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Validation of a species-specific *mtCOI* marker for the identification of cassava mealybug, *Phenacoccus manihoti* Matile-Ferrero (Hemiptera: Pseudococcidae)

JASTI SRI VISHNU MURTHY[1*], MANI CHELLAPPAN[1], RANJITH M.T.[1], SMITHA REVI[1], HARISH E.R. [2] and KIRAN A.G. [3]

[1]Department of Entomology, Kerala Agricultural University, Vellanikkara-680656, India.
[2]Division of Crop Protection, ICAR-Central Tuber Crops Research Institute, Thiruvananthapuram-695017, India.
[3]Centre for Plant Biotechnology and Molecular Biology, Kerala Agricultural University, Vellanikkara-680656, India.

***E-mail:** srivishnu.entomon@gmail.com

ABSTRACT: The invasive mealybug, *Phenacoccus manihoti* Matile-Ferrero (Hemiptera: Pseudococcidae), has emerged as a serious pest of the cassava crop, with its recent incursion into India prompting heightened concerns. This study validated a species-specific *mtCOI* marker (SS-*mtCOI*) to identify *P. manihoti* during its nymphal stage. A comprehensive survey was conducted across several districts of Kerala to collect specific samples of *P. manihoti* nymphs from cassava and alternative host plants. Subsequently, the SS-*mtCOI* marker was employed to evaluate the efficacy of this marker across various populations of *P. manihoti* in Kerala, utilizing extracted DNA and polymerase chain reaction (PCR) analysis for validation. This marker successfully identified all developmental stages (egg, first, second, third instar, and adult female), even at low DNA concentrations. This validation of the SS-*mtCOI* marker through PCR assay provides a quick, clear, and reliable method for identifying *P. manihoti*, eliminating the need for traditional slide mounting.

Keywords: Cassava Mealybug, *Phenacoccus manihoti*, species specific marker, PCR

INTRODUCTION

Cassava mealybug, *Phenacoccus manihoti* Matile-Ferrero (Hemiptera: Pseudococcidae), is native to South America and a major pest of cassava (*Manihot esculenta* Crantz) around the world (Cox & Williams 1981; Löhr *et al.,* 1990; Bellotti *et al.,* 1999). It causes about 80% reduction in yield, leading to annual economic losses surpassing $2B dollars (Herren 1981; Herren and Neuenschwander 1991; Neuenschwander *et al.,*1988; Nwanze, 1982). It successfully invaded at least 47 countries across South America, Africa and Asia, devastating cassava crops (Cabi, 2022; Morales *et al.,* 2016)). In Asia, it was first observed in Thailand in 2008 (Muniappan *et al*., 2009) and later spread to Cambodia, Indonesia, Laos, Vietnam (Bellotti *et al.,* 2012; Parsa *et al.,* 2012; Winotai *et al.,* 2010) and India in 2020 (Joshi *et al*., 2020) where it is causing a notable decline in cassava production. The thelytokous parthenogenesis reproduction in *P. manihoti* allows a single individual to establish a successful invasion (Parsa *et al.,* 2012). Its rapid spread, averaging 150 km per year, is facilitated by the attachment of eggs or ovisacs to carriers, along with the wind dispersal of both nymphs and adults over long distances (Liebhold and Tobin 2008; Winotai *et al.,* 2010). Along with cassava, Indeed, *P. manihoti* exhibits a broad palate, showing a preference for plants across nine different families (Cox and Williams, 1981; Morales *et al.,* 2016; Le Ru and Tertuliano, 1993). *P. manihoti* oligophagous behaviour allows it to thrive in various ecosystems, threatening cassava and ornamental plants. Its ability to spread quickly necessitates effective identification strategies for control measures (Parsa *et al.,* 2012). Identifying species through morphological features is mainly possible with adult females, while nymphs and ovisacs are challenging due to similarities among related species (Wang *et al.,* 2019). An urgent need exists for an effective diagnostic tool to manage further spread. The SS-*mtCOI* Marker PCR assay provides a straight forward solution for identifying species at any nymph stage, even for non-specialists (Jiang *et al.,* 2013; Rugman-Jones *et al.,* 2006; Zhang *et al.,* 2012). A PCR method was employed to monitor and identify *P. solenopsis* (Tian *et al.,* 2013) and *P. manihoti* (Wang *et al*., 2019) using *mtCOI* markers. Similarly, our study validated a specific *P. manihoti* marker for effectively identifying immature stages across various host plants and geographic areas.

