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Molecular Identification of Mealybug Species (Hemiptera: Pseudococcidae) Affecting *Theobroma cacao* for Improved Pest Management †

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Abstract: *Theobroma cacao* is affected by viruses on every continent where the crop is cultivated, with the best-known ones belonging to the *Badnavirus* genus. *Badnaviruses* are transmitted by several species of Pseudococcidae, a large, taxonomically diverse group of insects collectively known as mealybugs. Effective management of mealybugs depends on accurate identification of species present, as even closely related species have distinct life cycles and are vulnerable to different biological control organisms. This study compares the usefulness of the COI, ITS2, and 28S markers using the primer pairs (MFCO1/MRCO1, ITS2-M-F/ITS2-M-R, D10F/D10R, and D2F/D2R) to identify mealybugs associated with cacao plants in North America. All markers were informative for *Pseudococcus comstocki* (n=4) and *Maconellicoccus hirsutus* (n=8), but only CO1 provided unambiguous identification for *Pseudococcus jackbeardsleyi* (n=11). Primer pair D2F/D2R is not recommended for mealybug identification, as it frequently yielded sequences of *Anagyrus sp*., an Encyrtid parasitoid wasp commonly used for biocontrol. This study describes molecular diagnostic protocols for identifying cacao-associated mealybugs and detecting the presence of certain parasitoids. This information is essential for selecting the most effective interventions as part of an integrated pest management program.

Keywords: DNA barcoding; molecular markers; *Pseudococcus*; *Maconellicoccus hirsutus*; *Anagyrus*; mealybug; cacao; *Badnavirus*; virus vector; Florida

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1. Introduction

Mealybugs (Hemiptera: Pseudococcidae) are phloem feeders that use long, slender mouthparts to uptake plant fluids [1], which reduces the vigor of host plants. They can feed on all plant parts, and severe infestations cause defoliation and, eventually, plant death. Some species inject plant toxins during feeding causing twisted/stunted growth [2]. The damage generated varies among taxa and is determined by their reproductive potential, temperature tolerance, preferred feeding locations, the existence of effective control strategies, and their ability to transmit viruses [3].

On cacao, the primary economic impact of mealybugs is their ability to transmit viruses [4]. Viruses have been identified on every continent where cacao is grown commercially, most of which belong to the *Badnavirus* genus and are transmitted by several mealybug species [5,6]. Due to the high diversity of Pseudococcidae, each region has different species composition, and morphological differentiation of closely related species is challenging for non-specialists. On cacao, mealybug populations are composed of multiple species [4,7,8]. In West Africa, *Pseudococcus njalensis* (Laing) is the main vector, due to its

abundance in the area [9], while *Planococcus citri* (Risso) is the most significant vector of cacao virus in Trinidad [4].

Accurate taxonomic identification is a major barrier in research and management of mealybugs due to the specificity of commonly used biological control organisms [10]. Molecular approaches have been used successfully to identify mealybugs from France, Egypt, South Korea, South Africa, and Japan [11-13]. In addition to reducing the reliance on delicate morphological features, sequence-based identification also allows for the detection of cryptic species [14]. Genetic sequences from these projects have been deposited in GenBank, providing valuable reference material needed for the implementation of routine molecular identification for insects.

The purpose of this study was to develop molecular tools to determine the species composition of mealybug populations. This was done by evaluating the ability of three genetic markers and four primer pairs to identify the species affecting *T. cacao* (Linnaeus) in Florida. This information is essential for selecting the most effective management interventions.

