SHORT COMMUNICATION

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# MOLECULAR IDENTIFICATION OF A MEALYBUG SPECIES (HEMIPTERA: PSEUDOCOCCIDAE) INFESTING AN OIL PALM PLANTATION IN TAWAU, SABAH

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**ABSTRACT**

*Mealybugs are common insect pests that can cause significant damage to oil palms, including reduced yield and overall plant health. The mealybugs feed on the plant sap, which later excretes honeydew, leading to the growth of sooty mold, and impedes photosynthesis. The traditional morphological identification methods for mealybugs are very time-consuming and challenging, due to their diminutive size and similar characteristics between their developmental stages. In this research, we investigated the effectiveness of the Ef-1α and 28S rRNA genes for molecular identification of a mealybug species infesting oil palm plantations in Tawau, Sabah. The DNA was extracted from the mealybug samples and amplified with M51.9/rcM53-2, D2F/D2R, and D10F/D10R primers. The amplified regions of the identified* Nipaecoccus nipae *Tawau-isolate were sequenced and deposited in GenBank. Our results demonstrate the potential of molecular methods for quick and efficient identification of mealybug species, which can aid in developing appropriate control strategies.*

**Keywords:** 28S rRNA, Ef-1α, mealybugs, oil palm plantation.

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## INTRODUCTION

Mealybugs (Hemiptera: Pseudococcidae) are common insect pests that thrive on fruits, branches, leaves, roots, and trunks of the oil palm (Pacheco Da Silva et al., 2017). Despite their small size even as adults, they can cause direct harm to their hosts by extracting the plant sap and triggering the formation of sooty mould through their excretion of honeydew, which directly reduces the vigour of the host plants (Park et al., 2010). The black sooty mould fungus impedes photosynthesis by covering the leaves, and stem, thereby causing chlorosis, plant stunting and leaf defoliation, which eventually lead to the possible death of the host plant (Moniruzzaman et al., 2017; Williams & Watson, 1988).

Worldwide, there are about 2,005 species of mealybugs that have been described with 272 genera identified. They are extensively distributed across the tropical and subtropical regions, inflicting damage on various crops, including oil palm (Ahmad & Akhtar, 2016; Morales et al., 2016). For example, Mariau and Biggins (2001) reported that *Dysmicoccus brevipes* live on the roots of oil palms in Ecuador. *Pseudococus citricutus*, *Palmicultor* sp. and *D. brevipe* were reported to be infesting oil palm in India (Kalidas & Subbana, 2022). In Malaysia, Williams (1969) stated *Geococcus johorensis* as the first reported mealybug infestation in oil palm plantation in Johor. However, recently, there were several mealybug population outbreaks on the East Coast of Sabah, which were morphologically identified as *Nipaecoccus nipae* among the oil palm growers. Despite morphological identification, molecular techniques are crucial for confirming the species identity and detecting any cryptic species

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or genetic variations that might not be evident morphologically. The effect of sooty mould was often neglected until the yield reduction in this region became pronounced. After considering other aspects that can cause yield reduction, such as nutrient deficiencies, the possible effects of sooty mould on photosynthesis rates were explored. The disruption to the photosynthesis rate will gradually lead to the reduction of plant productivity and health. Currently, two treatments are being employed to control the mealybugs: 1) Trunk injection of acephate and 2) spraying of white oil and imidacloprid (Helmay et al., 2023). In addition, the lack of knowledge of mealybug species poses a threat to the implementation of integrated pest management strategies in the area.

Mealybugs have similar morphological characteristics, making it difficult to distinguish between their developmental stages due to their small size, thereby hindering the development of appropriate control strategies (Abd-Rabou et al., 2012). Accurate molecular identification is essential for enhancing the management of mealybugs due to the specificity of their control techniques (Chong & Oetting, 2007). Moreover, the acknowledgement of molecular techniques is increasing, emphasising their significance in discriminating species, especially when morphological identification techniques are ambiguous (Tahir et al., 2018). If the species is mistakenly identified, it can lead to ineffective pest management strategies, the use of inappropriate control methods and eventually leading to potential economic loss due to continued pest damage.

