

## Research Article

# New Report of *Dysmicoccus brevipes* Cockerell (1893) (Hemiptera: Pseudococcidae) on *Heliconia* sp., *Lagenaria* sp., and *Zea mays* L. Root in Bali Indonesia

I Putu Sudiarta<sup>1\*</sup>, Komang Saraswati Devi<sup>1</sup>, Gusti Ngurah Alit Susanta Wirya<sup>1</sup>, Putu Shinta Devi<sup>2</sup>, Febri Eka Wijayanti<sup>2</sup>, Made Getas Pudak Wangi<sup>1</sup>

1) Master Program of Agricultural Biotechnology Department, Faculty of Agriculture, Udayana University, Panglima Besar Sudirman Street, Dangin Puri, Denpasar, Bali, Indonesia, 80232

2) Bali Fish, Animal, and Plant Quarantine Center, Raya Sesetan Street No. 312, Pedungan, South Denpasar, Denpasar, Bali, Indonesia, 80222

\* Corresponding author, email: putusudiarta@unud.ac.id

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### ABSTRACT

The pink pineapple mealybug, scientifically known as *Dysmicoccus brevipes* (Cockerell) (Hemiptera: Pseudococcidae), can associate with all part of the plant, including the roots. Additionally, it plays crucial role as a carrier of plant viruses, highlighting its importance in relation to host plants. Reports of *D. brevipes* infestations on above-ground plant parts in Indonesia have been documented since 1917. However, there is a lack of data on the infestation of subterranean plant parts or roots by this organism which highlights the significance of this research. This article presents the identification of mealybugs on the roots of *Heliconia* sp., *Lagenaria* sp., and *Zea mays* L. from Bali, Indonesia, using a morphological method based on determination keys by Williams (2004) and a molecular method based on the MtCOI gene. The findings of this study suggested that the species observed on all three host plants was *D. brevipes*, making it the most recent record of *D. brevipes* presence on *Heliconia* sp., *Lagenaria* sp., and *Z. mays* roots in Bali, Indonesia.

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## INTRODUCTION

*Dysmicoccus* is a genus of mealybugs belonging to the Pseudococcidae, the family with the second most numerous species after Diaspididae in the Coccoidea (Morales et al. 2016). Most species of mealybugs from the genus *Dysmicoccus* are polyphagous and have the potential to cause economic damage to various host plants. Mealybugs can reside and reproduce on every component of plants, including the roots (Sartiami 2006). They survive by sucking plant nutrients, thereby disrupting the absorption of water and other nutrients. The presence of mealybugs on plants indirectly contributes to developing sooty mold. Sooty mold grows on the excretions of mealybugs, known as honeydew, and is a carrier for different plant viruses (Franco et al. 2009; Daane et al. 2012). Mealybugs can cause further symptoms such as leaf withering, leaf browning, hindered flower formation, and even the death of the host plant when the infestation is severe (Jansen 1995).

*Dysmicoccus brevipes* in Indonesia were first documented in 1917 through plant quarantine interceptions in Washington DC, USA, involving commodities from Bogor (Sartiami et al. 2016). This species has a widespread reputation for its ability to infest host plants, targeting various plant components such as leaves, stems, fruit, and even concealed plant structures. Kalshoven (1981) reported that *D. brevipes* found in Java live in colonies at the base of the fruit, leaf folds, and underground. Zarkani et al. (2023) reported the species commonly attacks watery rose apple, *Syzygium aqueum* Alston (Myrtaceae) in Bengkulu, Sumatera. This is supported by the statement by Williams and Watson (1988) that *D. brevipes* which belongs to the Pseudococcidae family is capable of inhabiting both above-ground and below-ground plant portions.

