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Research Reports

Isolation and Molecular Identification of Fungal Contamination on the Eggshells of *Lepidochelys olivacea L.* Collected from Coastal Waters

Isolasi dan Identifikasi Molekuler Kontaminasi Jamur pada Kulit Telur *Lepidochelys olivacea L.* yang Dikumpulkan dari Perairan Pesisir

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ABSTRACT

Background: One of the causes of the decline in the Olive ridley turtle population is the low egg hatching rate due to contamination by pathogenic fungi. **Purpose:** This study aims to evaluate the results of the isolation and molecular identification of fungal contamination on the eggshells of olive ridley turtles that failed to hatch from three coastal waters in Bali province. **Method:** Sampling was done using the purposive sampling technique. Samples of failed hatching eggshells from the coast were collected, as many as 2 eggs/semi-natural nest at TCEC. Samples were cultured on PDA media. Macroscopic and molecular identification methods were carried out to determine the species level of fungal isolates. **Results:** The results showed that 10 fungal isolates were successfully collected. Isolates KL1 and SG1 had white colony surface characteristics, isolates KL4, SY2 and SY3 showed black colonies, and isolates KL2, KL3, SG2, SG3, SY4 had green colonies. Three similar isolates were sent for further molecular identification. The BLASTn results of the nucleotide sequences of isolates SY2 and SG3 showed a similarity to *Aspergillus niger* strain PHY105 (91%), while fungal isolate KL1 had a similarity to *Fusarium solani* strain 87, by 98%. **Conclusion:** This study provides new information about the pathogenic fungi, *Fusarium solani* strain 87 and *Aspergillus niger* strain PHY105, which are suspected to be the cause of the hatching failure of olive ridley turtle eggs.

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ABSTRAK

Latar Belakang: Salah satu penyebab menurunnya populasi Penyu 'Olive ridley' adalah rendahnya tingkat penetasan telur akibat adanya kontaminasi jamur patogen. **Tujuan:** Penelitian ini bertujuan untuk mengevaluasi hasil isolasi dan identifikasi molekuler cemaran jamur pada kerabang telur Penyu Belimbing yang gagal menetas dari tiga perairan pantai di Provinsi Bali. **Metode:** Pengambilan sampel dilakukan dengan teknik purposive sampling. Sampel kerabang telur gagal menetas dari pesisir pantai dikoleksi sebanyak 2 butir/sarang semi alami di TCEC. Sampel dikultur pada media PDA. Metode identifikasi makroskopis dan molekuler dilakukan untuk mengetahui tingkat spesies isolat jamur. **Hasil:** Hasil penelitian menunjukkan bahwa sebanyak 10 isolat jamur berhasil dikoleksi. Isolat KL1 dan SG1 memiliki karakteristik permukaan koloni berwarna putih, isolat KL4, SY2 dan SY3 menunjukkan koloni berwarna hitam, dan isolat KL2, KL3, SG2, SG3, SY4 dengan koloni berwarna hijau. Tiga isolat yang sejenis selanjutnya dilakukan identifikasi molekuler. Hasil BLASTn terhadap sekuens nukleotida isolat SY2 dan SG3 menunjukkan kemiripan dengan *Aspergillus niger* strain PHY105 (91%) dan isolat jamur KL1 memiliki kemiripan dengan *Fusarium solani* strain 87 yaitu sebesar 98%. **Kesimpulan:** Penelitian ini memberikan informasi baru mengenai jamur patogen *Fusarium solani* strain 87 dan *Aspergillus niger* strain PHY105 yang diduga sebagai penyebab kegagalan penetasan telur penyu belimbing.

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Kata kunci: Amplification; *Aspergillus niger*; *Fusarium solani* ; Jamur Patogen; Kulit telur penyu

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INTRODUCTION

Sea turtles are a kind of marine reptile that can travel up to 2,094 kilo meters from their feeding grounds along the Indian and Pacific oceans to their nesting grounds. They can live up to 110 years (Harmino *et al.*, 2021). Sea turtles are listed as an endangered species worldwide in Appendix 1 of the Red Book Data by IUCN (The International Union for Conservation of Nature and Natural Resources), based on The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). In P.106/SETJEN/KUM.1/12/2018, the Republic of Indonesia regulates "Protected Plant and Animal Species" (Ayuningtyas *et al.*, 2019).