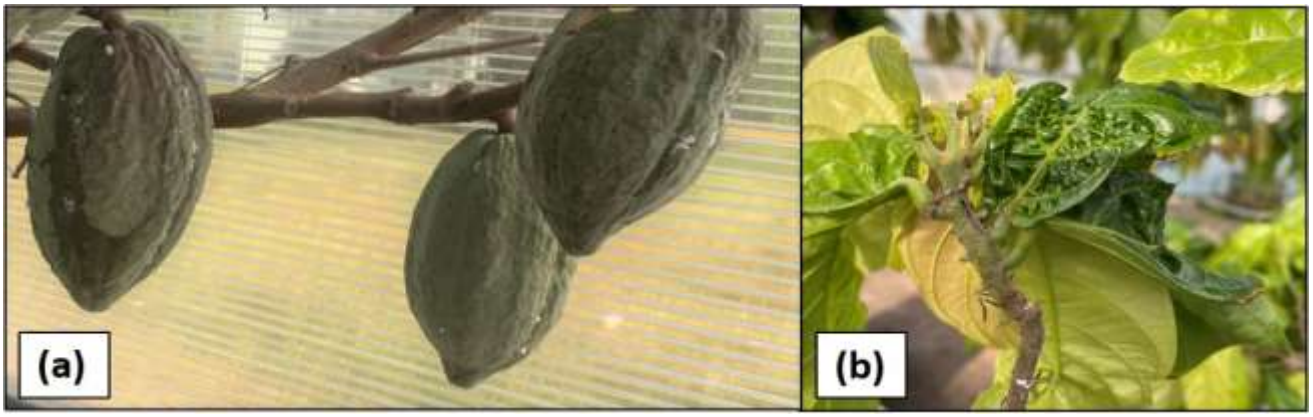

Figure 1. Signs of mealybug infestations on *Theobroma cacao*: (**a**) Mealybugs and eggs on pods and (b) leaf distortion characteristic of feeding by *Maconellicoccus hirsutus* (Green).

2. Materials and Methods

2.1. Insect sampling and DNA extraction

In January 2021, female mealybugs were collected from pods, stems, leaves and flowers of four randomly selected *Theobroma cacao* trees in a greenhouse in Miami, FL. Up to 5 specimens were collected from each tissue type (pods, stems, leaves and flowers), and stored in 70% ethanol at 23°C (±1°C) for three to four weeks before processing.

DNA was extracted from individual specimens using the Qiagen DNeasy Blood and Tissue Kit with a shortened, 10min, lysis step, as described in Albo et al. (2019)[15]. The final resuspension step was done with 50 µL of elution buffer for adults, and 30µL for the smaller nymphs. A Qubit 4 Fluorometer and the 1x dsDNA High Sensitivity Assay Kit (Life Technologies Corp., Carlsbad, CA, USA) were used to quantify DNA.

2.2. PCR amplification and sequencing

To identify mealybug species, four primer pairs, ITS2-M-F/ITS2-M-R, D10F/D10R, D2F/D2R, and MFCO1/MRCO1 were chosen based on published reports of successful amplification and sequencing of mealybugs (Table 1). Reactions with ITS2-M-F/ITS2-M-R, D10F/D10R, and D2F/D2R were run with 12.5 µL 2x Immomix Red (Bioline), 1 µL each of 10µM forward and reverse primers, 1 µL of DNA template, and 9.5 µL sterile nuclease-free water (25 µL reaction volume). PCRs with MFCO1/MRCO1 contained 12.5 µL 2x Immomix Red, 1.5 µL each of the forward and reverse primers (10 µM) and 1.2 µL DNA template (25 µL reaction volume). PCRs were performed on a Bio-Rad C1000 Touch thermal cycler (Hercules, CA, USA) using programs developed in this study (Table 2).

Amplification was visualized on a 1% (w/v) agarose gel at 150V (for 35 minutes). PCR products were purified with Qiagen PCR Purification Kit (Hilden, Germany) and sent to Eurofins for Sanger sequencing.

Table 1. Genes, primer sequences, and amplicon sizes for the markers targeted in this study.

Gene	Primer	Sequence (5′-3′)	Amplicon Size (bp)	Reference
COI	MFCO1 MRCO1	ATA-TCTCAAATTATAAATCAAGAA ATTACACCTATAGA-TAAAACATAATG	379	[8]
ITS2	ITS2-M-F ITS2-M-R	CTCGTGACCAAAGAG-TCCTG TGCTTAAGTTCAGCGGG-TAG	~800	[14]
28S	D10F D10R	GTAGCCAAATGCCTCGTCA CACAATGATAGGAA-GAGCC	738-767	[16]
28S	D2F D2R	AGAGAGAGTTCAAGAG-TACGTG TTGGTCCGTGTTTCAA-GACGGG	310-356	[17, 14]

