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Short Communications

The First Report of the Occurrence of the Root Mealybug *Ripersiella multiporifera* Jansen (2008) (Hemiptera: Coccoidea: Rhizoecidae) in Indonesia

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ABSTRACT

Ripersiella multiporifera is a root mealybug species within the family Rhizoecidae that has distinctive bitubular pores on the dorsal and ventral. This species was first discovered by Jansen (2008) in the Netherlands during an import interception of *Sansevieria* sp. from Indonesia and *Hoya kerrii* from Thailand. This species was also found during inspections in Sicily (Italy) on the roots of *Sansevieria trifasciata*. In Indonesia, there are no reports of the existence of *R. multiporifera*, emphasising the need for research on its presence. This work was conducted the morphological method based on modified determination key and the molecular method based on MtCOI gene. The identified species was *R. multiporifera*, and this finding represents the first evidence of *R. multiporifera*'s presence in Bali (Indonesia) which can be used as a reference for future research, especially in population control approaches.

Mealybug is a species of fauna that has a propensity for inhabiting and proliferating inside soil environments, often establishing colonies on plant roots. Awareness regarding the presence of root mealybugs within plant roots remains limited due to their concealed habitat and the constraints imposed by existing research approaches. Root mealybugs are known to infest both monocot and dicot plants, causing various symptoms, including leaf wilting, alterations in leaf colour, inhibited flower growth, and even the death of the host plant when the infestation is severe (Jansen 1995).

In Indonesia, a total of 370 species of mealybugs have been identified, 30 species of which are recognized as root mealybugs that inhabit plant roots, including family of Rhizoecidae (sixteen species), Pseudococcidae (eight species), and Xenococcidae (six species). *R. multiporifera* is a root mealybug species from the Rhizoecidae family which was initially documented as a novel species (Jansen 2008). The first report of this species was the import interceptions of the Dutch Plant Protection Service on *Sansevieria* sp. from Indonesia and *Hoya kerrii* from Thailand. Other data pertaining to this species was also found during inspections in com-

mercial nurseries by the Phytosanitary Service in Sicily (Italy) on the roots of *Sansevieria trifasciata* Prain (Mazzeo et al. 2023). Before being reported by Jansen (2008), the existence of *R. multiporifera* in Indonesia had not been documented, including "The Pests of Crops in Indonesia" (Kalshoven 1981). The existence of the root mealybug *R. multiporifera* species in Indonesia appears highly probable. Nevertheless, no official reports have proven its presence in this country. Therefore, this work is really important to conduct as an initial report on the species and its relationship with mealybug from other countries.

This research was carried out from March to May 2023, starting with a purposive sampling method on the roots of *Adenium* sp. plants in Denpasar City, Bali Province, Indonesia. Regarding Indonesia's tropical climate, the development of the mealybug occurs throughout the year. However, it tends to be higher during the dry season than rainy season with an estimated eight to eleven generations during February to November and two to three generations from November to January (Mani & Shivaraju 2016). The selection of *Adenium* sp. as the host plant is based on personal communication in late 2022 with several farmers and enthusiasts of *Adenium* sp. plants in Denpasar regarding the occurrence of mealybug infestations on the plant roots. The purposive sampling method was employed by obtaining 20-25 female imago mealybugs using a brush on *Adenium* sp. that exhibited symptoms characterized by white wax filaments around the root plants. The female imago mealybug root specimen was taken to the Plant Quarantine Entomology and Biomolecular Laboratory of the Denpasar Class I Agricultural Quarantine Centre and then slide-mounted for morphological identification. Ten specimens were successfully slide-mounted and identified using an Olympus CX21 compound microscope at the Plant Pest and Disease Laboratory, Faculty of Agriculture, Udayana University. Subsequently, the morphological characteristics of the specimen were compared with the determination key based on Mazzeo et al. (2023), adapted from Marotta (1992), Russo & Mazzeo (1992), Jansen (2008), and Jansen & Westernberg (2015). Molecular identification initiated by isolating female mealybug root DNA (Doyle & Doyle 1987), then continued with the PCR method with mitochondrial cytochrome oxidase subunit I (MtCOI). The MtCOI sequence's ability to successfully identify specific species in taxonomic groups in the Animalia kingdom has been verified; thus, it is possible to utilize it as a basis for a DNA barcoding marker (Hebert et al. 2003; Rahayuwati et al. 2016).

