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Molecular Identification of *Planococcus citri (Hemiptera: Pseudococcidae*)

Khadem, Ahad Gul[1*], Lau, Wei Hong[2], Mokhtar, Anis Syahirah[2], Chin, Nyuk Ling[3]

[1]Plant Protection Department, Agriculture Faculty, Paktia University, Gardez, 2201, Paktia, AFGHANISTAN

[2]Plant Protection Department, Agriculture Faculty, Universiti Putra Malaysia, Serdang, 43400, Selangor, MALAYSIA

[3]Department of Process and Food Engineering, Engineering Faculty, Universiti Putra Malaysia, Serdang, 43400, Selangor, MALAYSIA

*Corresponding author: ahadgulkhadem@yahoo.com

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Abstract: *Planococcus citri* is an important phytophagous pest in different agroecosystems. The species is very difficult to distinguish by morphological features. The goal of this research was to present basic data on the molecular data to confirm *P. citri*. The primers used in the molecular identification of *P. citri* consisted of mitochondrial cytochrome c oxidase subunit 1 (*COI*), *ribosomal small subunit RNA* (18S rDNA) gene, and *internal transcribed spacer 2* (ITS2) gene. The 1% (w/v) agarose gel showed the presence of successful amplification of the *COI*, *18S rDNA,* and ITS2 genes indicated with positive PCR products. The *Internal transcribed spacer (ITS2)*, *18S ribosomal* and *Cytochrome c oxidase I* (*COI) mitochondrial* genes used in this study confirmed the identity of mealybugs as *P. citri*. The *18S ribosomal* gene showed 98% nucleotide sequence homology to the *P. citri* USA isolate while the *COI gene* and *ITS2 gene* showed less than 95% nucleotide sequence homology to the *P. citri* USA isolate and *P. citri* Israel isolate, respectively. At present, the nucleotide sequence data of mealybugs is still lacking; consequently, mealybug species must be correctly identified for the proper management of this pest.

Keywords: *Planococcus citri, COI*, 18S rDNA gene, ITS2 gene, USA, Israel

1. Introduction

Citrus plants are targets of multiple insect pests, including citrus mealybug *(Planococcus citri).* Apart from citrus plants, *P. citri* attacks numerous other crops and ornamental plants throughout the world (Cox, 1981; Tóbiás et al., 2012). This mealybug *(Planococcus citri)* has been recorded as the most harmful mealybug of citrus (Panis, 1977). It can also be difficult to isolate *Planococcus citri* from *Planococcus ficus, Planococcus halli* , and *Planococcus mali* with morphological characteristics is problematic when using high magnification for studying slide-mounted female mealybugs (Cox, 1989). The potential method of identifying species that satisfy all of the above criteria is molecular identification. This method has been effectively applied to insect species with challenging morphological identification (Besansky *et al.* 2003). The ribosomal DNA (subunits 18S, 28S, internal transcribed spacers), cytochrome oxidase I (COI), and cytochrome oxidase II (COII) of mitochondrial DNA are the common genes used in identifying insects (Simon et al., 1994; Rokas et al., 2002; Li et al., 2005). Beuning et al. (1999) defined four different species of mealybug based on sequence differences in ITS1 and ITS2 regions. Recently, the *COI* gene has been recognized as a mitochondrial gene reference for using DNA barcoding to identify and classify a species (Hajibabaei et al., 2006; Linares et al., 2009). To avoid confusion between *Planococcus citri* and other closely related species such as *P. ficus, P. halli, P. kenyae, P. lilacinus, P. mali*, and *P. minor,* it is necessary to use molecular data to confirm the identity of the mealybugs prior to further experiments. The aim of this research is to utilize molecular analysis for the identification and explanation of citrus mealybugs (*P. citri*).

**Corresponding author: ahadgulkhadem@yahoo.com*

2. Materials and Method

2.1 Collection and Rearing of *P. citri*

Citrus mealybugs (*P. citri*) were collected from the infested pomelo trees (*Citrus maxima*) cultivated at the campus of Universiti Putra Malaysia (UPM). The infested leaves and petioles were put into plastic bags and brought to the Insect Pathology Lab, Faculty of Agriculture, UPM for rearing as described by the method of Khadem et al. (2022). Round pumpkin fruits (*Cucurbita pepo)* and sprouted potatoes (*Solanum tuberosum*) were bought from a market. To ensure their cleaning, they were disinfected through 0.5% (v/v) Clorox for a duration of five minutes. Subsequently, they were rinsed twice with distilled water. After that, they were air-dried at room temperature for four h and then placed in plastic containers measuring (38 L ×28 H ×21 W cm). The infested leaves and petioles were placed on top of the pumpkin fruits and sprouted potatoes. A few days later, mealybugs were transferred from the infested leaves and petioles onto the pumpkin's fruits and sprouted potatoes using a camel hairbrush. *Planococcus citri* were then reared on the pumpkin fruits and sprouted potatoes in the laboratory with a temperature range of 24 - 27°C ±2°C and a relative humidity range of 67 to 72±5%.

