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Research Article

# First Report of *Cladosporium dominicanum Zalar*, de Hoog & Gunde-Cim. Infecting Whitefly on Ornamental Plants in Bali, Indonesia

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## ABSTRACT

As natural enemies, entomopathogenic fungi are essential for controlling certain plant pests, such as whitefly. Many types of entomopathogenic fungi can infect whiteflies, including those from the genera *Aschersonia*, *Metarhizium*, *Beauveria*, *and Cladosporium*. Currently, there is great interest in using entomopathogenic fungi as an environmentally friendly pest control in organic and sustainable agricultural systems. This study aimed to identify entomopathogenic fungus associated with whiteflies. Identification of entomopathogenic fungus was performed morphologically and molecularly using DNA barcoding with ITS-1 and ITS-4 as primers, in addition to sequencing and phylogenetic tree analysis. Whiteflies infected with entomopathogenic fungus were discovered on ornamental plants (*Premna serratifolia*, *Ficus religiosa*, *and Ficus rumphii* ) in Denpasar, Bali, Indonesia from January to March 2023. We found that samples from the field had mycelium that was unbranched or sparingly branched with solitary conidiophores arising terminally from ascending hyphae or laterally from plagiotropous hyphae. Samples from PDA media had ramoconidia with darkened septa, straight and conidia in long branched chains, branching in all directions, conidia were obovoid, ovoid to limoniform. The fungal colonies had the characteristics of being grey-olivaceous, olivaceous-black, and the margins were grey-livaceous to white, gray olivaceous in colony center due to abundant sporulation. In addition, based on the BLAST nucleotide sequence from NCBI GenBank, the insect pathogen phylogenetic tree found in Denpasar, Bali, is in the same clade as *Cladosporium dominicanum* strain SCAU014, accession number KY827344.1. Based on morphological and molecular analysis the fungus that infects whiteflies on ornamental plants is *C. dominicanum.*

## INTRODUCTION

Whiteflies are significant crop pests that cause direct and indirect damage (Lee et al. 2014). There are many species of whiteflies, (Hidayat et al. 2018) reported a dichotomous identification key of whiteflies, completed based on morphological characteristics of 35 collected species in Bogor, Indonesia. Whiteflies are also reported to be vectors of several plant viruses in tomatoes (Ghanim 2014) and chili peppers (Ayu et al. 2021; Temaja et al. 2022; Selangga et al. 2023). Whiteflies are reported to be polyphagous, attacking multiple kinds of vegetables, fruits, and ornamental plants (Syafitri et al. 2017; Hidayat et al. 2018). However, according to Mrosso et al. (2023), farmers' knowledge to control whitefly on tomato plants varies widely, so practical research is needed to determine the best management practices to control this pest. To control whiteflies the majority of farmers continue to use synthetic chemical insecticides. However, the application of these insecticides on a regular basis and without proper adherence to label requirements is detrimental to the environment and humans (Bajwa & Sandhu 2014). Consequently, more eco-friendly control measures are required.