Based on the results of matching mealybug preparations with the determination key provided by Jansen (2008) (Table 1), there was one type of mealybug found on the roots of *Adenium* sp. plants, namely *R. multiporifera*. The general characteristics of *R. multiporifera* were an elongated to slightly oval body, white to yellowish white covered in white wax powder with brown antennae and legs. Morphological observations were carried out after slide mounting of female mealybug imago based on modified method work instructions published by Agricultural Quarantine Centre Test Laboratory of Indonesia in Tanjung Priok in 2016. In this method, specimens were cleared using chloroform and Essig's solution in the Syracuse dish, then heated and stained with acid fuchsin. Subsequently, the specimens were transferred to an object glass treated with heinz solution and then secured with a cover glass.

The identification results showed that the specimen has an average body length of 1.4 mm with an average width of 0.6 mm (Figure 1B). The female *R. multiporifera* imago described by Jansen (2008) has a length of 1.3 mm and a width of 0.7 mm (Figure 1A), had no eyes on the head

(Figure 1D), had an antenna 250–267 µm long, five-segmented and biceps (Figure 1E). There were two pairs of spiracles with a length of 41 µm with a peritreme width of 28 µm (Figure 1I). It has many trilocular pores spread across the dorsal and ventral parts with a size of 3.5–4 µm and 40-45 multilocular pores with an average diameter of 13 µm (Figure 1C). The labium with three joined segments is 100–127 µm long, and the clypeolabral shield is 145–167 µm long. It has two circles in the ventral abdominal segments II and III with a diameter of 43–52 µm (Figure 1G). Legs fully develop with hind trochanter + femur length 51–69 µm; hind tibia + tarsus 50–70 µm; and hind claw 9–11 µm long (Figure 1J). The anal ring is 63–71 µm wide with six setae 112-127 µm long (Figure 1H), a general characteristic of the *Rhizoecini*, such as the *Rhizoecus* and *Ripersiella*. The unique characteristic of mealybug root identification was the presence of small bitubular pores on the ventral and dorsal parts measuring 4.3–4.8 µm (Figure 1F). Based on Kozár & Benedicty (2004), the presence of bitubular pores on mealybug roots was the main characteristic in distinguishing the genus *Rhizoecus* and *Ripersiella*, which still come from the same family, namely *Rhizoecini*. The presence of multilocular pores on the ventral and dorsal parts of the head, as well as several bitubular pores on the ventral side, were also found in the identified specimen. Additionally, Jansen (2008) reported that *R. multiporifera* closely resembles to *R. saintpauliae* (Williams), but distinguishes itself by featuring up to 45 multilocular disc pores arranged in one to occasionally two rows on the posterior edges and single ones on the rest of segment. In contrast, *R. saintpauliae* has multilocular disc pores in small numbers, up to ten per segment, on the thorax and sixth abdominal segments. Furthermore, the distribution of multilocular disc pores in *R. multiporifera* apart from *R. hibisci*. *R. multiporifera* set out these pores on the head and single rows on the thorax and the initial two abdominal segments, accompanied by two circuli, whilst *R. hibisci*, potentially having 0-2 circuli, lacks multilocular disc pores on the head and shows single pores on the thorax and the first two abdominal segments. All of those characteristics were also found in the identified specimen and closely resemble those observed in the species *R. multiporifera*. However, morphological characters are complicated to ascertain, prompting the need for additional investigation to gather information about molecular characteristics.