The mitochondrial cytochrome c oxidase subunit I (COI) gene sequence has been widely and extensively utilised as a DNA barcode for animals. Unfortunately, its universality faces challenges, as amplifying the barcode region for the insect family Pseudococcidae has proven to be difficult (Palma-Jiménez et al., 2018; Park et al., 2010, 2011; Sethusa et al., 2014). In contrast, Ef-1$\alpha$ and 28S ribosomal RNA (28S rRNA) genes have been successfully used for the identification of mealybugs and other insect scales (Puig et al., 2021). Thus, in this research, we examine the effectiveness of the D2 and D10 regions of 28S rRNA gene for species identification of mealybugs in Tawau, Sabah.

## MATERIALS AND METHODS

### Sample Collection and DNA Extraction

In November 2023, some mealybugs were randomly collected from 14 year old oil palm fronds in KLK Plantation, Tawau, Sabah (4°26'47.5"N and 118°12'33.3"E), from 25 oil palms that were randomly chosen within 2 ha of the oil palm plantation. The samples were pooled to 0.05 g/tube making it one replicate. The pooled samples were then immersed in RNAhold to retain the DNA quality and kept in a -20ºC freezer. Total DNA extraction was carried out on three replicates by using the InnuPREP DNA Mini Kit according to the manufacturer's instructions (Analytikjena) with some modifications involving liquid nitrogen during the cell lysis process. The final resuspension was done by adding 150 µL of elution buffer, followed by storage at -20ºC freezer. Following the extraction, DNA quantification was determined using a NanoPhotometer spectrometer (IMPLEN P300 NanoPhotometer, CA, USA) and the Qubit™ dsDNA BR Assay Kit on Qubit 2.0 Fluorometer (Life Technologies, CA).

### Polymerase Chain Reaction (PCR) Amplification of EF-1$\alpha$ and 28S rRNA Genes

Three samples of mealybugs and three primer pairs, M51.9/rcM53-2, D2F/D2R and D10F/D10R were chosen in this study (*Table 1*). These primers were selected based on their effectiveness in amplifying EF-1$\alpha$ and 28S rRNA genes in the mealybug species (Downie & Gullan, 2004; Puig et al., 2021). Each 25 µL PCR reaction mix contained 2.5 µL of 10x reaction buffer, 0.3 µL TransStart *Taq* DNA Polymerase (TransGen Biotech Co., Ltd, Beijing, China), 1.0 µL of each primer (0.2 µM), 0.5 µL of 2.5 mM dNTPs, 1.0 µL BSA (Bovine Serum Albumin), 2.5 µL $MgCl_2$, 5 µL DNA template, and 11.2 µL of ultrapure water. The PCR analysis was performed using Biometra TADVANCE (MATRIOUX) under the following conditions: 4 min at 94ºC, 40 cycles of 1 min at 94ºC, 1 min at 54ºC, 1.30 min at 72ºC, and 1 min at 72ºC and held at 4ºC. All PCR products were visualised at 1% agarose gel stained using TransGen GelStain. The bands were excised and purified using EasyPure® Quick Gel Extraction kit (Transgen Biotech, China). The purified DNA samples were sent to Apical Scientific Sdn. Bhd., laboratory for Sanger sequencing.

### Sequence Similarity

Nucleotide sequences were assembled and edited where necessary, using Sequence Scanner 2 (ThermoFisher Scientific). The sequence similarity search was conducted using the Basic Local Alignment Search Tool (BLAST) with BLASTn as the database at the National Center for Biotechnology Information (NCBI) and the top match was selected based on the highest percentage identity. The EF-1$\alpha$, D2 and D10 amplified region sequences were deposited in Genbank with Accession Number: PP814618, PP158656 and PP158655, respectively.