In late 2022, several farmers in Badung Regency, Bali, Indonesia, reported the occurrence of mealybugs on the root systems of crops, specifically *Heliconia* sp., *Lagenaria* sp., and *Zea mays* L. However, the presence of subterranean mealybugs complicates identifying the specific mealybug species. There is currently a lack of reports on mealybug attacks on plant roots in Indonesia. Although there have been various reports on the level of damage caused by mealybug on plant roots in Indonesia as reported by Adus and Pu'u (2021) level of damage caused by mealybug on ginger plant (*Zingiber officinale* Rosc) reached a severe intensity of 66-84 %. Additionally, Akhsan and Dewi (2024); Lisnawita et al. (2023) reported that Indonesia as a pineapple exporter with an export value of US\$102.67 million (80.06 thousand tons) is experiencing disruptions from mealybug which are known to be vectors of the Pineapple Mealybug Wilt-Associated Virus (PMWaV) shows a very high level of damage ranging from 73-92.3 % across various plant ages. This emphasises the significance of conducting research to determine the diversity of mealybug species in Indonesia and develop population control technology is crucial due to the potential economic losses it can cause.

## MATERIALS AND METHODS

### Sample collection

Specimens were collected from Badung Regency, Bali, Indonesia (8.82867°S 115.21582°E), in December 2022 after conducting personal interviews with farmers. Mealybugs sampling was carried out using the purposive sampling method. The brush was rubbed on the roots of *Heliconia* sp., *Lagenaria* sp., and *Zea mays* L., which showed symptoms of white wax filaments around the roots. Twenty to twenty-five female mealybug adults were collected from each host plant and preserved in 95 % alcohol.

### Morphological identification

The collected specimens of female mealybug imago were then taken to the Entomology and Biomolecular Laboratory of the Bali Fish, Animal, and Plant

Quarantine Centre to be prepared to be used as observation preparations. A total of 10 female mealybug from each host plant were successfully slide-mounted using modifications to the work instructions by the Agricultural Quarantine Center Test Laboratory of Indonesia (2016). This technique used chloroform and Essig's solution to remove wax and fat from the specimen, followed by heating and staining with acid fuchsin. The specimen was then placed on an object glass given Heinz solution and covered with a cover glass. Sample slides were then identified using an Olympus CX21 compound microscope at the Plant Pest and Disease Laboratory, Faculty of Agriculture, Udayana University.

Morphological characters that were used for parameters in this research included body shape and size, number of antennae, presence of eyes, shape and size of various types of pores, description of limbs, size of mouthparts, anal lobe, as well as a description of seta, circulus, ostioles, and cerarii. The morphological characteristics of the specimens obtained were then compared with the key determination of "Mealybugs of Southern Asia" by Williams (2004).

### Molecular Identification

One female imago from each host plant was selected for molecular identification conducted at the Plant Disease Laboratory, Faculty of Agriculture, Udayana University. The identification process started by isolating the total DNA using cetyltrimethylammonium bromide (CTAB), following the method established by Doyle and Doyle (1987). In this protocol, CTAB and  $\beta$ -mercaptoethanol solutions were prepared for each sample, and the mealybug sample was ground and powdered using liquid nitrogen. The CTAB buffer was added, and the sample was incubated for 60 minutes. Chloroform: isoamyl alcohol was added to separate fat, protein, and polysaccharides. The supernatant was transferred to a new tube, and sodium acetate, isopropanol, or absolute ethanol were added. The sample was then centrifuged at 12,000 rpm for 10 minutes, washed with 70 % ethanol, centrifuged at 8000 rpm for 5 minutes, and dried. DNA was resuspended using TE buffer or nuclease-free water and stored at -20 °C.

The process of DNA amplification was conducted in the mitochondrial cytochrome c oxidase subunit I (MtCOI) gene, which has proven to be effective in the identification of different insect species (Nurbaya et al. 2022; Sudiarta et al. 2023). The pair of primers used in amplification were designed by Park et al. (2011), namely PcoF1 5'CCTTCAACTAATCATAAAAA-TATYAG3' and LepR1 5'TAAACTTCTGGATGTCCAAAAAATCA3' which amplified at 649 bp.