The isolated DNA was then multiplied using Veriti™ Thermal Cycler by the PCR method using one pair of MtCOI primers that successfully amplified mealybugs DNA at 649 bp, namely the forward primer PcoF1 5'CCTTCAACTAATCATAAAAATATYAG3' and the reverse primer LepR1 5'TAAACTTCTGGATGTCCAAAAAAATCA3' (Park et al. 2011). The composition of PCR products in molecular identification was carried out in a total volume of 20 µL consisting of 10 µL PCR master mix, 1 µL forward primer PcoF1, 1 µL reverse primer LepR1, 7 µL nuclease-free water, and 1 µL DNA template. Meanwhile, 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 the final stage at 72°C for 7 minutes. The PCR products were then visualized by electrophoresis using a 2% agarose gel made by mixing 60 ml of TAE 1X buffer, 1.2 g of agarose gel, and 5 µL of gel stain. Electrophoresis was carried out for 1 hour at 80 volts (Figure 2).

The PCR product that was successfully amplified was then sequenced to obtain the base sequence of the mealybug root species found in the roots of the *Adenium* sp.. The base sequences obtained were then analysed for homology in GenBank using BLAST. BLAST results with similar homology to the root mealybug species from Bali were collected

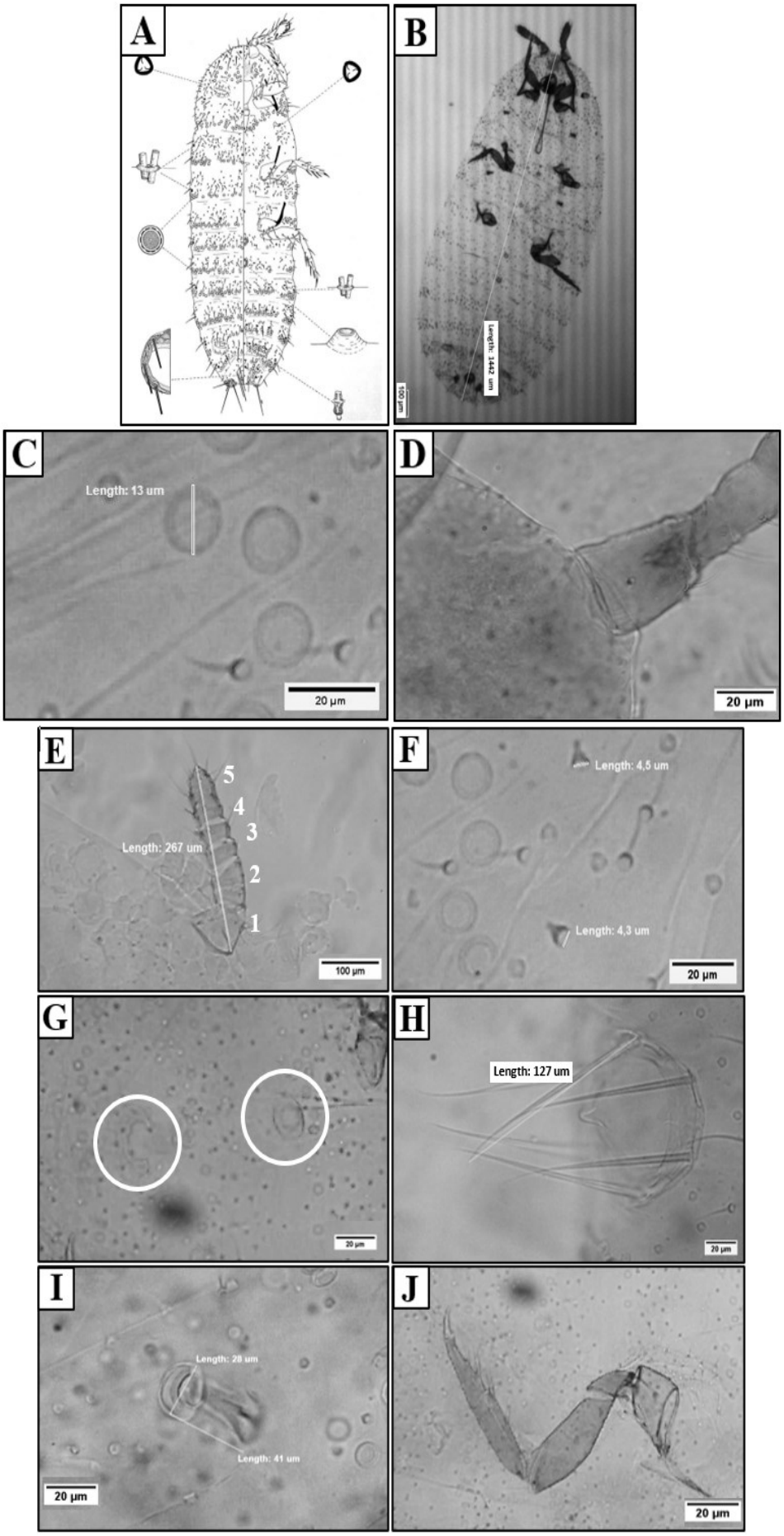

Figure 1. Morphological identification of *R. multiporifera* specimens. **A**. Detailed image. **B**. Female imago. **C**. Multilocular pores on the ventral side. **D**. Do not have eyes. **E**. Five-segment antenna. **F**. Bitubular pores on the ventral side. **G**. Two circulii in the second and third abdominal segments on the ventral. **H**. Six setae on the anal ring. **I**. Size of ventral spiracles on ventral. **J**. Limbs. Images were taken using an Olympus CX21 compound microscope at 4 x 10, 10 x 10, and 40 x 10 magnification.