**TABLE 1. PRIMER PAIR SEQUENCES OF ELONGATION FACTOR 1-A AND 28S RIBOSOMAL RNA GENES USED IN SPECIES IDENTIFICATION**

| Gene | Primer | Sequence (5′-3′) | References |
|---|---|---|---|
| Elongation factor 1-$\alpha$ | M51.9/rcM53-2 | F: CARGACGTATACAAAATCGG<br>R: CCGCGGCTGCTGGCACCAGA | Downie and Gullan (2004) |
| 28S ribosomal RNA | D2F/D2R | F: AGAGAGAGTTCAAGAGTACGTG<br>R: TTGGTCCGTGTTTCAAGACGGG | Puig et al. (2021) |
| | D10F/D10R | F: GTAGCCAAATGCCTCGTCA<br>R: CACAATGATAGGAAGAGCC | |

## RESULTS AND DISCUSSION

Considering the morphological identification of the pests is time-consuming due to their small size, identification using molecular techniques offers a promising alternative with quicker and more efficient results (Mwanauta et al., 2021). The cytochrome c oxidase subunit has proven to be difficult to amplify barcode regions in mealybugs (Malausa et al., 2011). The utilisation of molecular identification for insects heavily depends on the availability of reference libraries within the Genbank database.

*Nipaecoccus nipae* is commonly known as spiked mealybug, nipa mealybug, avocado mealybug, coconut mealybug, sugar apple mealybug, or Kentia mealybug due to the wide host range of this pest (Espinosa et al., 2009) described the size of the adult female mealybug range between 0.059 and 0.98 inches (1.5 and 2.5 mm) long, featuring an oval shape, exhibiting hues ranging from reddish-brown to orange. The surface is coated with yellowish-orange thick wax, with 10-12 pairs of marginal pyramid-shaped wax filaments (*Figure 1*). The honeydew excretions by this species during feeding can lead to the growth of the black sooty mould, which diminishes photosynthesis and attract ants that eventually protect the mealybugs from predators or parasitoids (Josephrajkumar et al., 2012). The insect is easily carried by the wind and transported by ants within and between different plant species (Mani et al., 2012). Aside from the COI gene's limitations to molecularly identify *N. nipae*, the ITS gene was also considered uninformative due to the absence of this gene in Genbank. Conversely, with its highly informative and conserved gene, the translation elongation factor 1-$\alpha$ (EF-1$\alpha$) was used instead within the diverse insect species (Cho et al., 1995). In addition, gene 28S rRNA gene sequence which is highly conserved also offers a potential utility in distinguishing the mealybugs species (Lang, 2023).

In this study, three primer pairs (M51.9/rcM53-2, D2F/D2R and D10F/D10R) of EF-1$\alpha$ and 28S rRNA gene produced a clear and single band in all tested replicates (*Figure 2*). Species identification using BLAST showed that the isolated mealybugs have a high percentage similarity with EF-1$\alpha$ and 28S rRNA gene from other *N. nipae*, ranging from 82.90%-92.79% (*Table 2*). Identification using EF-1$\alpha$ showed 86.08% identicality to *N. nipae* of Genbank Accession Number: AY427245.1, collected from South Africa. Meanwhile, D2 and D10 region amplification showed 86.69% and 92.79%, respectively to *N. nipae* of Genbank Accession Number: JQ651292.1 and AY427421.1, also from South Africa (Sethusa et al., 2014). Therefore, the mealybugs collected in this study were molecularly characterised as *N. nipae*.

In addition, our findings seemed to align with previous research that highlights the challenges of using COI and ITS genes for mealybug identification (Malausa et al., 2011). The consistent amplification and the high identity percentage obtained in this study using EF- 1$\alpha$ and two regions of 28S rRNA genes underscore their utility as reliable markers for *N. nipae* identification. Moreover, expanding the reference libraries in GenBank to include more sequences from diverse geographic regions and species will enhance the accuracy and reliability of molecular identification, facilitating a better pest management strategy. Precise identification allows for targeted control measures, reducing the spread and impact of *N. nipae* in oil palm plantation, particularly.