MATERIALS AND METHODS

Mealybug collection

A survey was undertaken in different districts of Kerala with the aim of collecting *P. manihoti*

specimens from cassava, *Manihoti esculenta* and other alternative hosts (Table 1 and Fig 1). Immature and adult stages of *P. manihoti* were collected from Indoor rearing at Kerala Agricultural University, Thrissur. *P. manihoti* specimens were identified and validated by morphological characteristics as described by (Williams and de Willink 1992; Parsa *et al.,* 2012; Joshi *et al.,* 2020). The other mealybug species were also collected and recognized by taxonomic keys before molecular research (Tang, 1992). All samples were preserved in 95% ethanol at -20°C until the DNA was isolated, and voucher specimens were deposited at the Kerala Agricultural University, Thrissur.

Table 1. Collection Details and Host Plant Information of *Phenacoccus manihoti*

District	Latitude	Longitude	Host plant
Thrissur	10.54931	76.28319	*Manihot esculenta, Talinum triangulae, Blumea lacera, Synedrella nodiflora, Lantana camera*
Ernakulam	10.14697	76.35342	*Manihot esculenta*
Kannur	11.79556	75.57417	*Manihot esculenta*
Palakkad	10.68221	76.51466	*Manihot esculenta*
Malappuram	10.98425	76.18517	*Manihot esculenta*
Kottayam	9.534239	76.53211	*Manihot esculenta*
Alappuzha	9.145715	76.5367	*Manihot esculenta, Grass*
Pathanamtittha	9.304222	76.73044	*Manihot esculenta*
Trivandrum	8.334488	77.09431	*Manihot esculenta*
Kollam	8.842853	76.75152	*Manihot esculenta*
Kozhikode	11.33821	75.92294	*Manihot esculenta*
Idukki	9.822318	76.69965	*Manihot esculenta*
Kasargod	12.34186	75.112	*Manihot esculenta, Alternenthera sessilis*