One of the environmentally friendly controls is the application of natural enemies such as insect pathogens. Bacteria, fungi, and viruses play an important role as insect pathogens (Lovett & St. Leger 2017). There are many genera of fungi infecting insects such as whitefly. *Aschersonia, Metarhizium, Beauveria, and Cladosporium* are the common genera of fungi that infect whiteflies. The entomopathogenic fungi of the genus *Aschersonia* play a crucial role in controlling whitefly populations in subtropical and tropical regions worldwide (Sudiarta et al. 2019; Bhosale et al. 2020; Prayogo & Bayu 2020). In addition, the *Aschersonia placenta* was reported to infect the whitefly in citrus plants in Bali, Indonesia (Sudiarta et al. 2019; Suputra et al. 2019). An in vitro study was carried out to assess the pathogenicity of an *A. aleyrodis* isolate against blackfly and whitefly (Bhosale et al. 2020). On the other hand, *Metarhizium* has reportedly been highly effective in controlling whiteflies (Paradza et al. 2021, 2022). The pathogenicity of dry conidia and fungal suspensions of ten strains *Metarhizium anisopliae* and six strains *Beauveria bassiana* strains were evaluated against adults and second instar nymphs of the greenhouse whitefly (Paradza et al. 2021). In addition, *B. bassiana* was reported as a biological control of whiteflies. The effectiveness of *B. bassiana* against cotton whitefly (*Bemisia tabaci*) was analysed. *B. bassiana* isolate (Bb-01) was found to be the most effective with an LC50 value of (2.4×107 spores/ml) which caused the highest mortality of eggs (65.30%) and nymphs (88.82%) (Zafar et al. 2016). At the same time, the *Cladosporium* genus has also been reported to be able to infect whiteflies in several countries such as Bangladesh and Egypt (Abdel-Baky & Abdel-Salam 2003; Islam et al. 2019; Islam 2022). According to Islam et al. (2019), *Cladosporium cladosporioides* isolated from rice brown planthopper can control whitefly (*B. tabaci*). However, the information of *Cladosporium* infecting whiteflies in Indonesia is still limited, especially on ornamental plants. Therefore, additional research must be conducted on exploring, identifying, and applying *Cladosporium* as a biological control for whiteflies The aim of this study is in order to identify the entomopathogenic fungi *Cladosporium*, which was found on infected whitefly on ornamental plants (*Premna serratifolia, Ficus religiosa, and Ficus rumphii*). The identification was based on morphological and molecular characteristics using general DNA barcoding with primers ITS-1 and ITS-4. According to Sudiarta et al. (2019) the DNA barcoding has been used successfully to confirm the species of entomopathogenic fungi.

## MATERIALS AND METHODS

### Study location

Research exploring entomopathogenic fungus on whiteflies was conducted at the Experimental Garden of the Faculty of Agriculture, Udayana University, and bonsai hobbyists in Denpasar City, Bali, Indonesia from January to March 2023. Denpasar City, Bali, Indonesia is located in the lowlands with an altitude of ±100 meters above sea level. The observed host plants of whitefly were ornamentals (*Premna serratifolia*, *Ficus religiosa*, *and Ficus rumphii*). Samples of insects infected with the fungus were observed, photographed, and then taken to the Laboratory of Plant Diseases, Faculty of Agriculture, Udayana University.

### Morphological identification

Photos of living individuals of whitefly in the host plant and nature infection of *Cladosporium* in whitefly were taken with an Olympus OM-D camera, E-M 1, 50 mm Macro lens. Infected insect samples were observed under an Olympus CX21 Microscope, 40X/0.25, Optilab before isolating the entomopathogenic fungus on artificial media. The suspected entomopathogenic fungus of whitefly was isolated by washing the sample using sterile water and 70% alcohol and rinsing again with sterile water before planting on Potato Dextrose Agar (PDA) media. After 1-2 weeks, the fungus was sub-cultured to obtain pure cultures. Microscopic examination was conducted to describe the morphological characteristics of the fungal isolate (Bensch et al. 2018).

### Koch's postulate test

Furthermore, Koch's postulate test was carried out to ensure the fungus was an insect pathogen. The *Cladosporium* Bali isolate was cultured in Liquid Potato Dextrose media and then the conidia were examined under a microscope. The conidia were sprayed on the whitefly nymphs on the *Ficus religiosa* plants. While the control was sprayed with sterile water containing 0.02% tween 80. Whiteflies that have been treated were incubated for 20 days at 25OC. After 20 days of whitefly nymphs being observed, whitefly nymphs infected with the fungus were observed under a microscope to ensure the true presence of the *Cladosporium.*