Several types of sea turtles that live in waters around the world include green turtles *(Chelonia mydas)*, hawksbill turtles *(Eretmochelys imbricata)*, olive ridley turtles *(Lepidochelys olivacea L.)*, Olive ridley turtles *(Dermochelys coriacea)*, flatback turtles *(Natator depressus)* and loggerhead turtles *(Caretta caretta)* (Widaysari *et al.*, 2023). Olive ridley turtles *(Lepidochelys olivacea L.)* are one of the sea turtles that can be found in tropical waters such as in Indonesia, including in Bali Province (Bahri *et al.,* 2023). *Lepidochelys olivacea L.* or "Olive ridley turtles" are one of the types of turtles that are often found around the Seminyak Beach area of Badung Regency, Serangan, Denpasar City, and Watu Klotok, Klungkung Regency, Bali Province.

Olive ridley turtles continue to experience population declines in the wild due to various factors, including the influence of various human activities and the resulting consequences. According to Stanford *et al.,* (2020), the poaching of eggs, disruption of feeding and breeding habitats, and unintended bycatching are only a few of the anthropogenic threats that olive ridley turtles are extremely vulnerable to. Conservation efforts focus on protecting female turtles and their breeding grounds, and treating sick and injured turtles in an effort to alleviate some of the stress that turtles currently experience in the wild. Building on aquaculture and pathogen-oriented veterinary research, research on the gut microbiome, skin, eggs, and nests of turtles has become a leading force in turtle conservation research, aimed at improving the rehabilitation and conservation of endangered wildlife (Dallas and Warne, 2023; Ebani, 2023).

The failure of turtle eggs to hatch is one of the factors contributing to the fall in the population of olive ridley turtles *(Lepidochelys olivacea L.)* in their natural habitat. One of the many reasons why turtle eggs don't hatch is microbial contamination, which can infect turtle nests and their eggs (Nursanty *et al.*, 2019). Similar studies have stated that turtle eggs are very susceptible to contamination by the bacterial families Aeromonadaceae, Pseudomonadaceae, Enterobacteriaceae, and that hatching failure is especially caused by *Fusarium spp.* (Pace *et al.*, 2019; Blasi *et al.*, 2020; Dallas and Warne, 2023; Ebani, 2023). Research has also revealed that widespread soil fungi belonging to the *Fusarium solani, Fusarium falciforme,* and *Fusarium keratoplasticum* species complex can have significant impacts on hatching success (Martínez-Ríos *et al.*, 2022). These organisms are also involved in "sea turtle egg fusariosis" (STEF), a fungal disease that causes egg mortality in natural to semi-natural turtle nests (Smyth *et al.*, 2019).

Another issue in Indonesia is the existence of fungi that contaminate the eggs of olive ridley turtles *(Lepidochelys olivacea L.)*, particularly at the TCEC Serangan Bali Turtle Conservation. According to studies by Praja *et al.* (2023), infection by microorganisms on the eggshells accounts for up to 80% of the reasons why olive ridley turtle eggs in semi-natural nests fail to hatch. Furthermore, the quantity of fungi on the eggshells of olive ridley turtles *(Lepidochelys olivacea L.)* has been shown to influence the success of turtle egg hatching (Ayuningtyas *et al.*, 2019).

It is imperative to diagnose the diseases caused by microorganisms through the use of rapid, sensitive, and low contamination technologies in order to identify the presence of fungal infections and enable appropriate prevention and control actions (Fang *et al.,* 2023). Based on this, this study was conducted to identify the fungal contamination infecting olive ridley turtle eggs *(Lepidochelys olivacea L.)* causing hatching failure through macroscopic, microscopic and molecular identification. The data obtained is expected to be used as a reference when carrying out treatment and prevention measures according to the type of fungus infecting turtle eggs in semi-natural nests, increasing the success of turtle conservation efforts (Gleason *et al.,* 2020).

MATERIALS and METHODS

Materials

The tools used in this study are as follows Oven (MemertTM UN55, Germany), beaker glass (Iwaki CTE33 Asahi Glass, Thailand), Erlenmeyer flask (AGC Iwaki CTE33, Thailand), petri dish (OneMed PS90 mm, Indonesia), scales (Joil, Indonesia), stirrer bar (Cimarec ELED SP142020-33Q, USA), hotplate (Cimarec ELED SP142020-33Q, USA), glass stirrer rod (IWAKI), ose needle, autoclave (ALL American 25X, USA), refrigerator (Polytron, Indonesia), tweezers (OneMed, Indonesia), laminar airflow (Innotech-Biobase, China), bunsen, blue tip and yellow tip (OneMed, Indonesia), plastic bag, cooler box (Lion Star, Indonesia), cotton (Bunda Biru, Indonesia), aluminum foil (Best Fresh, Indonesia), thermal cycler (BioRad), and documentation camera.

