BIODIVERSITAS
Volume 25, Number 11, November 2024
Pages: 4067-4073

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d251106

Natural association of the entomopathogenic fungi *Aschersonia placenta* with spiralling whitefly (*Aleurodicus dugesii*) in Bali, Indonesia

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Manuscript received: 4 June 2024. Revision accepted: 8 November 2024.

Abstract. *Sudiarta IP, Suputra IPW, Mertaningsih NP, Wirya GNAS, Selangga DGW, Utama IWEK, Gargita IWD, Klett K, Yudha IKW. 2024. Natural association of the entomopathogenic fungi* Aschersonia placenta *with spiralling whitefly (*Aleurodicus dugesii*) in Bali, Indonesia. Biodiversitas 25: 4067-4073.* The whitefly is one of the most common pests in agriculture, and one highly polyphagous species is the spiral whitefly (*Aleurodicus* sp.). The fungal genus *Aschersonia* can be used to control *Aleurodicus* sp.. This research has potential applications in agriculture because it can produce a new environmentally friendly method to control whitefly infestations. The aim of this study was to analyze the morphology and molecular characteristics of *Aschersonia* and their hosts in Bali. The samples were taken from several areas in Bali Province. Morphological identification was carried out at the Plant Pathology Laboratory, Faculty of Agriculture, Universitas Udayana. The conidia of *Aschersonia* were fusoid with tapered ends, 9-16 µm long and 1.5-2 µm wide, and the color of the culture was white to yellowish white. Molecular analysis exhibited that DNA bands measuring between 500-600 bp were successfully amplified with universal primers ITS1/ITS4. *Aschersonia* infected spiralling whiteflies has never been reported in mulberry plants. Further phylogenetic analysis showed that *Aschersonia*-infected spiralling whiteflies were in the same group as *Aschersonia placenta*/ *Hypocrella raciborskii* isolates from Thailand and India. *Aleurodicus dugesii* Cockerell 1896 had morphological characteristics, such as vasiform orifice with lingula extending beyond the borders of orifice, compound pores present on puparia, and thoracic legs with claws. The results of morphological analysis showed that *A. dugesii* from Bali (LC491422) had the closest kinship to *A. dugesii* from the USA (AY521251). This is the first report of the identification of *Aschersonia* placenta associated with *A. dugesii* in Indonesia.

Keywords: *Aschersonia placenta*, biological control, insect pest, molecular, morphological, whitefly

INTRODUCTION

Whitefly is one of the most common pests in agriculture. Whiteflies are polyphagous insects with a very broad host distribution; these insects are widespread in the tropics and subtropics. The host range of these pests is very broad, ranging from citrus and vegetables to ornamental plants (Francis et al. 2016; Sulistyo and Inayati 2016; Hidayat et al. 2020). One of highly polyphagous species is spiralling whitefly (*Aleurodicus* sp.). It is more commonly known worldwide as spiralling whitefly because it lays eggs in a typical spiral pattern (Kumashiro et al. 1983; Hidayat et al. 2018). In Indonesia, spiralling whitefly attack 22 plants from 14 families, including ornamentals, shade and fruit trees, and annual crops (Kajita et al. 1991). The farmers basically use insecticide to control spiralling whitefly. However, this approach has many negative impacts. Therefore, more friendly control of this species is needed, such as the use of natural enemies from the parasitoid, predator and insect pathogen. At the end of 2017, in Pancasari Village, Buleleng District, Bali, Indonesia, entomopathogenic fungi were found infecting whitefly on mulberry plants. The species of whitefly, based on its morphology, was identified as spiralling whitefly. In addition, based on the shape and color of stroma, the fungal species belongs to genus *Aschersonia*.