### Molecular identification

After the fungus was confirmed to be an insect pathogen, molecular characterization was carried out. First, DNA extraction was completed before the fungal DNA was amplified by PCR. Extraction of total DNA was conducted on a single colony of fungal growth on a PDA medium on a modified method performed using CTAB Doyle and Doyle method (Sudiarta et al. 2019). Total DNA was used as a template for the Polymerase Chain Reaction (PCR). DNA amplification used primer set ITS-1 Forward (5'TCCGTAGGTGAACCTGCGG-3') and ITS-4 Reverse (5'-TCCCTCCGCTTATTGATATGC-3') targeting ± 560 bp of the ITS rDNA of Fungi (Sudiarta et al. 2019). The 1 µl of PCR product (plus 2 µl of loading dye) was electrophoresed in 1% TBE agarose gel. Electrophoresis was carried out for 30 minutes at 100 volts. The DNA that has been electrophoresed was then visualized with a UV transluminator. PCR products were then sequenced at 1st BASE Laboratories (Malaysia). Phylogenetic analysis was conducted by comparing the sample sequences and entomopathogenic fungi sequences and an outgroup comparison from GenBank. A phylogenetic tree of the entomopathogenic fungi was constructed using the Clustal X program (Thompson et al. 1997), Bio Edit 7.2.5 (Genious), and MEGA 6.06 (Tamura et al. 2013). The phylogenetic tree was constructed using Mega 6.06 (Algorithm Neighbor Joining with 1.000 bootstraps replicates) (Tamura et al. 2013; Nurbaya et al. 2022).

## RESULTS AND DISCUSSION

### Natural incidence of Cladosporium dominicanum in whitefly

This study was conducted on ornamental plants (*Premna serratifolia*, *Ficus religiosa*, *and Ficus rumphii* ) attacked by whitefly. Based on the insect morphology, it can be ascertained that the observed insect was a whitefly (Figure 1). According to Hidayat et al. (2018), whiteflies (Hemiptera: Aleyrodidae) are a group of small insects with a white color and soft body. In Bahasa Indonesia, this insect is called "kutukebul" because when flying in groups it looks like smoke ("kebul" in Javanese means smoke). The size of the whitefly nymph in the final instar was ±0.8-1 mm, while that of the imago was ±1-1.2 mm. Ornamental plants attacked by whiteflies were *Premna serratifolia*, *Ficus religiosa*, *and Ficus rumphii.* Those ornamental plants are a popular material for bonsai. The whitefly attacked the leaves of ornamental plants on the lower leaf surface (Figure 1), with symptoms of yellowing, mosaic and curling leaves. These whiteflies were not identified using the determination key or DNA barcoding. Further research is needed to confirm the identity of this whitefly species. However, evidence shows whiteflies are polyphagous, feeding on food crops, vegetables, fruit, and ornamental plants (Hidayat et al. 2018; Syafitri et al. 2017). After knowing that the insect attacking the leaves of the ornamental plant is a whitefly, observations were made of the fungus infecting the whitefly.

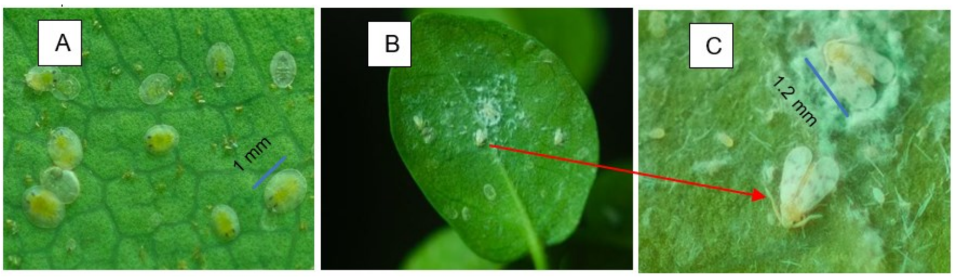

**Figure 1.** Living individuals of whitefly in the host plant (*Premna serratifolia*, *Ficus religiosa*, *and Ficus rumphii* ). A) Whitefly nymphs; B,C) Whitefly imago.

