont *T. princeps* was found to exhibit host symbiont co-speciation (Downie and Gullan, 2005). In contrast, *Moranella endobia* and other secondary gammaproteobacterial endosymbionts are of polyphyletic evolutionary origins (Thao et al., 2002). According to Jose et al. (2020), the endomicrobial community differs significantly between cassava mealybugs, *Paracoccus marginatus*, and *Ferrisia virgata*. Cotton mealybug, *Phenacoccus solenopsis* acquired from 10 different sources in India showed the presence of important secondary endosymbionts *Cardinium, Rikettsia, Wolbachia,* and *Arsenophonus* (Singh et al., 2012). The presence of *Wolbachia* is also reported in hibiscus mealybug, *Maconellicoccus hirsutus* (Husnik & Filip, 2016).

Even though, chemical insecticides provide reasonable control against mealybugs, repeated use can be ineffective, and endosymbionts present in the insects can be one of the reasons for that. They have already been reported to provide insecticidal resistance to insects (Kikuchi et al., 2012). The objective of present study was to identify specific endosymbionts of cassava mealybugs, and the strategy was- without employing costly NGS (Next-Generation Sequencing) approach- combining Sanger sequencing for the identification of culturable endosymbionts and diagnostic PCR with specific primers for the important unculturable ones.

Materials and Methods

Common mealybugs infesting cassava in Kerala, *Paracoccus marginatus* and *Ferrisia virgata* (Hemiptera: Pseudococcidae) were collected from different cassava fields in Kerala (10.8505° N, 76.2711° E) during March-December 2021. Selected mealybugs were transferred to sterile Eppendorf tube (2ml) containing 0.01 % streptomycin as a surface sterilizer. Mealybugs were removed from the tube containing antibiotics after one minute, and then transferred to another Eppendorf tube containing sterile distilled water. Thorough washing was done with sterile distilled water twice. After surface sterilization, mealybugs were transferred to a tube containing 2ml nutrient broth. Mealybugs were crushed with a micro pestle and incubated at room temperature overnight in a rotating shaker. After one overnight incubation, one ml of culture from each sample was taken, and serial dilution was performed until 10^{-6} dilution was obtained.

From the results obtained from a field study on the effectiveness of insecticides on the mealybug population, the most effective and commonly used systemic and contact insecticides (thiamethoxam and malathion, respectively) were used in the subsequent study. Nutrient agar media was prepared

by dissolving 28 g of nutrient agar in 1000 ml of distilled water. The media were autoclaved at 121°C temperature and 15 lbs pressure for 20 minutes. Under bearable temperature, insecticides thiamethoxam 25%WG and malathion 50% EC, 0.3 g L^{-1} and 2 ml L^{-1} were respectively added to separate flasks containing the nutrient agar media. Each culture sample was plated on labelled nutrient agar media under different treatments. After sufficient incubation, the plates were observed for bacterial colonies. Bacterial culture took minimum 24 hours for growth. Different colonies were streaked separately into new agar plates. The pure cultures were maintained in nutrient agar slants. The colony morphology of bacteria of each plate was observed under a stereo microscope. The Gram staining was performed using a Hi-Media kit (Hi-Media Laboratories Pvt. Ltd., India) according to the manufacture's protocol. The results were observed using Leica DMLB compound microscope (100x).

For molecular identification of promising-sub cultured bacteria, 1.5 ml of overnight grown bacterial cultures were taken in centrifuge tubes, and the contents were centrifuged at 12000 rpm for 10 minutes. The supernatants were discarded, and the pellets were suspended in 400µl TE buffer. After vortexing, 50µl of 10% SDS was added, and the contents were mixed by using a pipette. 500µl of phenol: chloroform: iso-amyl alcohol (25:24:1) was added and mixed well. The contents were centrifuged at 12000 rpm for 10 minutes and carefully collected the supernatant into a fresh tube, 500µl chloroform was added, mixed well, and centrifuged again at 12000 rpm for 10 minutes. DNA was precipitated after adding 25µl of 5M NaCl and 1 ml of 95% ethanol and incubated overnight at -20°C. Next day, centrifuged for 10 minutes at 12000 rpm, the supernatant was discarded, and pellets were washed with 70% ethanol. The contents were centrifuged for 10 minutes at 12000 rpm. The pellets were air dried at 37°C for 30 minutes, dissolved in water (30µl), and kept in a refrigerator at -20°C for storage.