Entomopathogenic fungi of the genus *Aschersonia* play an important role in controlling whitefly populations both in subtropical and tropical regions all around the world (Sani et al. 2020; Sudarjat et al. 2024). Indonesia, which belongs to a tropical country, is a suitable location for the growth of these fungi. Several types of whiteflies have been reported, which act as an important pest on various plants (Hidayat et al. 2018; Kurniawan and Fitria 2021; Selangga et al. 2023; Taufik et al. 2023). No studies have been reported on the origin of *Aschersonia placenta* in Indonesia. Four species of *Aschersonia* and their *Hypocrella* teleomorphs are *Aschersonia aleyrodis; Aschersonia andropogonis*, *Aschersonia placenta* and *Aschersonia* sp., *Hypocrella rhombispora* sp. nov (Ascomycota, Hypocreales, Clavicipitaceae) (Liu et al. 2006). *A. placenta* species are distributed in Indonesia, especially in Bali, and are found associated with whiteflies in citrus plants (Sudiarta et al. 2019; Suputra et al. 2019).

Aschersonia is an entomopathogenic fungus with considerable potential for controlling whiteflies (Qiu et al.

2013; Sudiarta et al. 2019). The pathogenicity of *Aschesonia* in controlling whiteflies has been studied for a long time. Wang et al. (2013) mentioned that certain types of *Aschersonia*, dosage, and condition, can cause 69-75% death in whitefly. It is important to identify *Aschersonia* and its host, the white fruit fly in Bali, Indonesia. This is in line with efforts to find safe and environmentally friendly methods to control white fruit fly pests. The aim of this study was to analyze the morphological and molecular characteristics of the insect pathogen *Aschersonia* and its host in Bali. The results of this study can also be used as a reference in utilizing the fungus as a biopesticide to control white fruit flies.

MATERIALS AND METHODS

Isolation of *Aschersonia*

This research was conducted in Pancasari Village, Sukasada Sub-district, Buleleng District (8°15'05.0"S 115°08'51.0"E), Belantih Village, Kintamani Sub-district, Bangli District (8°14'01.0"S 115°15'58.0"E), Buahan Kaja Village, Payangan Sub-district, Gianyar District (8°21'11.0"S 115°15'10.0"E), Cempaga Village, Bangli Sub-district, Bangli District (8°26'50.0"S 115°21'44.0"E), Kerta Village, Payangan Sub-district, Gianyar District (8°21'01.0"S 115°16'50.0"E), Sekaan Village, Kintamani Sub-district, Bangli District (8°19'01.0"S 115°19'50.0"E), South Batur Village, Kintamani Sub-district, Bangli District (8°15'55.0"S 115°20'20.0"E), Bali Indonesia (Figure 1) and Plant Pathology Laboratory, Faculty of Agriculture, Universitas Udayana. Isolation of *Aschersonia* sp. from host insects was conducted at the Plant Pathology Laboratory, Faculty of Agriculture, Universitas Udayana. *Aschersonia* was isolated according to the method of Liu et al. (2006) with slight modifications. For this, infected samples were dipped with distilled water (DW), so that conidia were dispersed in water. The dispersed conidia were transferred with a sterile Ose needle to the antibiotic-treated water agar. Plates were incubated for 5-6 days, and conidia of *Aschersonia* were transferred to PDA media. The culture of PDA was then incubated for 21 days at 23°C.

Morphological identification and characterization of *Aschersonia*

Aschersonia was identified morphologically on the basis of shape and color of the stroma, conidia, and culture characteristics on PDA plates, according to Liu et al. (2006).

Scanning electron microscope (SEM)

SEM procedure of *Aschersonia* samples infecting whiteflies using the method developed by Pusposendjojo (1985) with the following steps: Dried preparations were fixed with 2% glutaraldehyde solution and 4% osmium tetraoxide (OsO_4). Dehydration process used ethanol (70-100%), which was dried using an evaporation vacuum. The dried material was then attached to the specimen holder (aluminum stub) with colloidal silver paste and coated with gold metal (Au) (the thickness of the metal was approximately 15 nm) by following the evaporation process; then observed using a scanning electron microscope-SEM (Hitachi S 520). Observations were made visually on the photomicrograph results, which were processed with black and white Fuji film photos.