Infected whiteflies were covered with a bluish-gray stroma with varying degree of mycosis (Figure 2) wherein some infected nymphs were mummified with light fungal growth (Figure 2a) and profuse uniform growth of grey-black stroma containing spores at a later stage of infection (Figure 2b,c) in comparison with healthy whiteflies (Figure 2d). Observations were made under a light microscope to clarify the presence of mycelia on whitefly collected from the field. The symptoms of fungal infection are clearly visible outside the nymph's body (Figure 3A,B), the appearance of these symptoms after the pathogenic fungal hyphal comes out of the insect cuticle. The mechanisms are highly complex involving gene interactions between insect pathogenic fungi and insect hosts. Wang & Wang (2017) reported by using species in the *Beauveria* and *Metarhizium* genera as models, molecular biology studies have revealed the genes that function in fungus-insect interactions and thereby contribute to fungal virulence. The observations revealed that black-green mycelia and conidia could be seen emanating from the body of the whitefly. Unlike entomopathogenic bacteria and viruses that mode of entry to cause diseases after oral ingestion, entomopathogenic fungi infect insects via direct penetration of the surface of the insect (cuticle) and enter the body of the insect, multiplying in the body cavity (hemocoel). The contact mode of entry of entomopathogenic fungi is dependent on a biochemical mechanism using enzymes such as chitinase (Wang & Feng 2014; Lovett & St. Leger 2017).

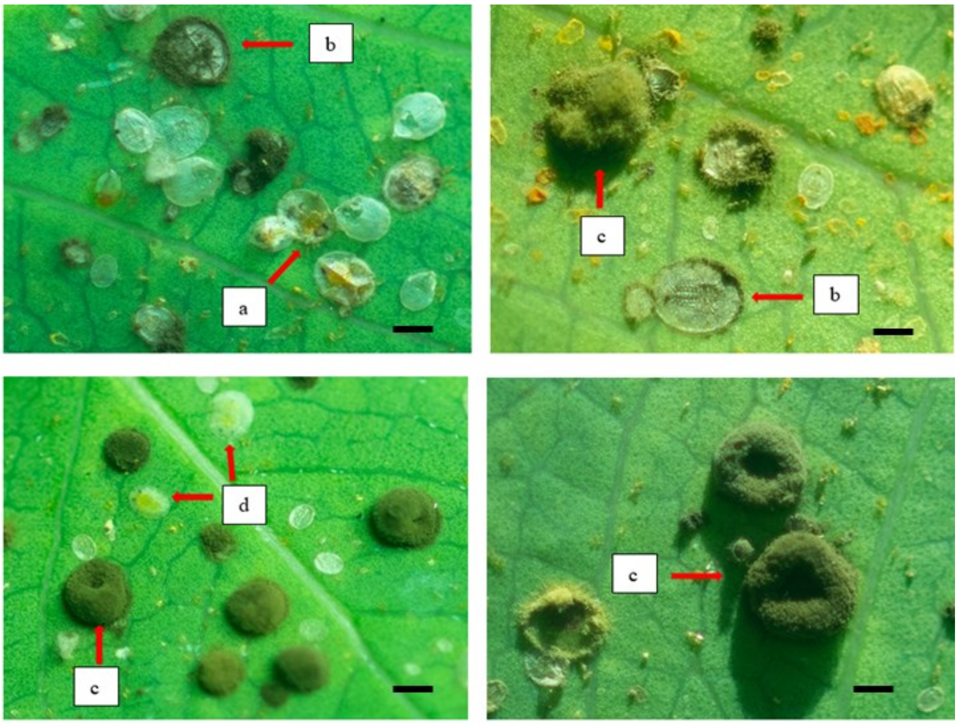

**Figure 2.** The variation of nature infection of *Cladosporium dominicanum* in whitefly. a) nymph death, nymphs were infected in the early phase; b) mycelia with spores have begun to appear in nymphs; c) infected nymphs in the late phase (stroma); d) healthy nymph, scale bars 0.5 mm.