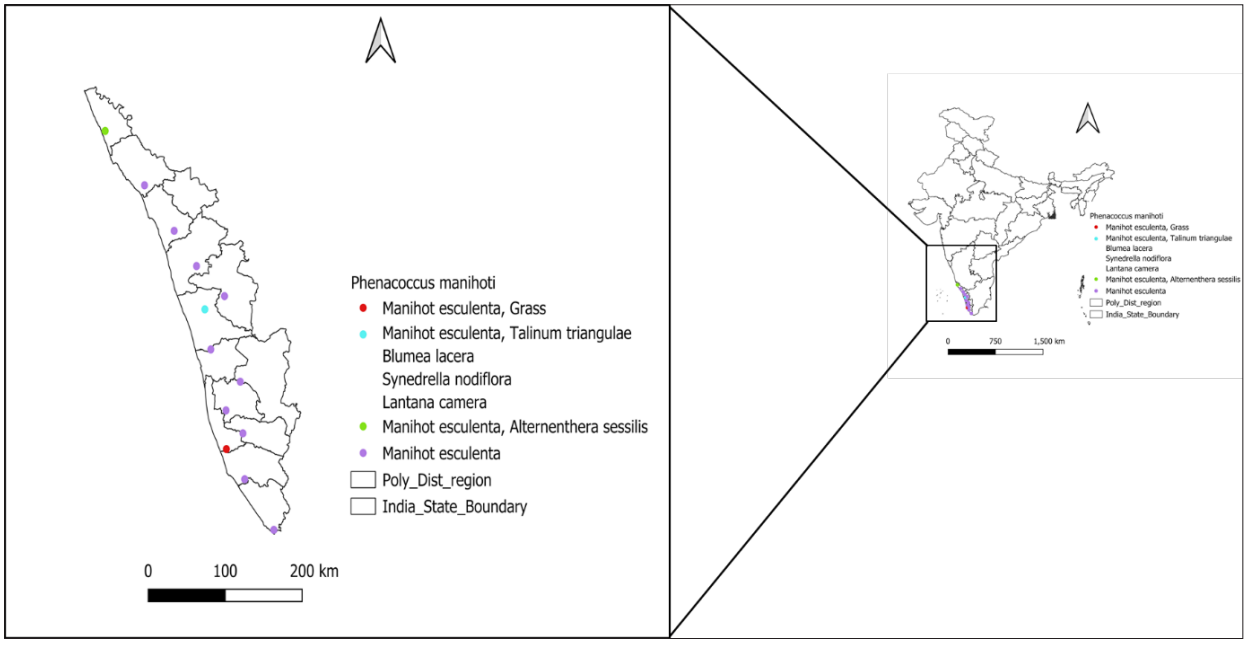

Fig.1. Collection Details of *Phenacoccus manihoti* in Kerala

DNA extraction

The genomic DNA from *P. manihoti* was extracted using the DNeasy® Blood & Tissue kit protocol (Qiagen, Germantown, MD, USA; catalog # 69504). The extracted DNA was then assessed by running it on a 1.2% agarose gel electrophoresis setup with 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA) for 30 minutes. Ethidium bromide (0.5 mg/ml) was added during its preparation. The gel was then visualized and documented using a Bio-Rad Gel EZ Imager.

Amplification and sequencing

We amplified the DNA using a Veriti 96-Well PCR Thermal Cycler (Applied Biosystems). A total of 20µL reactants were used, which includes PCR-grade water (7.8µL), master mix (2X EmeraldAmp Takara with Dye) (10µL), forward and reverse primers (0.6µL each), and DNA template (1µL). The pair of *mtCO1* primers used FP (5'-CTTGATAAAACAGGAATTGAG-3') and RP (5'-CCTTTGATGATTTCTTCTTCT-3') (Wang et al., 2019). The PCR process involved the following steps: initial denaturation for 2 min at 95°C, then 30 cycles of denaturation (94°C-30 sec), annealing (50°C-30 sec), and chain extension (72°C-30 sec). There will be a final extension at 72 °C lasting for five minutes. The 5 µL PCR products were resolved in a 1.2% agarose gel at 80 V for 30 min. We sequenced each positive product in both directions at Gene Spec Pvt. Ltd., Cochin, India, to verify that the products amplified by the specific markers originated from the gene *mtCOI*.

RESULTS AND DISCUSSION

Molecular analysis of *P. manihoti* and its immature stages from cassava

Genomic DNA extracted from adult and immature stages of *P. manihoti* (including 3rd, 2nd, and 1st instar nymphs and eggs) was amplified using the SS-*mtCOI* marker. Gel electrophoresis revealed a consistent presence of a 355 bp targeted fragment of *P. manihoti* across entire replicates, even at small concentrations. The band for *P. manihoti* (mtDNA) from various mature and immature stages is illustrated in lanes 1 to 5 in (Fig. 2).

SS-*mtCOI* marker specificity and stability in *P. manihoti* across Kerala

Genomic DNA extracted from *P. manihoti* specimens and other mealybugs of cassava collected from thirteen districts of Kerala was examined to assess its specificity and stability. The SS-*mtCOI* marker successfully amplified DNA from all samples, consistently producing a 355 bp fragment in gel electrophoresis, even at low concentrations (Fig 5.). Because of the marker's specificity, it was unable to detect other mealybug species (Fig 3.).

Specificity of SS-*mtCOI* marker from alternative hosts of *P. manihoti*

The DNA extracted from *P. manihoti* found on alternative hosts *(Alternanthera sessilis*, *Talinum triangulare*, *Blumea lacera*, *Synedrella nodiflora*, *Lantana camara*, and grass) was amplified, resulting in the detection of a 355 bp product (Fig 4.).

SS-*mtCOI* marker analysis

The DNA band observed in the PCR products indicated the presence of the CO1 gene, spanning 355 base pairs in length (Fig 2,3,4 and 5.) The BLAST analysis of the CO1 gene sequence revealed a perfect match with 100% identity and coverage. The sequences have been submitted to GenBank under the accession numbers (PP660149.1, PP660148.1, and PP660147.1). The sequences of mealybug samples are cent per cent identity with sequences from India (MT895817 and MW039322), china (KY611346, KY611348, KY611347 and KY611349) and also with other accessions OK172179 OK173048, OK172562, OK172342, OK174324, OK172561).