Morphological identification of whiteflies

Morphological identification of whiteflies using the puparial stage, and sample preparation began with mounting the slide. The morphological identification of whiteflies was mentioned in An Identification Guide to The Whiteflies (Hemiptera: Aleyrodidae) of The Southeastern United States by Gregory et al. (2005).

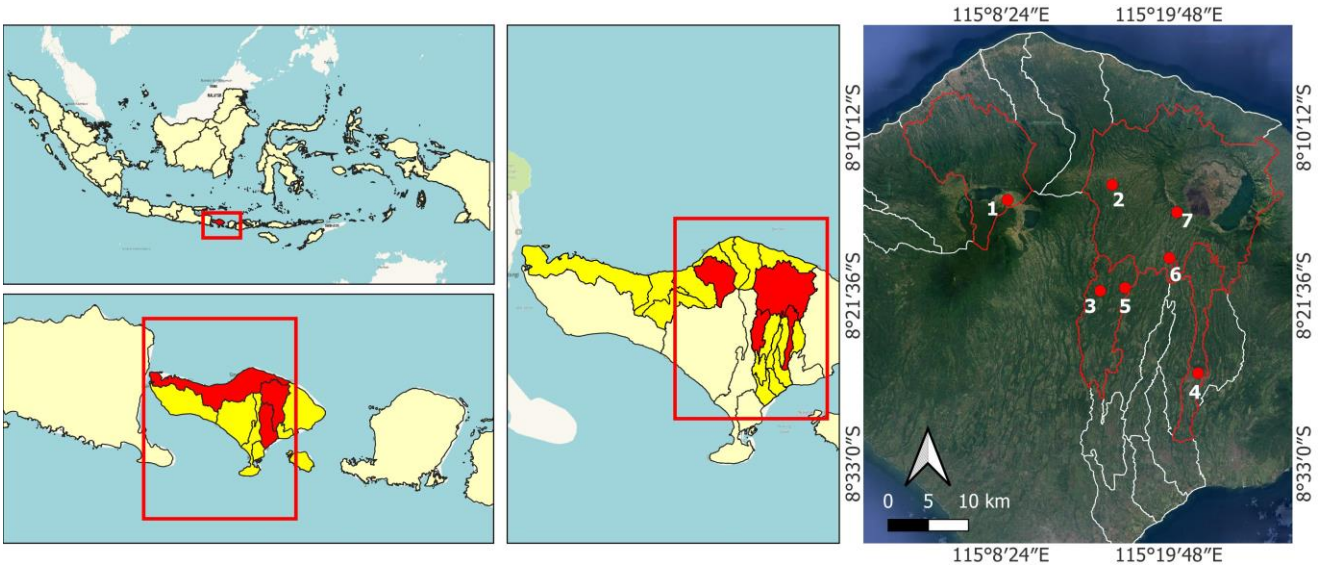

Figure 1. Distribution of *Aschersonia* in Bali, Indonesia. 1. Pancasari, 2. Belantih, 3. Buahan Kaja, 4. Cempaga, 5. Kerta, 6. Sekaan, 7. South Batur

Molecular identification

After the morphological identification of *Aschersonia* and whitefly, molecular identification was carried out. The molecular identification performed by PCR method was based on the large subunit (LSU) ribosomal DNA and mitochondrial cytochrome c oxidase subunit I (mtCOI) for entomopathogenic fungi and whiteflies, respectively.