The PCR reactions were conducted using a Veriti™ Thermal Cycler. The total reaction volume was 20  $\mu$ L, comprising of 10  $\mu$ L of 2X Vivantis RedTaq PCR master mix, 1  $\mu$ L of 10 pmol of each primer, 7  $\mu$ L of nuclease-free water, and 1  $\mu$ L of DNA template. The PCR program used was 94 °C for 5 minutes, 30 cycles at 94 °C for 1 minute, 52 °C for 35 seconds, 72 °C for 90 seconds, and continued with the final stage at 72 °C for 7 minutes. The PCR products were then visualized by electrophoresis using a 2 % agarose gel and 5  $\mu$ L of SMOBIO FluoroVue™ gel stain (10,000X) for 1 hour at 80 volts. The PCR product that was successfully amplified was then sent to FirstBase sequencing service (Malaysia) for purification and sequencing to obtain the nucleotide sequence of mealybugs found on the roots of *Heliconia* sp., *Lagenaria* sp., and *Zea mays* plants. The nucleotide sequences were further edited to remove regions with low-quality nucleotides using MEGA 11 software (Tamura et al. 2021). Next, the sample sequence was aligned with several sequences obtained from the National Center for Biotechnology Information (NCBI) GenBank® using the Basic Local Alignment Search Tool (BLAST). This process resulted in many reference sequences that exhibited similarity to

the sample sequence.

### Phylogenetic Analysis

The identification of mealybug species was conducted by comparing the nucleotide sequence of the sample with multiple reference sequences from GenBank. The proximity between the sample and the reference sequences was then calculated using a phylogenetic tree. Ten sequences of mealybugs of the genus *Dysmicoccus* and one sequence of *Bemisia tabaci* from Nigeria (MN164777) were obtained from GenBank and then selected to create a phylogenetic tree. Subsequently, all sequences were aligned using the ClustalW program in the MEGA 11 software. The Maximum Parsimony (MP) technique was used for constructing a phylogenetic tree due to its suitability for examining a limited number of sequences. MP employs a straightforward algorithm to identify the optimal tree (Dharmayanti 2011). Furthermore, Saitou and Imanishi (1989) asserted that the MP approach is highly effective in evaluating sequences with a base length of approximately 600 bp.

## RESULTS AND DISCUSSION

### Sign and Symptoms of Mealybug

When collecting samples, a consistent sign was the presence of white wax filaments around the roots of *Heliconia* sp., *Lagenaria* sp., and *Zea mays* L. These filaments could be found both in the soil and on the surface of the soil, as shown in Figure 1. The observable symptom during sample collection was the wilting of infected host plants and the yellowing leaves. Other symptoms that have been reported to appear due to *D. brevipes* attacks on plant roots are the decline in ginger production in East Nusa Tenggara, wilting in several plants such as pineapple, coffee, sugar cane, and bananas in Costa Rica (Williams & Granara de Willink 1992; Adus & Pu'u 2021). However, the sign and symptoms of attacks on plants caused by mealybugs are similar to those of other insects, such as white wax filaments and leaf chlorosis caused by whiteflies (De Barro 1995; Setiawati et al. 2007). Hence, the symptoms exhibited by the host plant during an attack do not provide conclusive evidence for detecting the presence of mealybugs on the roots. This is because mealybugs tend to target inaccessible plant parts, necessitating additional research to verify the symptoms caused by mealybug infestations on plant roots and their impact on reducing plant yields.



**Figure 1.** Living individuals of mealybugs on the host plant roots in Bali, Indonesia. A. *Heliconia* sp.; B. *Lagenaria* sp.; C. *Zea mays* L.

### Taxonomic Identification

Preparations of root mealybugs found on the roots of *Heliconia* sp., *Lagenaria* sp., and *Z. mays* from Bali, Indonesia, are presented in Figures 2B, 2C, and 2D. The morphological characteristics of the mealybug samples showed an average body length of 2.1 mm and were very similar to *D. brevipes*. Therefore, an illustration of *D. brevipes* by Williams (2004) is also presented in Figure 2A.