Table 1. Determination key of mealybugs based on morphological identification (Mazzeo et al. 2023).

No.	Description	
1	Presence of bitubular or tritubular pores	2
	Absence of bitubular and tritubular pores ***Ripersiella potavae***	
2	Presence of bitubular pores and absence of tritubular pores	3
	Absence of bitubular pores and presence of tritubular pores	8
3	Multilocular disc pores present only on venter, with one circulus	4
	Multilocular disc pores present on venter and dorsum, with 0-2 circulus	6
4	With tubular ducts; bitubular pores short or long	5
	Without tubular ducts; bitubular pores of one size, scattered on dorsum ***Ripersiella maasbachi***	
5	Bitubular pores short, wide 2-3 times longer than wider ***Ripersiella periolana***	
	Bitubular pores long, narrow, 3-6 times longer than wide ***Ripersiella vidanoi***	
6	Circulus present, multilocular disc pores present on venter and dorsum, scattered on head and thorax, in rows across all abdominal segments ***Ripersiella lelloi***	
	Circulus present or absents, multilocular disc pores not with this combination of characters	7
7	Multilocular disc pores absent on head. Small type bitubular pores 5 µm wide present on venter and dorsum ***Ripersiella hibisci***	
	Multilocular disc pores present on head. Small type bitubular pores about 4 µm wide confined to venter ***Ripersiella multiporifera***	
8	Circulus present, multilocular disc pores absent	9
	Circulus absent, multilocular disc pores present	10
9	Labium 60-70 µm long ***Rhizoecus albidus***	
	Labium 75-90 µm long ***Rhizoecus cacticans***	
10	Antennae five-segmented ***Rhizoecus falcifer***	
	Antennae six-segmented	11
11	Tritubular pores of one size ***Rhizoecus dianthi***	
	Tritubular pores of 2-3 sizes	12
12	Tritubular pores of two sizes ***Rhizoecus latus***	
	Tritubular pores of three sizes ***Rhizoecus americanus***	