DNA extraction by PCR (Polymerase Chain Reaction)

DNA extraction followed the protocol based on ZR Fungal/Bacterial DNA Kit™ Catalog No. D6005 by ZYMO RESEARCH. PCR amplification was conducted using MyTaq HS Red Mix (Bioline), with the target of amplification between 500-600 bp and 700 bp for rDNA of entomopathogenic fungi and mtCOI of whitefly, respectively. The PCR was started by preparing the PCR master mix (9.5 µL ddH20, 12.5 µL MyTaq Red Mix [2×], 20 µmol forward primer, 20 µmol reverse primer (Primers used in this study are listed in Table 1), and DNA template. In addition, PCR was conducted instages, namely 1 cycle initiation at 95°C for 5 min, followed by 30 cycles consisting of denaturation at 95°C for 1 min,annealing at 54°C for 1 min, extension at 72°C for 1 min,and 1 elongation cycle at 72°C for 5 min. The resultsobtained were then electrophoresed using a 0.5% agarosegel suspension that had been given 1 mL of ethidiumbromide (ETBr; 10 mg/mL, per 20 mL agarose) at 55 V for 70 min. The findings were then visualized using a DigiDocUV transilluminator (UVP, USA).

Electrophoresis

1 µL of PCR product (plus 2 µL of loading dye) was electrophoresed in a 1% TBE agarose gel for 30 minutes at 100 volts. The DNA that was electrophoresed was then visualized with a UV translator.

Sequence analysis

Sequencing was done at the Laboratory of Genetika Science Indonesia. The sequence results were analyzed to determine the level of homology or alignment with a sequence of rDNA for entomopathogenic fungi and mtCOI gen of whiteflies that were published in GenBank with the Basic Local Alignment Tool (BLAST) Program NCBI (www.ncbi.nlm.nih.gov).

Phylogenetic analysis

Phylogenetic analysis was performed using the software Chromas Pro, Molecular Evolutionary Genetics Analysis (MEGA 5.05), PAUP, BioEdit and TreeGraph2 after sequencing nucleotide data.

RESULTS AND DISCUSSION

Identification of entomopathogenic fungi *Aschersonia*

Isolation of Aschersonia

The prevalence of *Aschersonia* was found to be high in the field during sample collection (Figure 2). The whitefly population explosion was most likely caused by the *Aschersonia* population, which indicates that natural enemy populations always follow the development of the number or population of a particular pest (Rizali et al. 2021; Tricahyati et al. 2022). *Aschersonia* was discovered accidentally in Pancasari village. *Aschersonia*-infected spiralling whiteflies have never been reported in mulberry plants. Although the presence of *Aschersonia* was very high in mulberry plants, and did not interfere with the host plant, in fact, with the presence of *Aschersonia*, whitefly pest attacks can be controlled. The result showed that it can provide an overview of *Aschersonia's* effectiveness in suppressing whitefly populations.

Table 1. Primers used in the identification of *Aschersonia*

Primers name	Sequence (5'-3')	DNA target
ITS-1 forward	TCCGTAGGTGAACCTGCGG	ITS rDNA of fungi
ITS-4 reverse	TCCTCCGCTTATTGATATGC	
LCO forward	GGTCAACAAATCATAAAGATATTGG	mtCOI of whitefly
HCO reverse	TAAACTTCAGGGTGACCAAAAAATCA	

Figure 2. A. *Aschersonia* infected whitefly on mulberry leaf; B. *Aschersonia* infected whitefly pupa; C. *Aschersonia* colony and spiralling whitefly imago

In the present study, *Aschersonia* was grown in water agar medium. The most common technique for cultivating *Ashcheronia* is the method of Liu et al. (2006). This method isolates *Aschersonia* quite differently from the one used to isolate normally insect pathogenic fungi or other fungi. The most obvious difference is the use of water agar as the media for conidia germination of *Aschersonia*. In this method, water agar is used for the germination of the conidia of *Aschersonia* before being transferred to the PDA medium.

Morphological identification and characterization of *Aschersonia*

The identification of *Aschersonia* was based on the shape and color of the stroma, and colony and microscopic morphological features.

Stroma

The shape and color of stroma can be directly observed in the field and is usually located under the leaves where the host lives. SEM observations revealed that the stroma of *Aschersonia* sp. was a pack of tightly woven fungi mycelia. Mycelia was irregular, partial, and branched (Figure 3).