2.2 DNA Extraction

The G-spin™ Total DNA Extraction Mini Kit (iNtRON Biotechnology, USA) was employed for extracting the DNA of the adult female *P. citri*. The entire body of the females was placed in the grinding jar (mortar) containing liquid nitrogen and homogenized completely. Using a spatula, 25 mg of ground sample was transferred to a microcentrifuge tube having 1.5 ml volume. 200 µl of Buffer CL, 20 µl of 20 mg/ml Proteinase K and 5 µl of RNase A Solution were added to this tube and the mixture was vortexed vigorously. The lysate was then incubated for 30 min at 56°C in Memmert WNB29 waterbath (Memmer GmbH + Co. KG, Germany) and inverted every 2 minutes to ensure complete lysis. Then, 200 µl of Buffer BL was added to the tube containing the sample, and the contents were mixed thoroughly. The resulting mixture was then incubated at 70°C for 5 min in a waterbath. The tube was centrifuged for 5 min at 13,000 *g* for removing un-lysed tissue particles such as cell debris. Following this, 350 µl of the supernatant was added to a new 1.5 ml microcentrifuge tube and 200 µl of absolute ethanol was added to the lysate. The mixture was then thoroughly mixed using pulse vortexing. Subsequently, the 1.5 ml microcentrifuge tube was centrifuged for a moment for eliminating any droplets that may have formed on the inner side of the lid. The mixture was loaded onto a spin column, which was placed on a 2 ml Collection Tube. The cap was closed, and both tubes were subjected to centrifuging for 1 min at 13,000 *g*. The resulting filtrate was discarded, and the Spin Column was returned to the 2 ml Collection Tube. 700 µl of Buffer WA was then added to the Spin Column, which was centrifuged again for 1 min at 13,000 *g*. After discarding the flow-through, the Collection Tube was reused and a total of 700 µl of Buffer WB was added to the Spin Column and centrifuged again for 1 minute at 13,000 g. The flow-through was discarded again, and the Spin Column was returned to the Collection Tube. To dry the membrane, an additional centrifugation step was carried out for 1 minute, and both the flow-through and Collection Tube were discarded. The Spin Column was then placed into a new 1.5 ml microcentrifuge tube, and 50 µl of Buffer CE was added to the membrane. Following a 1-minute incubation at room temperature, the mixture was centrifuged for 1 min at 13,000 *g* to extract the DNA. The resulting DNA sample was then stored at -20°C.

2.3 Primers

Four sets of primers were used in the molecular identification of *P. citri*. The first set and second set of primers were targeted for *COI* genes. The third set of primers was targeted for *ribosomal small subunit RNA* (18S rDNA) gene and the final set of primers was targeted for *internal transcribed spacer 2* (ITS2) gene. Table 1 shows the sequences of the primers used in this study.

Table 1: Particulars of primers used for the analysis of *COI, 18S rDNA* and *ITS2* genes

Gene	Primers	Sequence of the Primer (5'-3')	Annealing temperature (°C)	Amplicon size (bp)	Reference
COI	C1J2195 TL2N3014	5'-TTGATTYTTTGGTCATCCAGAAGT-3' 5'-TCCAATGCACTAATCTGCCATATTA-3'	67	840	Simon et al. (I1994)

continued

18S rDNA	18S-2880 18SB	5′-AGAATTAAGCCATGCATGTCTCAG-3′ 5′-TNGCCCTGCCTAATTGATCCTCG -3′	67	630	Malausa et al. (2011)
ITS2	ITS2-M-F ITS2-M-R	5'- CTC GTG ACC AAA GAG TCC TG -3' 5'- TGC TTA AGT TCA GCG GGT AG- 3'	55	800	Kol-Maimon et al. (2014)

2.4 Polymerase Chain Reaction (PCR)

To amplify the *COI*, *18S rDNA,* and *ITS2* gene of citrus mealybug, the Promega GoTaq® Green Master Mix (Promega, USA) was used. As shown in Table 2, the PCR reactions were conducted with a total volume of 25 µl. The PCR reaction was performed in an automated thermocycler Mastercycler® Pro S (Eppendorf Asia Pacific Sdn Bhd., Germany) with the programs as stated in Table 3. The PCR products were kept at 4°C prior to agarose gel detection.