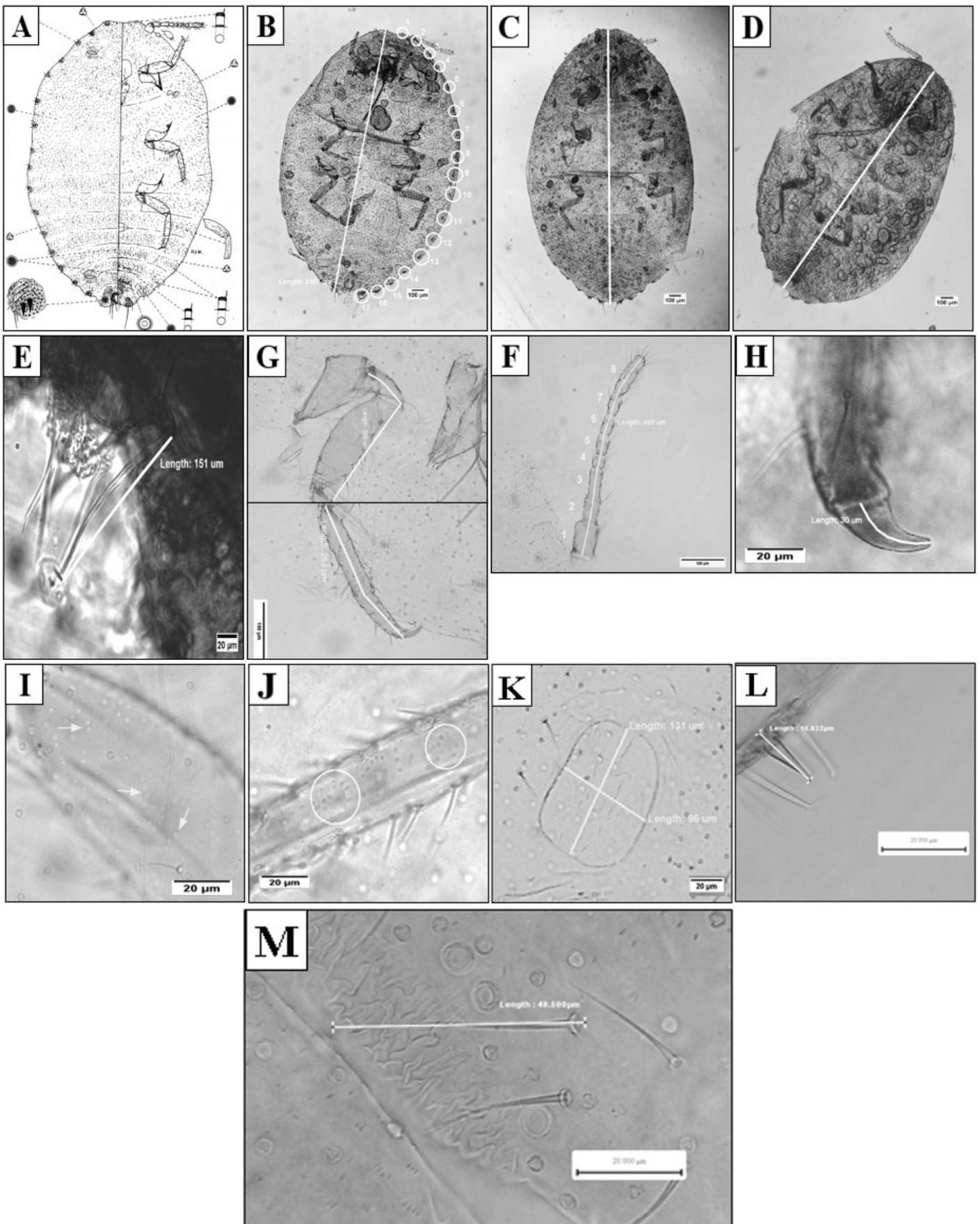
All samples showed the presence of 17 pairs of cerarii on the edge of the body (Figure 2B). The anal lobe was moderately developed and has setae on the ventral surface with an average length of 151  $\mu\text{m}$ . The anal ring had six setae with an average length of 151  $\mu\text{m}$  (Figure 2E); one pair of antennae, each with eight segments with a length of 435-465  $\mu\text{m}$  (Figure 2F); had eyes with 1-4 discoidal pores in each eye. The limbs were fully developed; the length of the hind trochanter + femur was around 306  $\mu\text{m}$ , and the hind tibia + tarsus was around 263  $\mu\text{m}$  (Figure 2G), and the claw was around 30  $\mu\text{m}$  (Figure 2H). Translucent pores were absent in the trochanter but very numerous in the hind femur and hind tibia (Figures 2I and 2J). It had a labium of about 290  $\mu\text{m}$ , which was longer than the clypeolabral shield. The circulus measured 96-131  $\mu\text{m}$  and was divided by intersegmenta lines (Figure 2K). Ostioles were fully developed. The cerarii of the anal lobe had two pointed setae 25  $\mu\text{m}$  long and 7.5  $\mu\text{m}$  wide at the base, six or seven auxiliary setae, and a group of trilocular pores. The anterior cerarii were smaller in size than the cerarii in the anal lobe, namely 17  $\mu\text{m}$  long with a basal width of 4.8  $\mu\text{m}$ . It had many long setae on both the dorsal and ventral parts of segment VIII, 40-80  $\mu\text{m}$  long. There were no multilocular pores on the dorsal side, but trilocular pores were evenly distributed. Discoidal pores had two sizes, large size with a diameter of 5  $\mu\text{m}$  in the subdominal area of segments V-VIII. The small size was spread out but not too much. On the ventral surface, there were multilocular pores with a diameter of 8  $\mu\text{m}$  on the posterior vulva to the edge of the abdominal segments VI and VII; sometimes 1-4 were found on the anterior abdominal segment VII. The trilocular pores were evenly distributed but were fewer in number than the dorsum.