The color produced by the conidial masses in the stroma was orange to yellowish orange. The stroma was flat with a slight convex portion. Similar characteristics are also observed by Liu et al. (2006) in *Aschersonia aleyrodis* and *A. placenta* (Zhang et al. 2018; Sani et al. 2020).

Conidial form

The results of microscopic observation revealed that *Aschersonia* conidia were fusoid with tapered ends, 9-16 µm long and 1.5-2 µm wide (Figure 4). The results of these morphological characteristics indicate that it belongs to the genus *Aschersoni*a. Ingle et al. (2022) isolated *Aschersonia* from citrus blacky nymphs, it has hyaline, septate, branched, and smooth mycelium. When the stroma is cut transversely, a group of flask-shaped perithecia and fusoid conidia are 11-12 long and 1.3-2.5 µm in size.

Cultural characteristics on PDA

On PDA media, it had a convex and round surface with smooth edges. The color of culture was white to yellowish white. The culture had a mass of conidia that were yellow to yellowish orange. The surface texture of the colony was like fine cotton (Figure 5). These results are in accordance with previous findings by Sudiarta et al. (2019), which stated that *Aschersonia aleyrodis* and *Aschersonia placenta* have a flat stroma shape. The conidia produced are orange to yellowish orange. Liu et al. (2006) stated that *Aschersonia* culture on PDA media showed several variations.

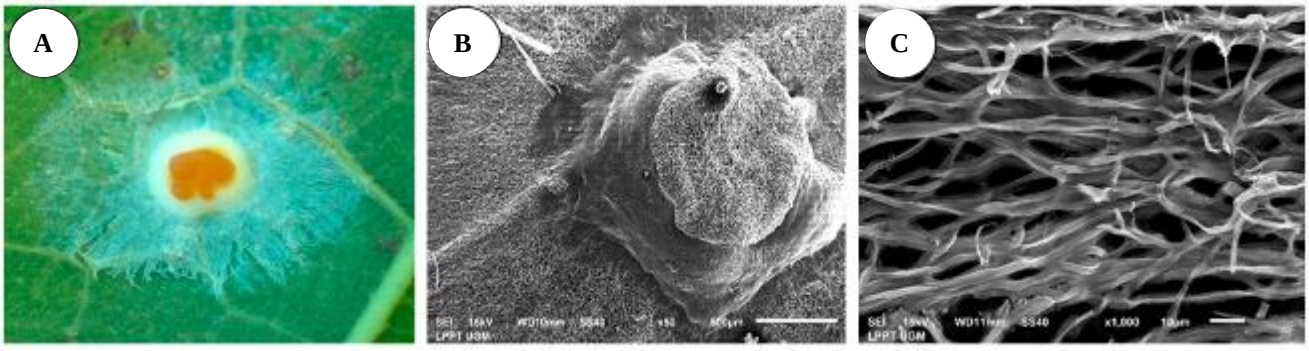

Figure 3. Stroma of *Aschersonia* on the fresh sample. A. Visual observation of stroma from the field; B. SEM observation of stroma; C. Pack of tightly woven mycelia