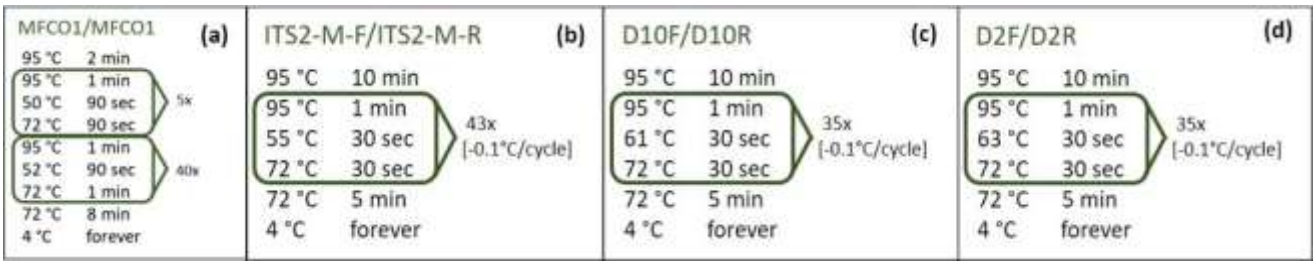

Figure 2. Thermal cycler programs optimized for amplification of mealybug DNA using primer pairs: (**a**) MFCO1/MRCO1; (b) ITS2-M-F/ ITS2-M-R; (c) D10F/D10R; (d) D2F/D2R.

2.3. Mealybug identification

The protocols developed during this study were tested on 23 mealybug specimens feeding on cacao in Florida. Sequences were edited and aligned using Geneious ®11.1.2 (Biomatters Ltd., Auckland, New Zealand), and a subset was deposited in GenBank. The usefulness of primers pairs for mealybug identification was determined by analyzing the resulting sequences in BLASTn. Top matches were selected based on max score, and if multiple species were among the top matches for a given sequence, the first two to three entries were recorded in the results table. Specimen identification was determined based on BLASTn results of the COI sequences, because this genetic region is considered the most biologically informative.

3. Results

Three primer pairs (MFCO1/MRCO1, ITS2-M-F/ITS2-M-R, D10F/D10R) produced clear, single bands in all species tested, and high-quality sequences (>75%) after aligning and editing. Sequences obtained with all three primer pairs yielded consistent species matches for *Pseudococcus comstocki* (Kuwana) (n=4) and *Maconellicoccus hirsutus* (n=8), with

coverage and identities ranging from 96-100% (Table 2). However, amplification with D10F/D10R was only achieved in half of the *P. comstocki* samples.

Table 2. BLASTn results for COI, ITS2, 28S sequences amplified and sequenced with primers MFCO1/MRCO1, ITS2-M-F/ITS2-M-R, and D10F/D10R, respectively. Matches obtained with COI sequences are given preference. Data shown are from one representative of each species used in this study.

	Marker	Seq. (bp)	Seq Quality (%)	Genbank match	Accession No.	% Ident.	Query cov. %
seudococcus comstocki	COI	371	98.9	*P. comstocki*	LC121496.1	98.9	100
	ITS2	643	96.9	*P. comstocki*	KU499509.1	96.3	100
	28S	840	98.6	*P. comstocki*	JF965413.1	99.8	98
Pseudococcus jackbeardsleyi	COI	370	86.8	*P. jackbeardsleyi*	KJ187489.1	99.5	100
	ITS2	679	98.7	*Pseudococcus viburni*	KF819654.1	79.2	90
	28S	801	100	*Pseudococcus viburni*	AY427376.1	99.1	99
				Oracella acuta	JF965418.1	98.9	99
				P. jackbeardsleyi	EU188510.1	99.9	95
Maconellicoccus hirsutus	COI	374	97.9	*M. hirsutus*	MK090645.1	100	100
	ITS2	755	94.4	*M. hirsutus*	KU883603.1	99.5	98
	28S	808	99.9	*M. hirsutus*	AY427403.1	99.5	96

For *Pseudococcus jackbeardsleyi* (Beardsley) (n=11), only CO1 sequences provided unambiguous identification. Sequences obtained with the D10F/D10R primer pair were close matches to three different species (*Pseudococcus viburni* (Signoret), *Oracella acuta* (Lobdell), and *P. jackbeardsleyi*) available in GenBank. In contrast, no highly similar sequences were found for ITS2 in GenBank. For each gene region, multiple alignments showed no nucleotide level variation among individuals of the same species.

Primer pair D2F/D2R is not recommended for mealybug identification, as it frequently yielded sequences of *Anagyrus sp*. (Howard) (Hymenoptera: Encyrtidae), parasitoids commonly used for biocontrol. Although these primers were developed for use in Hymenoptera [17], they were selected for this study because later work [8] found them to be more effective than D10F/D10R at amplifying the 28S region of Pseudococcidae. A subset of mealybug and parasitoid sequences generated in this study were deposited in GenBank (Tables 3 and 4).

Table 3. Mealybug sequences generated in this study and deposited in Genbank.