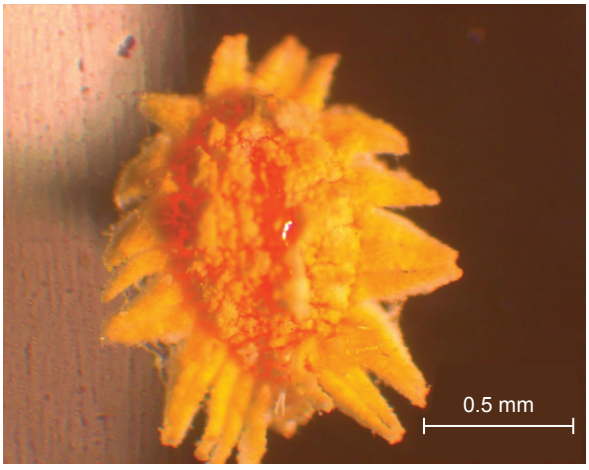


*Figure 1. A ventral view of a* Nipaecoccus nipae *isolate from Tawau under a compound microscope at 10× magnification.*

**TABLE 2. BLASTn RESULT FOR EF-1α AND 28S rRNA GENES AMPLIFICATION SEQUENCED WITH EF-1α, D2F/D2R AND D10F/D10R PRIMERS**

| Marker primer | Gene | Scientific name | Genus | Family | Query coverage (%) | Identity (%) | Accession |
|---|---|---|---|---|---|---|---|
| EF-1α | Elongation factor 1-α | *N. nipae* | *Nipaecoccus* | Pseudococcidae | 93 | 86.08 | AY427245.1 |
| | | *N. nipae* | *Nipaecoccus* | Pseudococcidae | 93 | 85.83 | EU188555.1 |
| | | *N. nipae* | *Nipaecoccus* | Pseudococcidae | 89 | 82.90 | AY427245.1 |
| D2 | 28S ribosomal RNA | *N. nipae* | *Nipaecoccus* | Pseudococcidae | 94 | 89.96 | JQ651292.1 |
| | | *N. nipae* | *Nipaecoccus* | Pseudococcidae | 94 | 86.33 | JQ651292.1 |
| | | *N. nipae* | *Nipaecoccus* | Pseudococcidae | 94 | 86.64 | JQ651292.1 |
| D10 | | *N. nipae* | *Nipaecoccus* | Pseudococcidae | 95 | 92.79 | AY427421.1 |
| | | *N. nipae* | *Nipaecoccus* | Pseudococcidae | 95 | 92.79 | AY427421.1 |
| | | *Nipaecoccus* aff. gilli PJG-2003 | *Nipaecoccus* | Pseudococcidae | 94 | 92.53 | AY427422.1 |

These findings highlight the effectiveness of using EF-1α and 28S rRNA gene for the molecular identification of *N. nipae*. Furthermore, the consistent amplification and success of the molecular approach used in this study suggest the applicability and reliability of these genes in the identification of mealybug species.

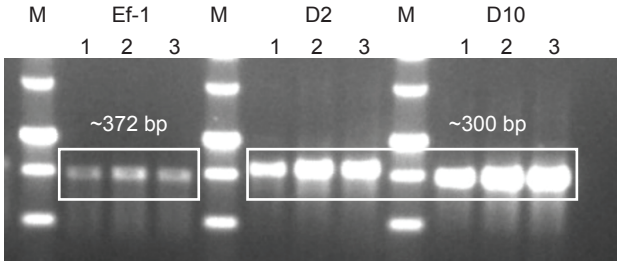


*Figure 2. Gel electrophoresis image displaying the amplified products of EF-1α and 28S rRNA genes from* N. nipae *isolate from Tawau. Lanes M indicate 1Kb ladder, while 1, 2, and 3 represent individual samples.*

## CONCLUSION

Although the techniques presented in this study were developed for *N. nipae* identification, they can also be utilised to identify mealybug species from other regions or for studying pest populations. The success of these molecular approaches, despite the limitations of the COI and ITS genes, demonstrate that alternative genes in this case, the EF-1α and 28S rRNA, have the potential to distinguish the mealybug species. To date, among the 2,005 identified mealybug species, 37 recognised species belong to *Nipaecoccus* sp. However, further research is necessary to acquire more DNA markers for the identification of mealybugs, particularly from diverse geographical regions.

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