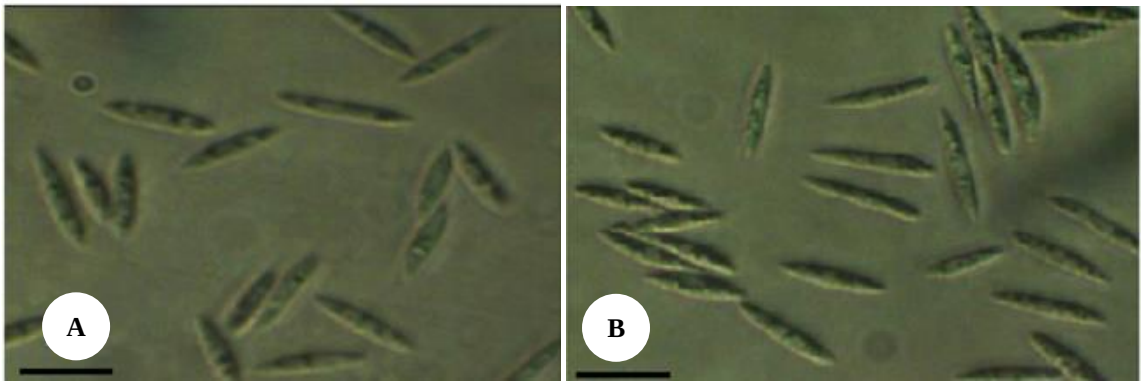

Figure 4. Fusoid conidia of *Aschersonia*. A. Conidia of *Aschersonia* from the field; B. Conidia of *Aschersoni*a from the growth medium. Bar: 10 µm

Many factors influence the development of *Aschersonia* in artificial media. Influential factors include the type of media used and temperature (Xie et al. 2016). The optimum temperature to grow *Aschersonia* on artificial media is 25°C (Sudiarta et al. 2019). This study also found that room temperature of approximately 28°C was unsuitable for the growth of *Aschersonia* on PDA media. At this temperature, *Aschersonia* did not grow well even after 3 weeks. The development of *Aschersonia* in artificial media is very important for its use as a biological agent to control whiteflies (Zhang et al. 2017; Pérez-González et al. 2022; Prayogo et al. 2022).

Molecular analysis of *Aschersonia*

The results of the molecular analysis showed that the amplified DNA target showed a base-length band between 500-600 bp. The emergence of DNA bands was important showing that PCR successfully carried out on the DNA sample was *Aschersonia* (Figure 6). The appearance of DNA bands is important information indicating that PCR has been successfully carried out on DNA samples believed to be *Aschersonia*. Based on molecular evidence amplified with the primer pair ITS-1 and ITS-4 (Ingle et al. 2022).

Phylogenetic analysis

The results of phylogenetic analysis on the sequences of *Aschersonia* culture from Jehem (LC489988) citrus whitefly and *Aschersonia* culture from Pancasari (LC489989) were in different clades. *Aschersonia* from Jehem (LC489988) and Pancasari (LC489989) formed a separate group but were still in the same group, indicating that the two samples were the same species. There was also an outgroup from the *Hypocrella raciborskii*/*A. placenta* group, indicating that the two species were from the genus *Aschersonia* and the species was *H. raciborskii*/*A. placenta* (Figure 7). Previous reports of the species *H. raciborskii*/*A. placenta* being an insect pathogen of *Dialeurodes* sp. (Sudiarta et al. 2019). Currently, the species *H. raciborskii*/*A. placenta* is known to have a distribution area covering: Cameroon, China, Ghana, India, India, Malaysia, New Guinea, the Philippines, Thailand, Indonesia, and Vietnam (Liu et al. 2006; Sudiarta et al. 2019).

Morphological identification of whitefly

It was observed that the full body shape of a whitefly was found on mulberry plants, as shown in Figure 8.

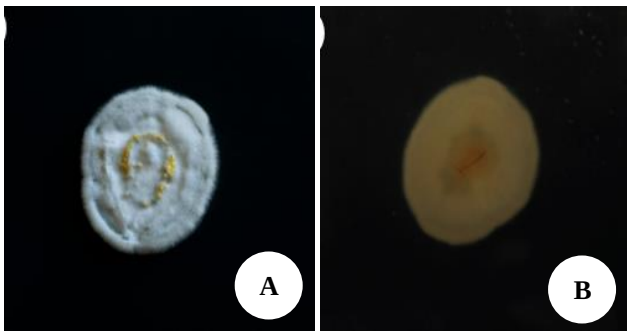

Figure 5. The colony of *Aschersonia* culture on PDA. A. *Aschersonia* front side; B. *Aschersonia* back side