Agarose gel electrophoresis was performed based on the method described by Sambrook et al. (1989) to check the quality of DNA. Also, the purity of DNA was checked using NanoDrop spectrophotometer (model–NanoDrop–1000, Thermo Scientific™). PCR was done using 25µl reaction mixture, which contains Thermo–scientific master mix (2X)– 12.5µl, distilled PCR water–9.5 µl, forward primer (16 S F- 10 µmol/L)–0.5 µl, reverse primer (16S R- 10 µmol/L)–0.5µl and DNA template (20 ng/µL) – 2µl. PCR conditions were set as following: initial denaturation of -92°C for 2 min 10 sec, 35 cycles of denaturation at 94°C for 1 min 10 sec, annealing temperature of primer at 49°C for 30 sec, extension at 72°C for 2 min, final extension at 72°C for 10 min and with a holding temperature of 4°C. PCR products were gel-purified using kit (Thermo Scientific™) and sent for sequencing. Sequencing was carried out in an automated sequencer (ABI Prism 310; Applied Biosystems, USA) using universal primers in both directions. The nucleotide sequence obtained was processed to remove low-quality reads and transformed into consensus sequences with Geneious Pro software version 5.6. The resulting high-quality sequences were analyzed with BLASTn (NCBI; http://www.ncbi.nlm.nih.gov) to confirm the authenticity of the isolate and the sequences obtained were submitted to the NCBI database.

For diagnostic PCR to confirm the presence of unculturable bacterial endosymbionts in mealybug samples, DNA was isolated from mealybugs using DNeasy blood and tissue kit (Qiagen®), followed the manufacturer's protocol and obtained good quality DNA. DNA of mealybug populations were diagnosed for the presence of different bacterial endosymbionts *Wolbachia, Rickettsia, Arsenophonus* and *Cardinium*. Specific bacterial primers were used for amplification of 16S rRNA bacterial gene (Subramanian et al., 2019). For each bacterial endosymbiont, PCR Master mix RTU 12.5µL (EmeraldAmp Max PCR Mix), forward and reverse primers (1µL each-10 µM), DNA template

(5µL @ 20 ng/µL)) and the final volume of 25µL were prepared with nuclease free water. PCR reaction include: denaturation at 94°C for 30 seconds. Annealing was carried out at different temperatures specific for each bacterial endosymbionts (*Wolbachia* 52°C, *Arsenophonus* 52°C, *Rickettsia* 55°C, *Cardinium* 50°C) for 30 seconds. The extension was carried out at 72°C for 40 seconds, with the final extension for 5 minutes at the same temperature. The total number of cycles for PCR reaction was 45. Both positive and negative controls were used for each reaction. The plasmids containing 16S rRNA gene of different bacterial endosymbiont were used as positive controls, while the reaction without any DNA was used as negative control. The PCR products were then checked on 1.8% agarose gel, and the PCR products for different bacteria exhibited bands of different size.

Results and Discussion

Isolation of bacteria from mealybugs

From different treatments, endosymbiotic bacteria of the cassava mealybugs were cultured in agar plates. After observing colony morphology, distinct bacterial colonies were sub-cultured and selected (Table 1) for further molecular identification procedures. Similar to the present study, Qamer et al. (2003) and Sousa et al. (2013) also performed detailed works on bacterial colony morphology.

Molecular identification of bacteria

The quantity of DNA from different samples varied from 249 ng μl^{-1} to 2644 ng μl^{-1} and the A260/A280 ratio of DNA samples (quality) ranged from 1.72 to 1.98 indicating sufficient purity of isolated DNA for further PCR amplification. PCR analysis was carried out for bacteria using 16S rRNA primer with an annealing temperature of 49°C, and in gel electrophoresis amplicons were yielded at 1500 bp (Figure 1).

Based on sequencing report, the bacteria were identified using NCBI-BLAST. Thiamethoxam 25%WG (0.3 g L^{-1}) resistant endosymbiotic bacteria *Pseudomonas oryzihabitans* and *Staphylococcus* sp.

Table 1. Colony morphology of endosymbiotic bacteria

Treatment	Size	Shape	Margin	Elevation	Opacity	Pigmentation	Consistency	Gram staining
PM-TM-S1	Small	Irregular	Undulate	Umbonate	Translucent	Yellow	Moist	Negative
PM-TM-S2	Large	Circular	Entire	Raised	Opaque	Dark yellow	Shiny	Positive
PM-TM-S3	Large	Circular	Entire	Raised	Translucent	Cream	Shiny	Negative
FV-MT-S1	Small	Circular	Entire	Flat	Opaque	White	Moist	Positive
FV-MT-S2	Small	Circular	Entire	Raised	Translucent	Cream	Moist	Negative
FV-MT-S3	Large	Irregular	Undulate	Flat	Opaque	White	Dry	Positive
FV-MT-S4	Large	Irregular	Undulate	Raised	Opaque	Orange	Shiny	Negative
FV-MT-S5	Small	Circular	Entire	Flat	Translucent	Nil	Shiny	Negative