The species *D. brevipes* is often discussed among entomologists because its morphological characteristics are similar to *D. neobrevipes*. Based on Williams and Granara de Willink (1992), several morphological characters are very similar in these two species, namely discoidal pores near the eyes, multilocular pores limited to the ventral segments VI, VII, and VIII, and the absence of oral rim tubular ducts throughout the body. The main difference between *D. brevipes* and *D. neobrevipes* is the body color of the adult female in real life. *D. brevipes* has a body color that tends to be pink, while *D. neobrevipes* has a gray body color. Beardsley (1992) stated that the main difference between *D. brevipes* and *D. neobrevipes* lies in the size of the dorsal abdominal seta in segment VII, which is longer than conical cerarian setae at around 45-80  $\mu\text{m}$  (Figure 2L and 2M), was also found in sample specimens of mealybugs from Bali, Indonesia, further strengthening the identification results. In addition, Yan-Biao et al. (2014) stated that *D. neobrevipes* tends to have sclerotisation or thickening on the ventral part, and the anal lobe tends to be elongated, which is not shown in the morphological characteristics of mealybug samples found in Bali, Indonesia.

### Molecular Analysis

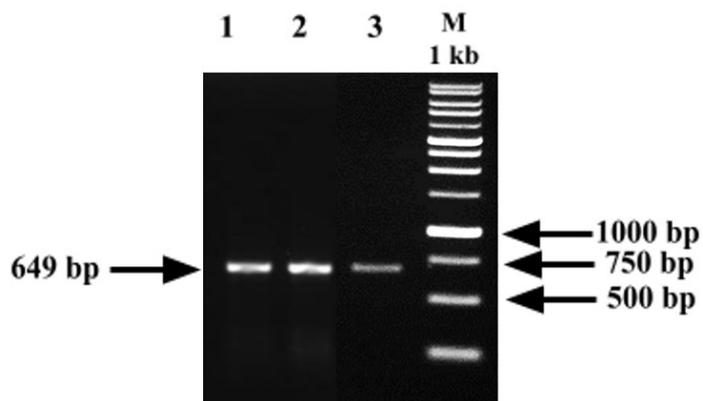
The accuracy of the morphological identification results was subsequently verified using molecular identification. The three mealybug DNA samples from Bali, Indonesia, were successfully amplified at a fragment length of 649 bp. The results of electrophoresis visualization are presented in Figure 3. The amplified DNA was then sequenced to obtain the nucleotide base sequence, which was then searched for species proximity using BLAST in GenBank. Based on BLAST results, other sequences close to the sample sequence were then used as reference sequences for phylogenetic analysis.