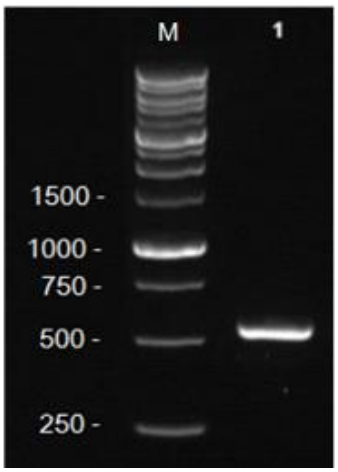

Figure 6. Result of DNA amplification of *Aschersonia*

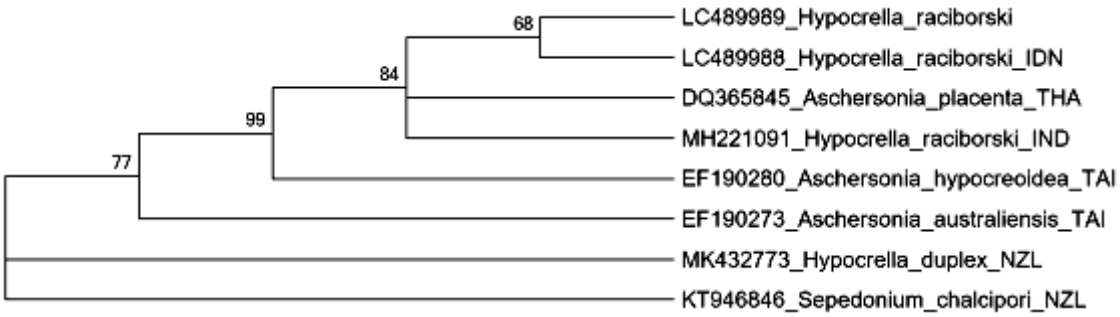

Figure 7. The phylogenetic tree arranged on the DNA composition of *Aschersonia* culture from Jehem (LC489988) of citrus whitefly as a control positive and *Aschersonia* culture from Pancasari (LC489989) with Maximum Parsimony method. The number in the branch represent the percentage of the group's trust level

The result of morphological characteristics of whitefly included following observations: (i) compound pores present on puparia with a central process (Figure 9.B); (ii) present thoracic legs, each with a claw (Figure 9.C); (iii) vasiform orifice with lingula extending beyond borders of orifice, lingula extending past vasiform orifice, 2 pairs of setae at the apex (Figure 9.A). Whitefly had 6 pairs of pores of the same size in segment III-VI with an elongated cone shape known as *A. dugesii.* Similar morphological characteristics were observed in *A. dugesii* (Hidayat et al. 2023).

Molecular identification of whitefly

Phylogenetic analysis

The sequence similarity between *Aleurodicus dugesii* from Bali (LC491422) and the order species from GenBank were analyzed. The results of phylogenetic analysis on *Aleurodicus dugesii* divide the sequence into several groups (Figure 10). *Aleurodicus dugesii* sequences form a separate group. The *Aleurodicus dugesii* sample then formed a wider branching with group AY521251 *Aleurodicus dugesii*, which indicates that both samples belonged to the same species. The highest nucleotide homology of the *A. dugesii* cytochrome oxidase (COI) DNA subunit I was found in eggplant plants in Bali, based on molecular evidence with amplification of LCO and HCO primer pairs (Yuliadhi et al. 2024). Hidayat et al. (2023) also reported that the partial COI sequence of *A. dugesii* Cipanas, West Java had more than 99% similarity to *A. dugesii* from the United States.