Table 2: PCR reaction mixture

PCR component	µl
Green Master Mix	12.5
Forward primer	2.5
Reverse primer	2.5
Nuclease free water	5.0
DNA template	2.5
Total	**25.0**

Table 3: PCR amplification profiles

Gene	Primers	PCR Condition
CO1	C1J2195 TL2N3014	Initial denaturation: 94°C, 2min 30 cycles: Denaturation: 94°C, 30 sec Annealing: 48°C. 1.5 min Extension:72°C. 1min 35s Final extension: 72°C, 5 min
18S rDNA	18S-2880 18SB	Initial denaturation: 94°C, 2min 30 cycles: Denaturation: 94°C, 30s Annealing: 67°C, 1min Extension: 72°C, 1min 30s Final extension: 72°C, 5 min
ITS2	ITS2-M-F ITS2-M-R	Initial denaturation: 95°C, 1min 30 cycles: Denaturation: 95°C, 1 min Annealing: 55°C, 1 min Extension: 72°C, 1 min 30s Final extension: 72°C, 5 min

2.5 Agarose Gel Electrophoresis

To make a 1% (w/v) agarose gel, 500 mg agarose powder was combined with 50 ml 1× TAE buffer and heated in a microwave for 2 min (shaken thoroughly after one min) until it was clear. Before the PCR products were loaded onto it, the resulting gel was loaded in the gel cast and left for cooling for a period of 25-30 minutes. Then, the gel was placed inside the gel tank, and 1× TAE buffer was loaded in the gel tank until it completely covered the agarose gel. PCR

products (2 µ) and CSL-MDNA-1KbPLUS DNA Ladder RTU (Cleaver Scientific Ltd, UK; 1µ) were loaded into each well of the agarose gel by a micropipette (Eppendorf Asia Pacific Sdn Bhd., Germany). The gel was run for a duration of 45 minutes with a voltage of 65 V, and after completion, the gel was stained for 20 minutes in a solution having 10 mg/ml of ethidium bromide. Following this, the gel was de-stained in distilled water for another 20 minutes. The resulting agarose gel was viewed through the Gel documentation system (Gel Doc XR, BioRad, USA), and an image of the gel was taken.

2.6 DNA Sequencing

Positive PCR products were chosen and forwarded to Apical Scientific Sdn. Bhd. Located in Selangor, Malaysia for nucleotide sequencing.

2.7 Data Analysis

The sequencing results retrieved in FASTA format were analyzed and aligned using Sequence Scanner 2, BioEdit and CLUSTAL OMEGA. Afterward, they were compared with sequences in GenBank through Basic Locus Alignment Search Tool (BLASTn) existing on the National Centre for Biotechnology Information website (https://www.ncbi.nlm.nih.gov/). Once the high identity score of the *P. citri* was attained, the sequence data were aligned by MUSCLE (Multiple Sequence Alignment by Log Expectation) software at the default parameter settings (Thompson *et al.,* 1994 and 1997; Jeanmougin *et al.,* 1998). The sequence quality was revised by removing the bases at the start and end of the sequence where the regions of uncertain alignment in the sequences were found (Swofford et al. 1996).

3. Results

The positive PCR products of *COI*, *18S rDNA* and ITS2 genes were detected on the 1% (w/v) agarose gel (Figure 1) with expected size as stated in Table 1. The nucleotide sequence of *COI, 18S rDNA* and *ITS2* genes have confirmed the identity of UPM citrus mealybugs were having sequence homology to other isolates of *P. citri* deposited in the repository of GenBank (Table 4). The *COI* gene of UPM *P. citri* showed 91.3% similarity in nucleotide sequence to the *COI* gene of *P. citri* USA isolate (Genbank accession number: MF952472). UPM *P citri* also showed similar result in *18S rDNA* gene of UPM *P. citri* with 98.4% sequence homology to the *18S rDNA* gene of *P. citri* USA isolate (Genbank accession number: AY426042). The *ITS2* gene of UPM *P. citri* has exhibited 92.2% sequence homology to the *P citri* isolate from Israel (Genbank accession number: KF738219).