## Morphological characteristics of pathogenic fungi on whitefly

Morphological identification was carried out by observing the shape and colour of the fungal colonies on PDA media, conidial development and branching patterns of conidial chains, and general morphology and size of conidiophores and conidia (Bensch et al. 2018). The morphological observation was carried out on the fungal-infected whiteflies upon field collection and on the isolated fungus grown on PDA. Samples taken directly from the field had mycelium that was unbranched or sparingly branched with solitary conidiophores arising terminally from ascending hyphae or laterally from plagiotropous hyphae (Figure 3D, E). Samples from PDA media had ramoconidia with darkened septa, straight (Figure 3F, Arrow) and conidia in long branched chains, branching in all directions (Figure 3C); conidia were obovoid, ovoid to limoniform (Figure 3F). In addition, the results of observations showed that the fungal colonies that attacked whiteflies on ornamental plants in Denpasar, Bali, which were cultured on PDA media, had the characteristics of being grey-olivaceous, olivaceous-black, and the margins were grey-livaceous to white, gray olivaceous in colony center due to abundant sporulation (Figure 4A,B). On PDA media, the size of the fungal colony after 14 days after planting was around 35 mm. The hyphae observed from PDA media were the same as hyphae from field collection (Figure 4C,D). Based on these morphological characteristics, the fungus that attack the whitefly is predicted as *Cladosporium* (Bensch et al. 2018; Islam et al. 2019). The similarity of conidiophores and conidia both in shape and size in the genus *Cladosporium* was so high that it was difficult to distinguish between species. There are three *Cladosporium* species complexes: *C. dominicanum*, *C. herbarum*, and *C. sphaerospermum* species complexes (Bensch et al. 2018). It is necessary to confirm identification with a DNA barcoding approach using ITS with design primers.

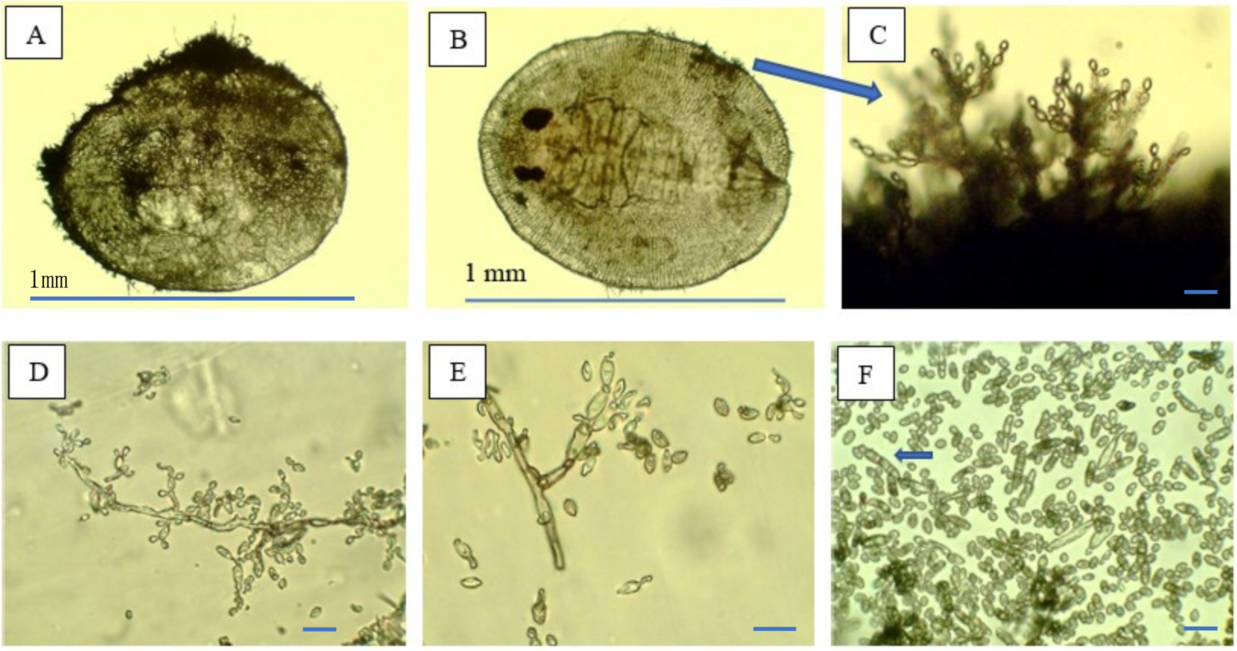

**Figure 3.** Microscopic observation of the fungus *Cladosporium* attacking whiteflies isolated directly from the field. A-B) Nymphs infected with *Cladosporium* fungus; C-F) Macronematous conidiophores and conidial chains. Arrow is ramoconidia.