Phylogenetic analysis began with the alignment of root mealybug sequences from Bali and several comparative sequences using MEGA 11 software with the ClustalW program. The results of this alignment were then seen at the homology level presented in Table 1. It presents the genetic dis-



**Figure 2.** Morphological identification of *D. brevipes* specimens. (A) Illustration of *D. brevipes* by Williams (2004), (B) Samples of mealybugs on the roots of *Heliconia* sp., (C) Sample of mealybugs on the roots of *Lagenaria* sp., (D) Sample of mealybugs on *Zea mays* roots, (E) Seta on the anal ring, (F) Eight segment antenna, (G) Legs, (H) Claws, (I) Translucent pore on the hind femur, (J) Translucent pore on the hind tibia, (K) Circulus which was divided by intersegmental lines, (L) Conical cerarian setae, (M) Dorsal abdominal seta in segment VII and VIII. 4x, 10x, and 40x magnification

tances created through pairwise distance analysis between mealybug samples from Bali, Indonesia, and mealybug sequences from other countries. Based on this table, it can be seen that mealybugs from Bali, Indonesia, which were obtained from the roots of *Heliconia* sp., *Lagenaria* sp., and *Zea mays*, show a high similarity to *D. brevipes* with a range of homology percentages, namely 99.3 % - 100 % with a genetic distance of 0.000 - 0.002. Therefore, it was verified that the species of mealybug discovered in Bali was *D. brevipes*. These results are supported by the statement of Ptaszyńska et al. (2012) that using MtCOI fragments in molecular identification will indicate the same species in the 95.1 % - 100 % range. A statement by Hebert et al. (2003) also supports the correctness of the identification results, namely that genetic distances between sequences with a value of less than 0.03 can be declared as the same species. On the other hand, *D. neobrevipes*, which has morphological characters very similar to mealybug samples from Bali, Indonesia, shows a homology percentage range of 92.5 % - 92.7 %. Thus, it can be ascertained that the sample mealybugs were not *D. neobrevipes*.

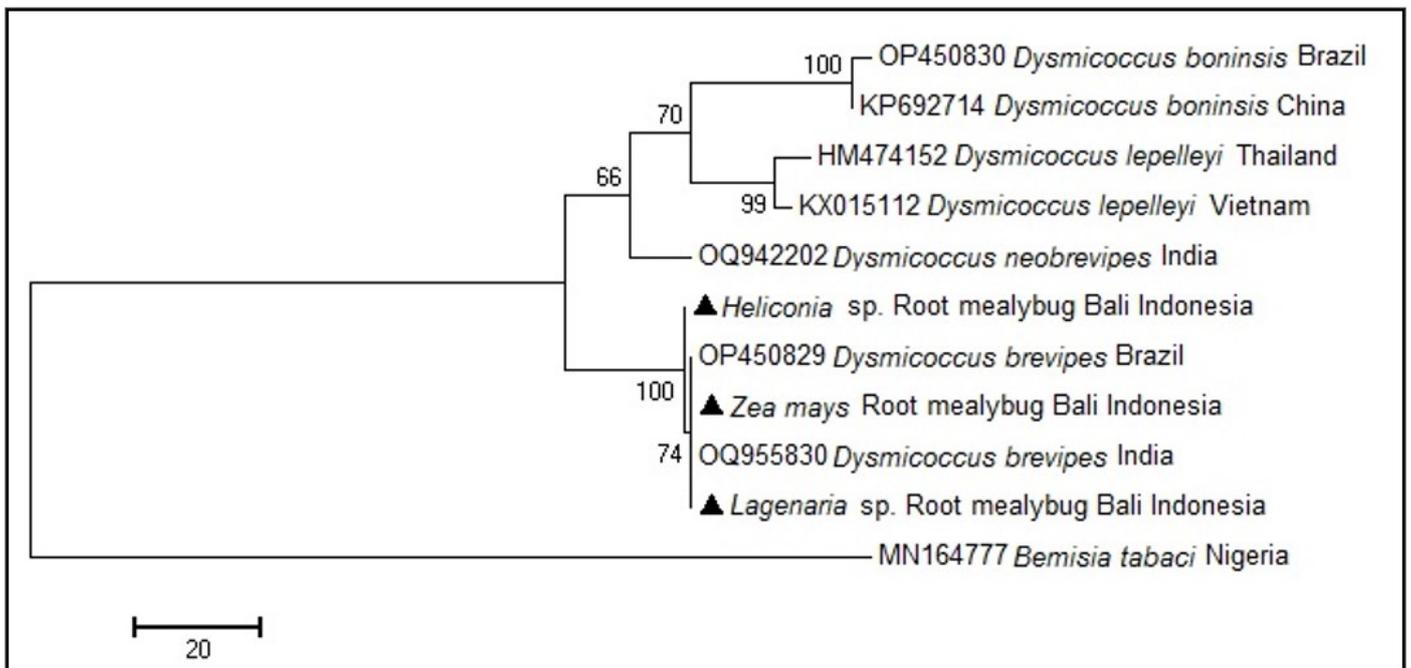


**Figure 3.** Results of *D. brevipes* DNA amplification using primers PcoF1 and LepR1 which were successfully amplified at 649 bp. Column 1 was a band of root mealybugs *Heliconia* sp., column 2 was a band of root mealybugs *Lagenaria* sp., and column 3 was a band of root mealybugs *Zea mays* L.