ID	Species	Host	Origin	Collected	COI	ITS2	28S (D10F/R)
MB6	*Pseudococcus comstocki*	*Theobroma cacao*	USA	Jan-2021	MZ312155	MZ229908	MZ264161
MB8	*P. comstocki*	*Theobroma cacao*	USA	Jan-2021	MZ312156	MZ229909	n/a
MB10	*P. comstocki*	*Theobroma cacao*	USA	Jan-2021	MZ312157	MZ229910	n/a
MB11	*P. comstocki*	*Theobroma cacao*	USA	Jan-2021	MZ312158	MZ229911	MZ264162
MB5	*P. jackbeardsleyi*	*Theobroma cacao*	USA	Jan-2021	MZ319383	MZ229922	MZ264173

MB9	*P. jackbeardsleyi*	*Theobroma cacao*	USA	Jan-2021	MZ319384	MZ229923	MZ264174
MB12	*P. jackbeardsleyi*	*Theobroma cacao*	USA	Jan-2021	MZ319385	MZ229924	MZ264175
MB17	*Maconellicoccus hirsutus*	*Theobroma cacao*	USA	Jan-2021	MZ312118	MZ229916	MZ264167
MB20	*M. hirsutus*	*Theobroma cacao*	USA	Jan-2021	MZ312119	MZ229917	MZ264168

Table 4. Parasitoid sequences amplified and sequenced from host DNA using D2F/D2R primers. GenBank accession numbers are shown in the last column.

ID	Species	Host	Origin	Collected	28S (D2F/R)
MB5	*Anagyrus sp.*	*Pseudococcus jackbeardsleyi*	USA	Jan-2021	MZ265304
MB7	*Anagyrus sp.*	*P. jackbeardsleyi*	USA	Jan-2021	MZ265305
MB8	*Anagyrus sp.*	*P. comstocki*	USA	Jan-2021	MZ265306
MB16	*Anagyrus kamali*	*Maconellicoccus hirsutus*	USA	Jan-2021	MZ265307

4. Discussion

Cytochrome c oxidase subunit I (COI) is considered the most informative marker for insects and most living organisms [18]. However, it has proven difficult to amplify in some groups, such as mealybugs, leading to the development of numerous different primer pairs [13,14]. The COI primers used in this study were designed by [8] and validated on taxa collected from cacao in Asia, Africa, and the Americas (*Planococcus*, *Ferrisia, Dysmicoccus*, and *Pseudococcus*). It amplifies a small section of the universal barcode region [18], but this fragment provided unambiguous identification in the species examined here.

Both *P. jackbeardsleyi and M. hirsutus*, have been reported affecting cacao in Africa and the Americas [19,20]. Neither has been tested for their ability to transmit viruses to cacao, but they are closely related to confirmed vectors, and have been detected on CSSV-infected cacao in Cote d'Ivoire [19]. *Pseudococcus comstocki* was one of the first confirmed vectors of a cacao virus in Trinidad [4] but no reports were found of it being used in transmission tests for other cacao viruses.

Accurate identification of species present in a population is essential for the selection of effective controls. Several parasitoids and predators are commercially available for controlling mealybugs, but they are not effective against all taxa. For example, a study investigating target species of the parasitoid *Anagyrus sinope* (Noyes and Menezes) found that it could only parasitize one of the five *Pseudococcus* species tested [10].

Anagyrus kamali (Moursi) has been effectively used to control *M. hirsutus*, in Egypt and the Caribbean [21,22], however, it is fairly host specific. Following the release of *A. kamali* in the Caribbean, Sagarra et al. (2001) conducted host range studies using nine mealybug species prevalent in the area [23]. Although *A. kamali* occasionally laid eggs in species other than *M. hirsutus*, it could not complete its life cycle on these hosts.

Parasitoid establishment and survival are additional obstacles to the successful use of biological control. The detection of *Anagyrus* sp. and *A. kamali* sequences in this study indicates that these organisms are established in the area, and additional releases would have little effect on pest populations.

The implementation of routine molecular identification for insects, relies on the availability of high-quality reference libraries against which sequences can be compared [24]. Low representation of an organism in GenBank results in ambiguous, or incorrect, identification [15]. In this study, ITS2 sequences were not considered informative for *P. jackbeardsleyi*, due to the absence of these sequences in GenBank. However, the ITS2 sequences

generated in this study were deposited in GenBank, making this marker valuable for future research.

Although the tools presented here were developed for cacao mealybugs, the species detected are highly polyphagous and affect a wide range of geographic locations. These protocols can be used by agricultural inspectors and scientists to identify mealybug specimens and study pest populations.

Supplementary Materials: Supplementary File 1

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