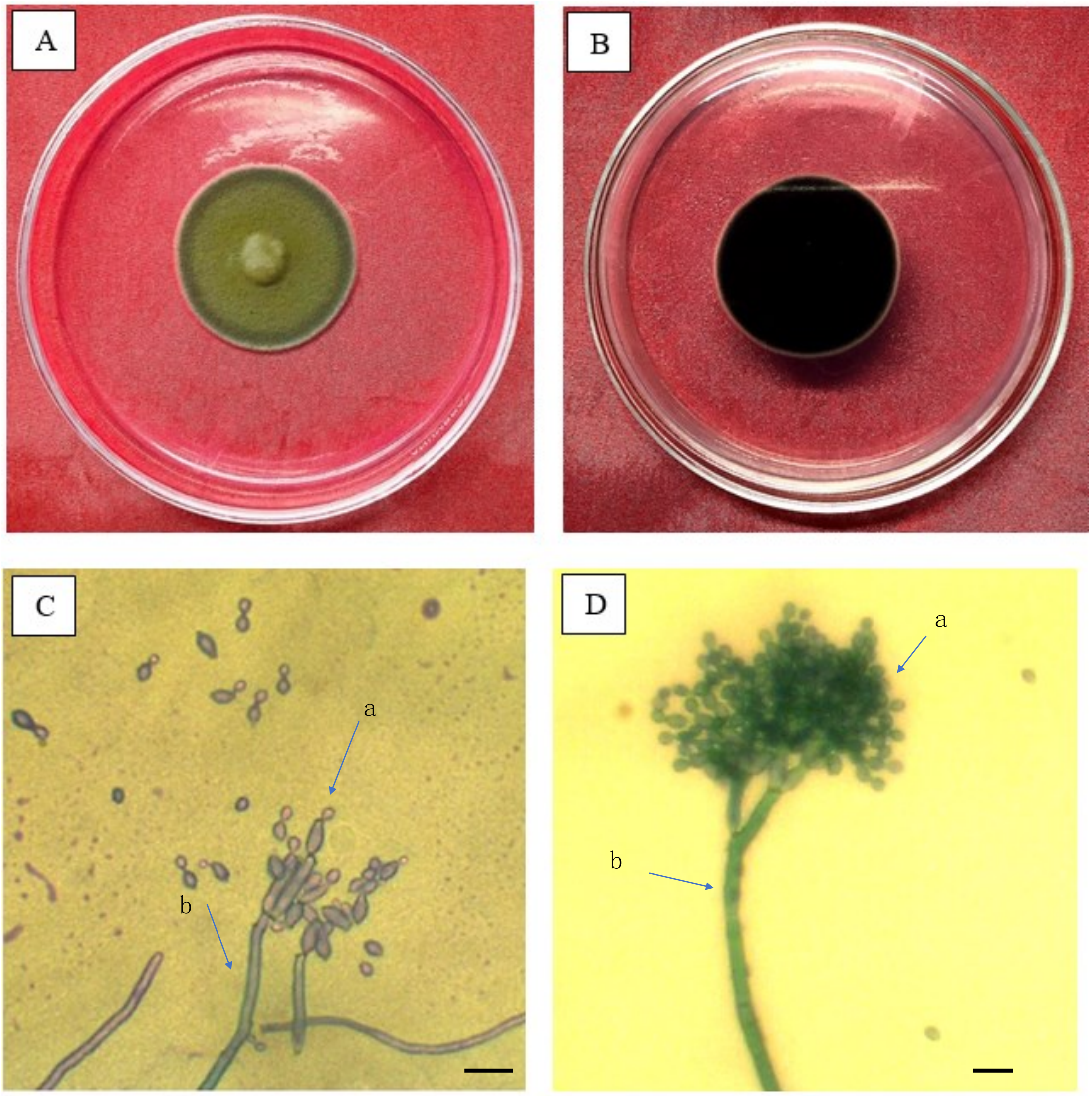

**Figure 4.** Culture of the fungus *Cladosporium* Bali isolate on PDA media 14 days after inoculation. A) Front view of fungal colony on PDA media; B) Rear view of fungal colony on PDA media; C,D) Conidia (a) and conidiophore (b), scale bars 10 μm.