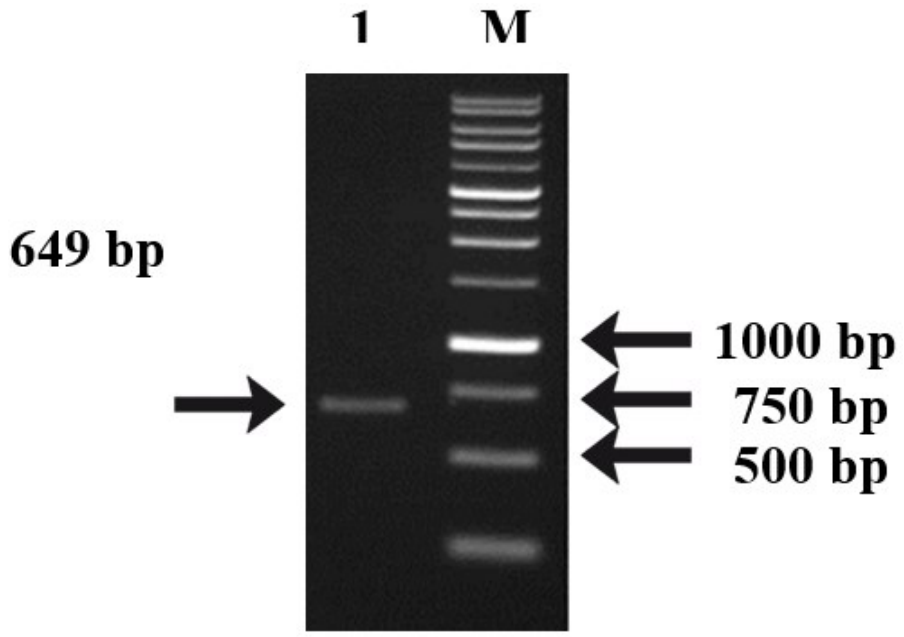

Figure 2. Results of DNA amplification of *R. multiporifera* using the PCR method with primers PcoF1 and LepR1. Columns 1 is the band of mealybug root DNA amplified at 649 bp. M = 1 kb.

for further alignment using BioEdit software with the ClustalW program. The obtained outcomes were subsequently transformed into an identity matrix displayed in Table 2.

The alignment of mealybug sequences with the MtCOI target gene obtained from Indonesia revealed 100% similarity with *R. multiporifera* Italy (OQ833547) and *R. multiporifera* Netherlands (KM453216). A phylogenetic tree was constructed based on sequencing data obtained through the utilization of MEGA 11 and BioEdit version 7.5.2, employing a bootstrap repetition of 1000 iterations using Maximum Parsimony method. According to the phylogenetic tree in Figure 3, the mealybug sequence found on the roots of *Adenium* sp. in Bali (Indonesia) is *R. multiporifera* based on GenBank. The findings of the identification matrix analysis indicate a significant degree of homology between *R. multiporifera* specimens from Bali (Indonesia) and those from Italy (OQ833547) and the Netherlands (KM453216). This further supports Jansen (2008) initial report regarding the finding of *R. multiporifera* in imported *Sansevieria* sp. from Indonesia in the Netherlands, as well as *R. multiporifera* found in Italy (Mazzeo et al. 2023) showing a homology percentage of 100% with *R. multiporifera* from Netherlands. Therefore, it is very likely that the root mealybug Italy specimen also came from Indonesia by imported plant since it was found in commercials nurseries (Mazzeo et al. 2023). Based on Ptaszynska et al. (2012) the MtCOI gene fragment indicated to have a similarity of species ranging from 95.1 to 100%. It can be concluded that the root mealybug found in Indonesia is *R. multiporifera*, which is the same as *R. multiporifera* found in the Netherlands and Italy. Therefore, this also clarify the low similarity value of *R. multiporifera* from Indonesia with *R. emarai* from Netherlands (82.1%) because they were different species.