Molecular identification was continued with phylogeny analysis as a cladogram (Figure 4), created using the Maximum Parsimony (MP) method with bootstrap repetitions 1000 times. The results of the phylogenetic tree construction showed two main clades, namely the clade consisting of the sequence groups *D. boninsis*, *D. lepelleyi*, and *D. neobrevipes*. The other clade con-

**Table 1.** Level of homology and pairwise distance analysis of root mealybug sequences from Bali, Indonesia with mealybug sequences from other countries. \*RM = Root Mealybug

Sequence	Homology (%); Pairwise Distance			Accession Number
	<i>Heliconia</i> sp. RM	<i>Lagenaria</i> sp. RM	<i>Zea mays</i> L. RM	
<i>Dysmicoccus brevipes</i> India	99.3; 0.002	99.5; 0.000	99.5; 0.000	OQ955830
<i>Dysmicoccus brevipes</i> Brazil	99.7; 0.002	100; 0.000	100; 0.000	OP450829
<i>Dysmicoccus neobrevipes</i> India	92.7; 0.081	92.5; 0.083	92.5; 0.083	OQ942202
<i>Dysmicoccus lepelleyi</i> Thailand	90.4; 0.110	90.4; 0.111	90.4; 0.111	HM474152
<i>Dysmicoccus lepelleyi</i> Vietnam	90.4; 0.110	90.4; 0.111	90.4; 0.111	KX015112
<i>Dysmicoccus boninsis</i> Brazil	87.4; 0.142	87.4; 0.142	87.4; 0.142	OP450830
<i>Dysmicoccus boninsis</i> China	87.8; 0.136	87.8; 0.137	87.8; 0.137	KP692714
<i>Bemisia tabaci</i> Nigeria	45.4; 2.012	45.2; 2.042	45.2; 2.042	MN164777



**Figure 4.** Phylogenetic tree of root mealybugs *Heliconia* sp., *Lagenaria* sp., and *Zea mays* from Bali, Indonesia, compared with mealybug sequences from other countries based on the MtCOI gene with a bootstrap repetition value of 1000 times.

sisted of sequences associated with *D. brevipes*. From the cladogram construction, it is evident that the two clades have a common ancestor in comparison to the outgroup sequence. It was verified that the root mealybug species found in Bali, Indonesia, was the *D. brevipes* species. *D. brevipes* attacked *Heliconia* sp. *Lagenaria* sp. and *Zea mays* have been reported worldwide, but mealybug samples did not come from plant roots (Graham 1983; Williams 2004; Matile-Ferrero & Étienne 2006). This also shows that this research is the first report of *D. brevipes* on plant roots in Indonesia.

## CONCLUSIONS

The mealybug presence on *Heliconia* sp., *Lagenaria* sp., and *Zea mays* root specimen from Bali, Indonesia, has been confirmed as *D. brevipes* based on morphological and molecular analysis. This is the first report of the finding of *D. brevipes* presence on *Heliconia* sp., *Lagenaria* sp., and *Zea mays* root in Indonesia. It can be used as an initial reference for future study in order to further comprehend the distribution and level of damaged of *D. brevipes* on these three host plants.

## AUTHOR CONTRIBUTION

I.P.S. and G.N.A.S.W. contributed to the conception of the study. K.S.D. and M.G.P.W. collected samples and obtained data. K.S.D and P.S.D. contributed to the morphological identification. K.S.D. and F.E.W. contributed to molecular and phylogenetic analysis. I.P.S. contributed to the final editing. All authors read, critically revised, and approved the final manuscript.

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## CONFLICT OF INTEREST

Authors declare that there is no competing interest regarding the publication of manuscripts.

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