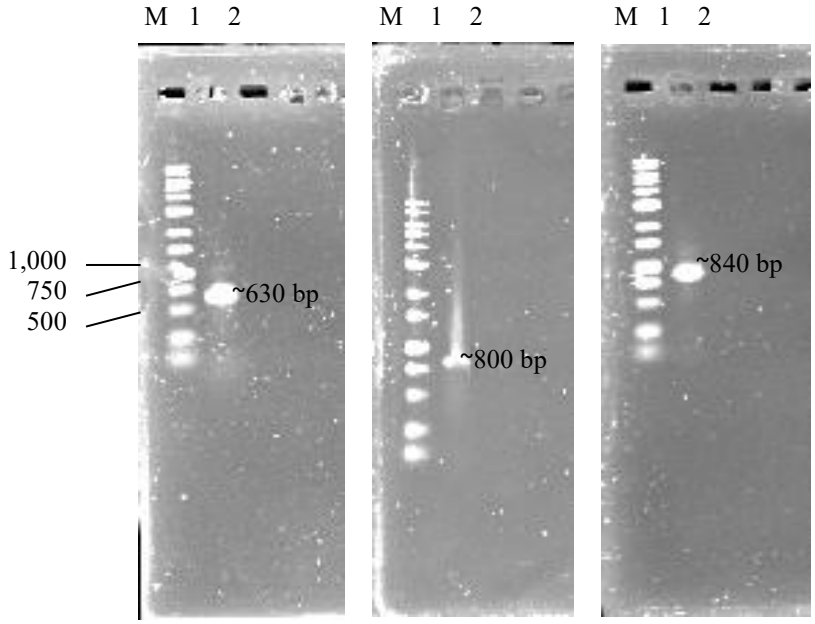

Fig. 1: Positive PCR products on 1% (w/v) agarose gel. (a) *18S rDNA* gene, (b) *ITS2* gene, and (c) *COI* gene. Lane 1: CSL-MDNA-1KbPLUS DNA Ladder RTU (Cleaver Scientific Ltd, UK); Lane 2: PCR product; Lane 2: Negative control.

Table 4: Nucleotide sequence score of UPM *P. citri* in comparison with other *P. citri* isolates in Genbank

Gene	Genbank Accession number	Nucleotide homology (%)	Country	Reference
COI	MF952472	91.32	USA	Assef and Malindzisa 2(018)
18S rDNA	AY426042	98.40	USA	Malausa et al. (2011)
ITS2	KF738219	92.20	Israel	Kol-Maimon et al. (2014)

4. Discussions

The goal of this research was to identify the citrus mealybugs (*P. citri*) in a molecular way which were collected from the pomelo plants (*Citrus maxima*) located in the campus of Universiti Putra Malaysia, Serdang. The *ITS2*, *18S ribosomal,* and *Cytochrome c oxidase I* (*COI) mitochondrial* genes used in this study confirmed the identity of mealybugs as *P. citri*. The *18S ribosomal* gene showed 98% nucleotide sequence homology to the *P. citri* USA isolate while the *COI gene* and *ITS2 gene* showed less than 95% nucleotide sequence homology to the *P. citri* USA isolate and *P. citri* Israel isolate, respectively. The low percentage of nucleotide sequence homology could be due to low purity of PCR products. The *ITS2, 18S ribosomal,* and *COI* genes of *P. citri* collected from the pomelo plants were effectively amplified by the primers used in this study (Coleman, 2003; Casteleyn et al., 2009; Ashfaq *et al.,* 2011; Kol-Maimon et al., 2014; Palma-Jimenez and Blanco-Meneses, 2016). The molecular data provided here could provide better information on the genetic population of mealybugs.

5. Conclusion

P. citri is a significant and prevalent pest that poses a serious threat to citrus plants in Malaysia. It is economically important in the citrus industry. Beside citrus, it could be found in ornamental plants, either in the greenhouses or orchards. The current study aimed to provide basic data on the molecular data for the confirmation of *P. citri*. The data achieved from the DNA analyses were completely reliable with the morphological description. At present, the nucleotide sequence data of mealybugs is still lacking. For the proper identification of mealybug species, it is essential to properly manage this pest in the field, as well as strengthen the quarantine and controlling programs so that the inspectors could correctly confirm the identity of mealybugs and take proper management decisions.

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Conflict of Interest

The authors declare no conflicts of interest.

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