PM–*Paracoccus marginatus*, F.V.–*Ferrisia virgata*, T.M.–Thiamethoxam 25%WG, M.T.–Malathion 50%EC, S–Subculture

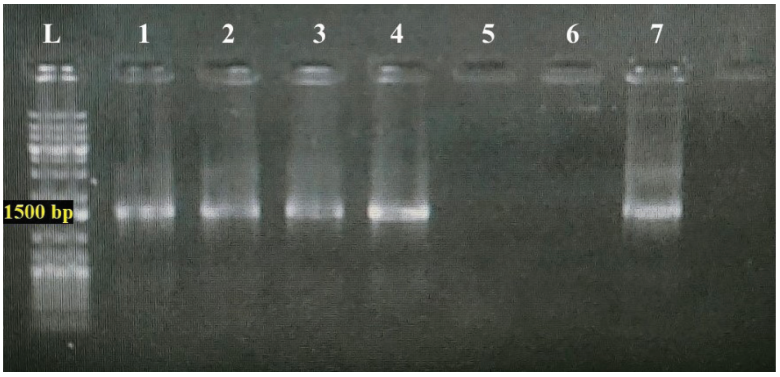

Figure 1. PCR analysis of bacterial isolates using 16S rRNA primers (L: 1Kb plus ladder, Lanes 1–4 and 7: bacterial endosymbionts of mealybugs)

Table 2. Endosymbiotic bacteria of mealybugs

Treatment	Organism identified	Percent identity with reference sample	NCBI accession number
PM-TM-S1	*Pseudomonas oryzihabitans*	92.09%	OP572218
PM-TM-S2	*Staphylococcus* sp.	88.09%	OP572193
FV-MT-S1	*Paenibacillus alvei*	96.15%	OP572215
FV-MT-S2	*Ralstonia* sp.	93.92%	OP572194
FV-MT-S3	*Clostridium lundense*	93.72%	OP572097

PM–*Paracoccus marginatus*, FV–*Ferrisia virgata*, TM–Thiamethoxam 25%WG, MT–Malathion 50%EC, S–Subculture

were identified from *P. marginatus*. Malathion 50%EC (2 ml L^{-1}) resistant *Paenibacillus alvei, Ralstonia* sp., and thiamethoxam 25%WG (0.3 ml L^{-1}) resistant *Clostridium lundense* were identified from *F. virgata*. The per cent identity with reference samples for these bacteria were 92.09%, 88.09%, 96.15%, 93.92%, and 93.72% respectively. The sequences were submitted to NCBI, and the accession numbers obtained are OP572218, OP572193, OP572215, OP572194, and OP572097 (Table 2).

Resistance development in insects, including mealybugs is one of the biggest threats to pest management strategies. Repeated application of similar insecticides helps insects to develop resistance. According to Kikuchi et al. (2012) endosymbionts can provide the resistance capability to host insects and they detailed the symbiont-mediated detoxification of fenitrothion by *Riptortus pedestris*. From the present study, we can see that the five mealybug endosymbionts are resistant against promising insecticides and may even contribute to the insects' insecticide detoxification capability.

According to Jose et al. (2020), endomicrobial community differs significantly between *P. marginatus* and *F. virgata*. Like in the present, study conducted by Megaladevi et al. (2020) also confirmed the presence of *Pseudomonas* and *Staphylococcus* in *P. marginatus*. Presence of *Pseudomonas* in *P. marginatus* is also reported by Abinaya et al. (2019). According to Saati-Santamaría et al. (2021), the bacteria assist bark beetles by supplying them with nutrients, shielding them from tree chemical defences, and antagonizing entomopathogenic fungi. Hashim et al. (2022) reported *Pseudomonas aeruginosa* in bioremediation of insecticidal (chlorpyrifos) toxicity. Similarly, Ibrahim et al. (2021) noted that the endosymbionts from citrus mealybug (*Planococcus citri*), *Pseudomonas putida* and *Bacillus cereus* are significantly efficient in the degradation of chlorpyrifos and polyethylene. The results of the study conducted by Rogowska-van der Molen et al. (2022) show that *Pseudomonas* sp. strain Nvir, from the insect gut system, can fully degrade nitropropionic acid (NPA)- a common, naturally occurring nitroaliphatic toxin produced by legumes and fungi. Leontopoulos et al. (2011) provided evidence that *Pseudomonas oryzihabitans* produces metabolites with nematostatic effects. *Pseudomonas* sp. composition and abundance correlated with brown planthopper survivability (Gupta et al., 2022).