The findings of the phylogenetic analysis indicate the presence of distinct group variations between *R. multiporifera* Bali and *R. hibisci* specimens collected from the Netherlands (KM453214), specifically 86.7%. Additionally, a lower degree of homology is observed, specifically 82.4%, between *R. multiporifera* Bali (Indonesia) and *R. dianthi* Netherlands (KM453217), as determined by sequence data obtained from GenBank. The percentage of similarity between *R. multiporifera* Indonesia and the genus *Rhizoecus* in Table 2 indicates higher results compared to *R. emarai* Netherlands (KM453217). This is attributed to discussion within Rhizoecidae, the genera *Ripersiella* and *Rhizoecus* have often been referred to synonymously for several times, making it highly likely that the genus *Rhizoecus* in the GenBank data used is either *Ripersiella* or vice versa (Mazzeo et al. 2023). However, currently both genera have been separated as a distinct genus in accordance with Choi & Lee (2022), who stated that the genus *Ripersiella* and the genus *Rhizoecus* form different groups because they are non-monophyletic or do not originate from the same ancestor. Meanwhile, the lowest percentage of homology and being an outgroup was shown by *R. multiporifera* from Bali with *B. tabaci* from Nigeria (MN164777) taken from GenBank, amounting to 46,6%.

The similarity of *R. multiporifera* from Bali (Indonesia) with *R. multiporifera* from Italy and the Netherlands is also proven by the pairwise distance analysis shown in Table 3. The results of the pairwise distance analysis show that *R. multiporifera* from Bali (Indonesia) has the smallest genetic distance value, namely 0,000 with *R. multiporifera* from Italy (OQ833547) and Netherlands (KM453216). Meanwhile, *R. multiporifera* from Bali (Indonesia) showed the largest genetic distance value with *Bemisia tabaci* from Nigeria (MN164777), namely, 0.961. The pairwise distance value indicates the genetic distance or level of similarity between

Table 2. Percentage of homology levels of *R. multiporifera* found in Bali (Indonesia) with other countries found in GenBank based on the MtCOI gene.

No.	Sequence	Homology (%)						
		1	2	3	4	5	6	7
1	Adenium root mealybug Bali Indonesia							
2	KM453216 *Ripersiella multiporifera* Netherlands	100						
3	OQ833547 *Ripersiella multiporifera* Italy	100	100					
4	KM453213 *Ripersiella emarai* Netherlands	82.1	82.1	82.1				
5	KM453215 *Rhizoecus hibisci* Netherlands	86.7	86.7	86.7	81.9			
6	KM453217 *Rhizoecus dianthi* Netherlands	82.4	82.4	82.4	85.1	81.9		
7	MN164777 *Bemisia tabaci* Nigeria	47.1	47.1	47.1	44.1	45.6	45.3	

Table 3. Pairwise distance value of *R. multiporifera* found in Bali (Indonesia) with other countries found in GenBank based on the MtCOI gene.

No.	Sequence	1	2	3	4	5	6	7
1	Adenium root mealybug Bali Indonesia							
2	KM453216 *Ripersiella multiporifera* Netherlands	0.000						
3	OQ833547 *Ripersiella multiporifera* Italy	0.000	0.000					
4	KM453213 *Ripersiella emarai* Netherlands	0.148	0.148	0.148				
5	KM453215 *Rhizoecus hibisci* Netherlands	0.210	0.210	0.210	0.220			
6	KM453217 *Rhizoecus dianthi* Netherlands	0.198	0.198	0.198	0.204	0.164		
7	MN164777 *Bemisia tabaci* Nigeria	0.961	0.961	0.961	1.026	1.127	1.054	