Fig.2. SS-*mtCOI* Marker PCR amplification across *P. manihoti* developmental stages

M, marker; 1–5, samples feeding on cassava from Kerala; 1-female Adult; 2-3rd instar nymph 1st instar nymph; 3, 2nd instar nymph; 4, 1st instar nymph; 5, egg

Fig.3. SS-*mtCOI* amplification in *P. manihoti* and other mealybug species from cassava

(M- Ladder; 1- P. *manihoti*; 2- *P. marginatus*; 3- *P. solenopsis*; 4- *F. virgata*; 5- *P. jackbeadslyi*)

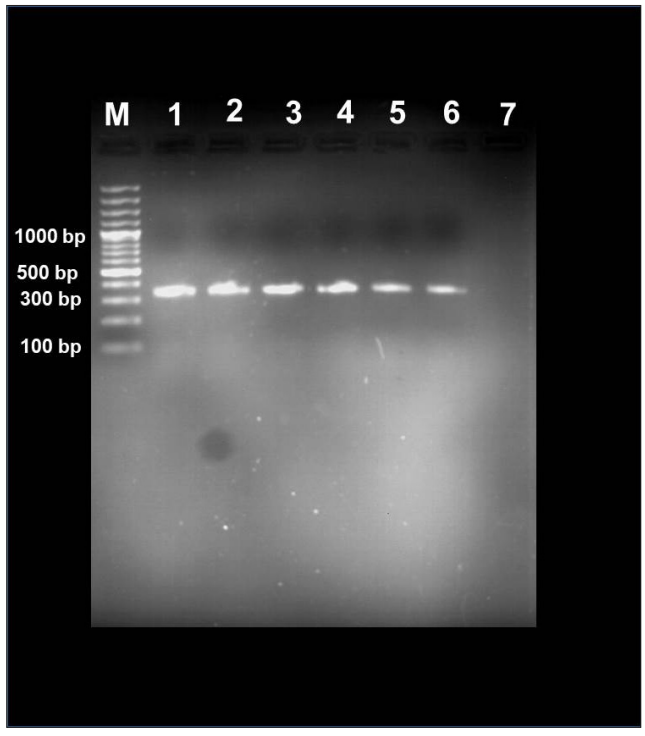

Fig.4. SS-*mtCOI*marker amplification pattern in *P. manihoti* from alternative hosts

(M- ladder; 1- Alternentherasessilis; 2- Talinum triangulae; 3- Blumealacera; 4- Synedrella nodiflora; 5-Lanatana camera; 6-Grass)

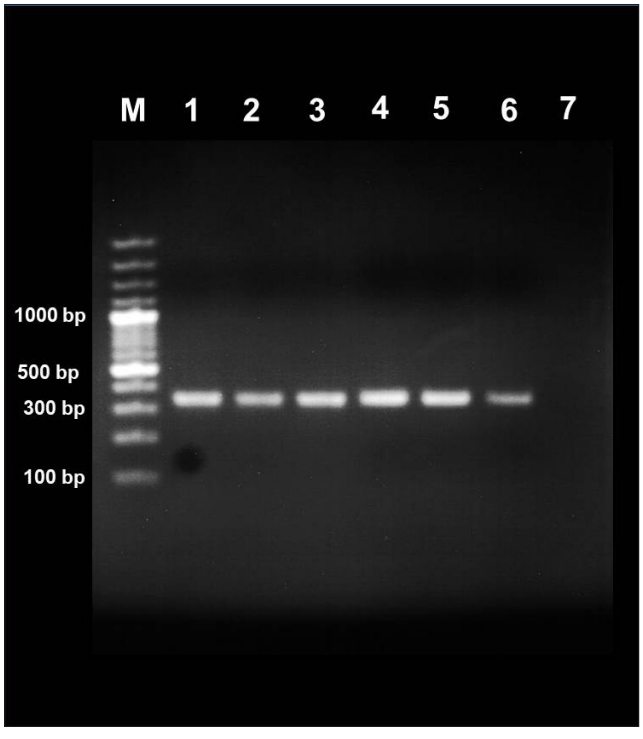

Fig.5. SS-*mtCOI* marker amplification in *P. manihoti* developmental stages from various districts of Kerala

(M – Ladder; 1-Thrissur; 2-Palakkad; 3-Ernakulam; 4-Malappuram; 5- Kozhikode; 6- Kannur; 8-Kottayam; 9-Alappuzha; 10- Pathanamthitta; 11- Kollam; 12- Idukki; 13- Thiruvananthapuram districts; 7 and 14 - blank.