## Koch's postulate assay

The observation was carried out after 20 days the insects treated with the *Cladosporium* showed symptoms similar to the field observation. The symptom was observed, some infected nymphs were mummified with light fungal growth and profuse uniform growth of grey-black stroma containing spores at a later stage of infection (Figure 5A) in comparison with healthy whiteflies (Figure 5B). Uninfected nymphs were emerged as adults, marked by the remaining pupae shown in Figure 5B. The data indicated the control was not infected by *Cladosporium.*

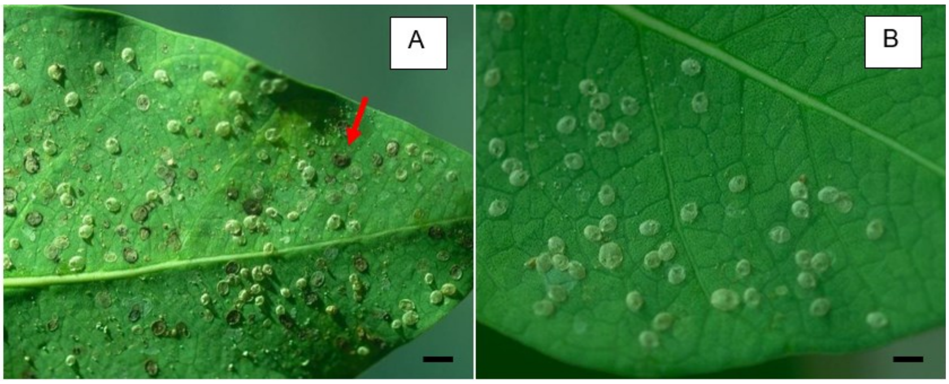

**Figure 5.** Koch's postulate assay result. A) The infected nymphs symptom (arrow); B) Healthy nymph, was emerging to imago, scale bars 2 mm.

## Molecular characters of pathogenic fungi on whitefly

PCR was performed with the Infinigen PCR machine, the ±560 bp ITS target gene was successfully amplified. PCR product results were visualized with an agarose gel size of 560 bp (Figure 6A). These PCR product was then used for sequence and phylogenetic trees analysis. From the results of the BLAST search, the entomopathogenic fungus found in Denpasar, Bali, has an affinity with *Cladosporium dominicanum* strain SCAU014, accession number KY827344.1 (Per. Ident and Query Cover 100%) and *C. dominicanum* strain SCAU099, accession number MF061761.1. The insect pathogen phylogenetic tree found in Denpasar, Bali, is in the same clade as *C. dominicanum* strain SCAU014, accession number KY827344.1 (Figure 6B). Based on those information the entomopathogenic fungus species associated with the whitefly from Denpasar, Bali is same to *C. dominicanum.* Barcoding DNA using ITS to confirm the morphological character of entomopathogenic fungi was commonly used, our previous study succeeded in identifying how the entomopathogenic fungi *Aschersonia placenta* infects the citrus whitefly using primers ITS-1 and ITS-4 (Sudiarta et al. 2019). According to Alfiky (2022) based on ITS sequence analysis and phylogeny, the entomopathogenic fungi were identified as *B. bassiana* (four isolates), *M. anisopliae* (two isolates), and one isolate of *Cordyceps javanica.* In addition, using ITS for identification, the *Cladosporium* species was reported. Ghiaie et al. (2017), reported four species of *Cladosporium* successfully, using ITS barcoding. The morphological methods are not specific enough to differentiate species of the genus, Therefore, the DNA barcoding approach is very helpful in increasing the accuracy of identification.

*C. dominicanum* is a species of fungus that belongs to the *C. sphaerospermum* species complex, found in the Dominican Republic, Iran, Philippines, USA, Aruba, Taiwan, and Bermuda (Zalar et al. 2007; Bensch et al. 2018). Information about *C. dominicanum* that infects whiteflies has not been widely reported in Indonesia, although in several countries, the genus *Cladosporium* has been reported to infect insects, including whiteflies