Lin et al. (2019) reported the probable role of *Staphylococcus* in conferring insecticide resistance to mealybugs. *Staphylococcus* is already known to have a role in insects for the production of medium length sugars from sucrose (Indiragandhi et al., 2010). Presence of *Staphylococcus cohnii* and *Clostridium* spp. were identified in the long-tailed mealybug *Pseudococcus longispinus* by Popova et al. (2016). Sreerag et al. (2014) and Anusree Padmanabhan et al. (2019) identified the presence of *Staphylococcus* in mealybugs, *Rhizoecus amorphophalli,* and *Phenacoccus solenopsis,* respectively. Engel and Moran (2013) also testified the presence of endosymbiont *Clostridium* in the insect gut. *Paenibacillus* was identified from cassava as an endophyte (Menpara & Chanda., 2013), whereas *Paenibacillus alvei* is common in honeybee larvae (Anderson et al., 2011).

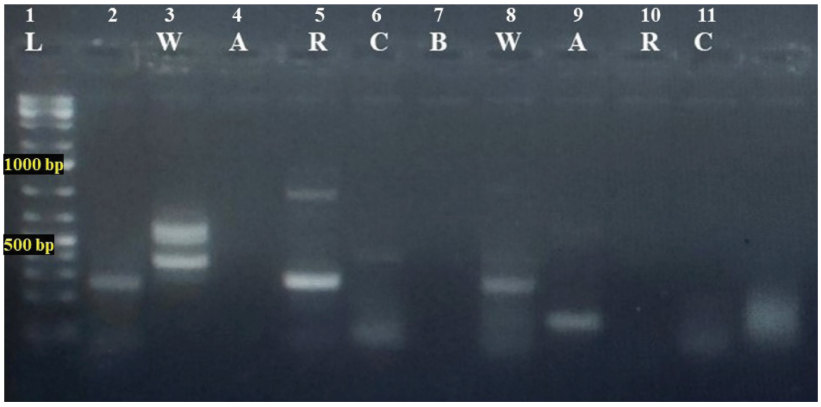

Figure 2. Diagnostic PCR for the presence of secondary bacterial endosymbionts in mealybug samples
Lane 3–6: bacterial endosymbionts of *F. virgata*; Lane 8–11: bacterial endosymbionts of *P. marginatus* (L- 1Kb plus ladder, *W- Wolbachia, A- Arsenophonus, R-Rickettsia, C- Cardinium,* B- Blank)

Diagnostic PCR for the identification of bacterial endosymbionts in mealybug samples

The quantity of DNA from *P. marginatus* and *F. virgata* were 57.91 ng μl^{-1} and 29.95 ng $\mu l^{-1,}$ respectively and were, with A260/A280 ratio of 1.98 and 1.82 (Figure 2).

In diagnostic PCR using specific bacterial primers, in accordance with the banding pattern suggested by Subramanian et al. (2019) for *Wolbachia, Arsenophonus, Rickettsia,* and *Cardinium*; presence of *Wolbachia* only is confirmed, and that was detected in mealybug *F. virgata*, at a fragment length of 650 bp (second band obtained in lane 3 and other bands in the gel image are non-specific bandings according to the standard list). The presence of secondary endosymbiont *Wolbachia* is already reported in many insects, including mealybugs, and Husnik and Filip (2016) informed its presence in mealybug *Maconellicoccus hirsutus*. Many studies were conducted to understand its role in insects. They probably help in resistance development of the host (Liu & Guo, 2019) and are described to cause cytoplasmic incompatibility in the parasitic wasp *Encarsia inaron* (White et al., 2009). According to Hosokawa et al. (2010) and Nikoh et al. (2014), *Wolbachia* strain associated with the bedbug *Cimex lectularius*, is essential for the host's growth and reproduction via provisioning of B vitamins. Studies by Teixeira et al. (2008) and Hedges et al. (2008) revealed that *Wolbachia* can protect *Drosophila melanogaster* against RNA viruses, whereas according to Bi and Wang (2020), *Wolbachia* impacts various behaviours of insect hosts. It was also observed that *Wolbachia* increases the fitness of insect host and provides some protection against parasitisation (Xue et al., 2012).

Various insecticide resistant endosymbiotic bacteria were detected in the present study in two important cassava mealybugs using conventional and molecular approaches. Also, diagnostic PCR technique was effectively utilized for the identification of an un-culturable endosymbiont in *F. virgata*.

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