Invasive insects pose significant threats to agriculture, contributing to global food shortages. Pacheco *et al.* (2014) also found that a multiplex PCR was a quick and cheap way to identify *P. solenopsis*, *Dysmicoccus brevipes*, *Pseudococcus viburni*, *Planococcus citri*, and *P. ficus*. Similarly, *P. manihoti*, an invasive pest with significant repercussions for cassava crops, is adept at long-distance dispersal, often through international trade (Parsa *et al.,* 2012). There is an urgent need for a rapid method to identify *P. manihoti* due to its significant impact. The PCR assay developed in this study utilizes the SS-*mtCOI* marker for accurate, species-specific detection. This method is fast, sensitive, and reliable, completing the process in 2.5 hours using DNA from any growth stage. A 353-bp segment confirms the presence of *P. manihoti*. Additionally, the assay eliminates the need for slide preparation, sequencing, or restriction digestion, making it accessible for non-specialists during plant quarantine inspections. Species-specific PCR with an SS-marker is a rapid tool for identifying species by detecting specific gel electrophoresis bands. It is effective for various species, including mealybugs (Zhang *et al.,* 2012; Saccaggi *et al.,* 2008).

Notably, multiplex PCR differentiated *Planococcus citri*, *P. ficus*, and *Pseudococcus longispinus* (Saccaggi *et al.,* 2008), while specific markers were developed for *P. comstocki*, *P. viburni*, and *P. citri* (Hosseini and Hajizadeh, 2011).Wang *et al.* (2019) evaluated the specificity of the SS-*mtCOI P. manihoti* marker set was thoroughly tested against 21 closely related mealybug species commonly encountered in Chinese ports or fields, including several quarantine pests and congeners. Encouragingly, no cross-reaction was observed with non-target species, confirming the primer pair's specificity. Moreover, this method proved effective across various developmental stages and different *P. manihoti* populations, and it consistently performed well across different PCR thermal cycler models, showcasing its stability.

This included four quarantine pests (*P. solenopsis*, *D. neobrevipes*, *P. lilacinus*, and *P. minor*), as well as three congeners (*P. madeirensis*, *P. solani*, and *P. solenopsis*). The absence of cross-reaction with non-target species, as indicated by the results shown in (Fig. 3), confirms the primer pair's specificity. Moreover, this approach precisely detected all stages of development and diverse populations of *P. manihoti* (see Fig. 2). Successful with a detection limit as low as 50pg µL–1 of DNA, this PCR assay demonstrates high sensitivity in identifying *P. manihoti*, surpassing the limitations of repeatability and reliability associated with RAPD analysis. Consequently, it presents a superior alternative for detecting *P. manihoti* in imported cassava sets and tubers, which may harbour various developmental stages and females, often resembling closely related species morphologically.Crucially, this approach is easy to apply and does not necessitate a deep understanding of taxonomy or molecular biology. However, in order to guarantee that the primer pair can be used in a wider range of similar mealybug species, it is advisable to conduct further tests to confirm its specificity.

CONCLUSION

In conclusion, developing a stage-independent identification method for *P. manihoti* utilizing species-specific markers holds significant promise for both invasion prevention and the management of other mealybug species. By employing markers that are not contingent on specific developmental stages, such as eggs or nymphs, this approach offers a versatile and robust means of accurately identifying *P. manihoti* across all life stages. Such precision in identification is crucial for implementing timely and targeted interventions to prevent invasions and mitigate the potential damage caused by this pest. Moreover, the applicability of species-specific markers extends beyond *P. manihoti*, providing a valuable tool for the identification and management of related mealybug species. This advancement represents a vital step forward in enhancing our capacity to safeguard agricultural and horticultural systems from the threats posed by invasive pests, ultimately contributing to the sustainability and resilience of global ecosystems.

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