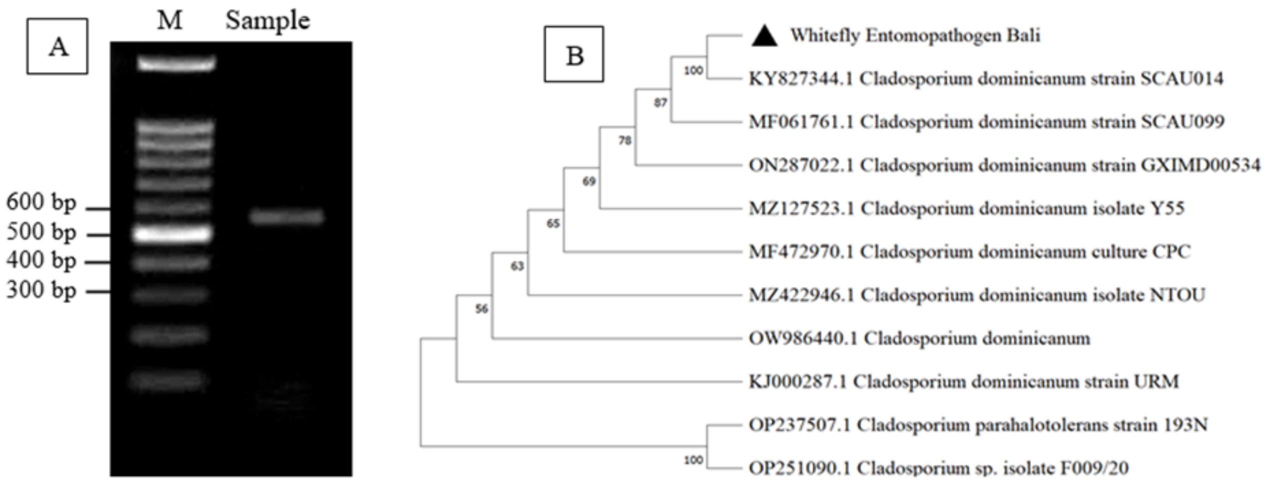

**Figure 6.** The molecular characterization of the fungus *Cladosporium dominicanum.* A) Agarose gel electrophoresis of PCR amplified products (size about 560 bp) using ITS1 and ITS4 PCR primer sets (volume of sample loaded per lane 1 µl). The marker was a 100 bp DNA ladder (M). B) Phylogenetic tree arranged based on DNA composition of *C. dominicanum* with the Maximum Parsimony method. The number in the branch was the percentage of the level of trust in the group.

(Abdel-Baky & Abdel-Salam 2003; T. Islam et al. 2019; M. T. Islam 2022). Compared with other entomopathogenic fungi (*B. bassiana* and *M. anisopliae*), *C. dominicanum* have not been utilized as a biological control in Indonesia. *B. bassiana* and *M. anisopliae* are common entomopathogenic fungi, and have been utilized as biological controls of insect pests (McGuire & Northfield 2020). This is the first report of *C. dominicanum* infecting whiteflies in Bali and Indonesia on an ornamental plants (*Premna serratifolia, Ficus religiosa, and Ficus rumphii* ). Based on this, the opportunity to use *C. dominicanum* as a bioinsecticide in the support of sustainable agriculture is extremely promising. The biotechnological application of *Cladosporium* as a microbial biopesticide is likely to be further developed (Kumar et al. 2019; Tripathi et al. 2019). Currently, there is limited information available to utilize the entomopathogenic fungus *Cladosporium* as a biological control, and this research will be essential information for future development including mass production, selecting an appropriate medium, and developing an application formulation.

## CONCLUSIONS

Based on morphological and molecular characteristics that *C. dominicanum* was identified as the entomopathogenic fungus that infected whiteflies on ornamental plants (*Premna serratifolia, Ficus religiosa, and Ficus rumphii* ) in Denpasar, Bali, Indonesia.

## AUTHOR CONTRIBUTIONS

IP.S., NN.P.S.W., D.S. and D.G.W.S. contributed to the article equally. IP.S. and G.N.A.S.W. conceptualized the research and collected the samples from the field. IP.S. carried out the morphological identification, D.G.W.S. performed the molecular analysis. IP.S. and D.G.W.S. prepared the manuscript and final editing by IW.D.G. and K.K.. All authors read and approved the final manuscript.

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## CONFLICT OF INTEREST

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

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