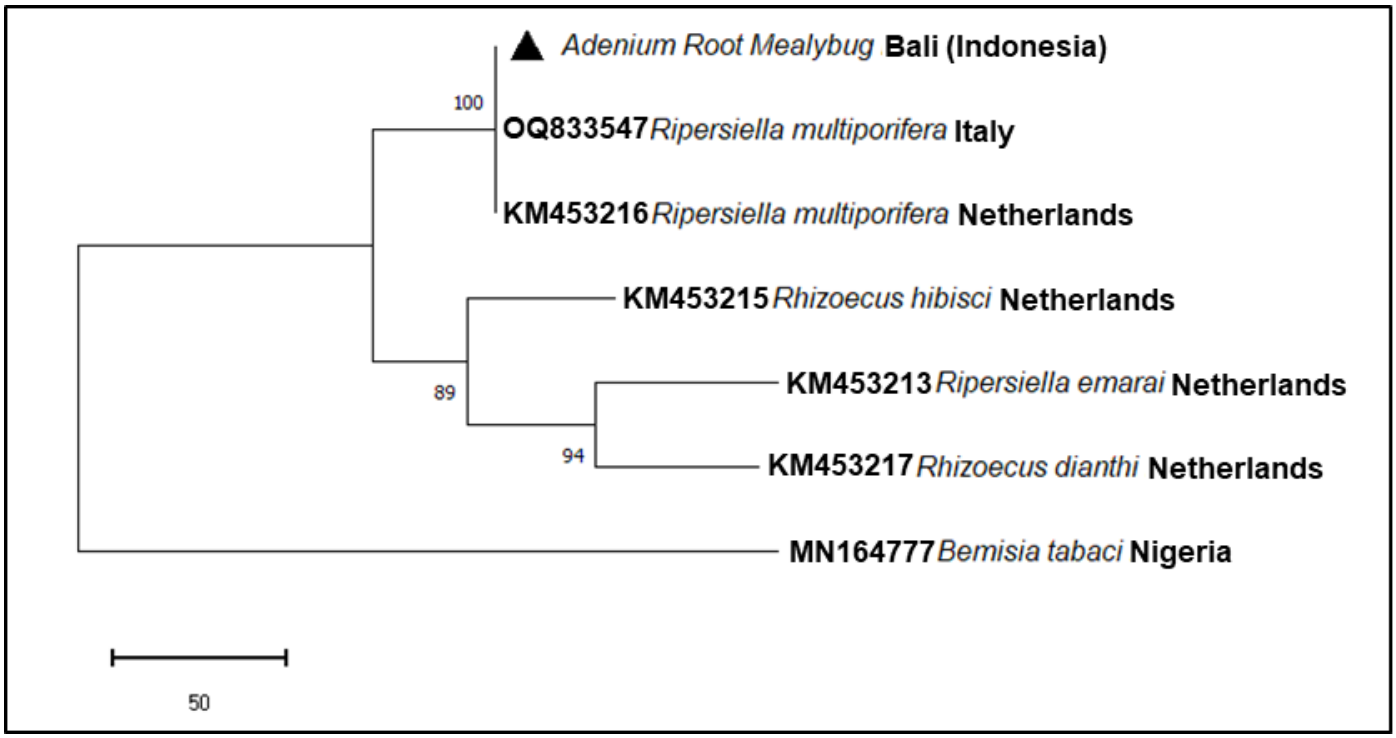

Figure 3. *R. multiporifera* Bali phylogeny tree is marked with symbols compared with several similar sequences taken from GenBank based on the MtCOI marker gene. ▲ *R. multiporifera* Bali (Indonesia); *R. multiporifera* Italy (OQ833547); *R. multiporifera* Netherlands (KM453216); *R. hibisci* Netherlands (KM453215); *R. emarai* Netherlands (KM453213); *R. dianthi* Netherlands (KM453217); *B. tabaci* Nigeria (MN164777).

sequences. A smaller genetic distance value indicates higher similarity between sequences. This was also stated by Hebert et al. (2003), who stated that the sequences with a genetic distance value less than 0.03 are considered to be the same species. This statement further strengthens that the mealybugs found on the roots of *Adenium* sp. in Bali (Indonesia) is *R. multiporifera*.

AUTHOR CONTRIBUTIONS

K.S.D., I.P.S., A.A.A.A.S.S., and G.N.A.S.W. contributed equally to the writing of the article. K.S.D. and I.P.S. collected samples from the field and finalisation of the manuscript. K.S.D and P.S.D contributed to the morphological identification stage. K.S.D and F.E.W. contributed from molecular identification to bioinformatics analysis.

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CONFLICT OF INTEREST

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

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