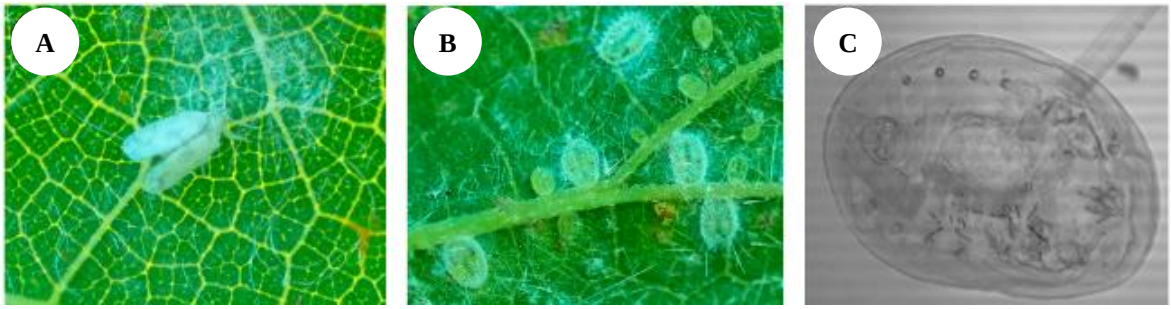

Figure 8. Full body shape of *Aleurodicus dugesii*. A. Imago of *Aleurodicus dugesii*; B. Nymph of *Aleurodicus dugesii*; C. Full body shape of *Aleurodicus dugesii* after preparation. Magnification 10x using stereo microscope. Bar: 100 µm

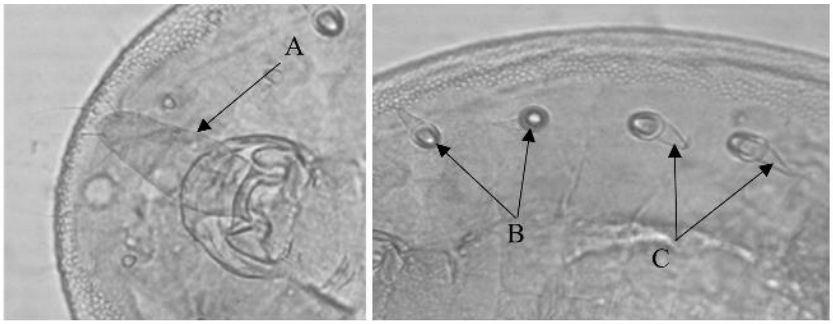

Figure 9. *Aleurodicus dugesii* after preparation. A: Vasiform orifice with lingula extending beyond borders of the orifice, B: Compound pores present on puparia, C: Thoracic legs with claws. Magnification 40x using a stereo microscope. Bar: 100 µm

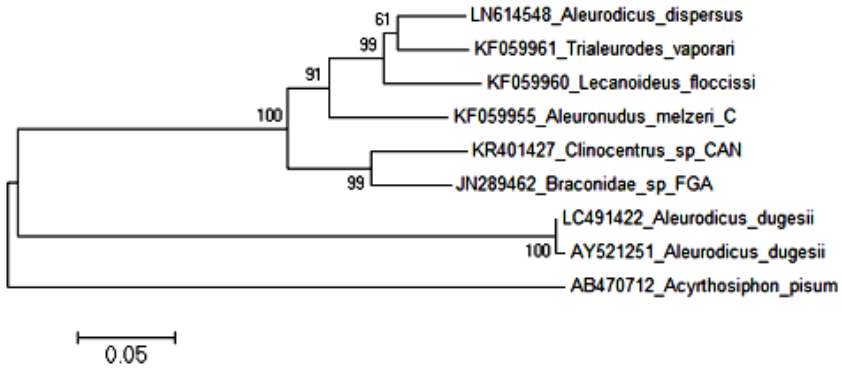

Figure 10. The phylogenetic tree was constructed based on the DNA composition of *Aleurodicus dugesii* with the Maximum Parsimony method. The number in the branch represents the percentage of the group's trust level

Based on the morphological characteristics and molecular analysis of rDNA sequences, it was concluded that the fungal species that infected spiralling whitefly on the mulberry plant in Pancasari Bali, Indonesia, was *Aschersonia placenta*. Based on morphological characterization and molecular analysis of MTCOI sequences, the host insect of *A. placenta* obtained from mulberry plants was identified as *Aleurodicus dugesii*.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to the Research and Community Services Institution, Universitas Udayana, Bali, Indonesia, which funded the research.

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