Olive ridley turtle eggs that failed to hatch, distilled water (SmartLab, Indonesia), 70% alcohol (OneMed, Indonesia), instant Potato Dextrose Agar (PDA) media (Merck, Germany), Lactofenol cotton blue (HIMEDIA S016, Indonesia), Choramphenicol antibiotic (Bernofarm, Indonesia), DNA extraction reagent (Jena Bioscience Yeast DNA Preparation – Column Kit, Germany), and the Internal Transcribed Spacer (ITS1 and ITS4) primer set (Jena Bioscience, Germany) were additionally used.

Study Area

The sampling of the research was conducted at the Turtle Conservation and Education Center (TCEC) Serangan Bali

with the egg origins consisting of three semi-natural coastal nest locations in Bali including Seminyak Beach, Serangan Beach and Watu Klotok Beach. This observation activity was carried out for 4 months inclusive of the preparation stage, sample collection, isolation of fungi on the eggshells, characterization of the fungal colonies, and molecular identification.

Turtle Egg Sampling Procedure

Sampling was conducted during the rainy season at TCEC Serangan Bali at 3 semi-natural nests, namely the semi-natural nests of Seminyak Beach (Badung Regency), Serangan (Denpasar City), and Watu Klotok (Klungkung Regency). The selection of the 3 nests was due to the high tourism activity, turtle nesting, and the alleged high activity of selling turtle meat and eggs. Samples were collected by taking eggs that failed to hatch from each different nest aseptically, before being separated from the sand attached to the shell using a sterile plastic bag, which was later put into a coolbox containing ice gel. Isolation of the contaminant fungi was carried out at the Basic Science Laboratory of Dhyana Pura University.

The samples used in this study were olive ridley turtle eggshells that failed to hatch with the criteria of not hatching after being incubated for ± 45-60 days at the Turtle Conservation Center TCEC Serangan Bali, with the sampling locations coming from Seminyak Beach, Serangan Beach and Watu Klotok Beach. The physical characteristics included the eggshells shrinking so that the shell shape was irregular, the color of the shell being cloudy yellowish to black, and damaged eggs.

The population in this study was 86 olive ridley turtle eggs from the semi-natural nesting site of Seminyak Beach, 82 olive ridley turtle eggs from the semi-natural nesting site of Serangan Beach and 82 olive ridley turtle eggs from Watu Klotok Beach at the TCEC Serangan Bali Turtle Conservation Center. Samples of eggs that failed to hatch were then selected using random sampling techniques for each beach, so that the number of samples of eggs that failed to hatch collected was 2 eggs/beach.

PDA Media Creation

A total of 39 grams of instant PDA media was put into a 1000 mL Erlenmeyer to be dissolved using 1000 mL of distilled water, before being heated and homogenized on a hotplate at a temperature of 95°C. The media was then sterilized in an autoclave at a temperature of 121°C for 15 minutes using a pressure of 1 atm. Chloramphenicol antibiotics were added at a dose of 250 mg by as much as 1 tablet/90 ml of distilled water, before being pipetted into each petri dish in a laminar air flow which aimed to prevent bacterial contamination. The media was then poured into a disposable petri dish and left to solidify (Azzahra *et al.*, 2019).

Sterilization of Tools and Materials

Sterilization was carried out on glass-based equipment and microbial growth media using an autoclave with a pressure of 1 atm at a temperature of 121°C for 15 minutes. The purpose of sterilization was to prevent the glass-based equipment and materials (microbial growth media) used in this study from being contaminated and not causing contamination.

Fungi Isolation

Fungal isolation on the shell was carried out using a direct planting technique on the surface of Potato Dextrose Agar (PDA) media. Eggshell fragments were planted directly on a petri dish that had been filled with PDA media first, for it to be identified following two steps, namely macroscopic and molecular observations.

Purification of Fungal Isolates

The purification of the fungal isolates was done with the aim of separating the endophytic colonies that have different colony morphologies. Purification was done by transferring one fungal colony to the medium. Each part of the fungus that had a different macroscopic colony morphology was transferred to sterile PDA media and re-incubated for 5-7 days to obtain pure isolates (Wiradana *et al.*, 2024).

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Macroscopic Identification

Macroscopic identification was carried out based on colony observations including colony color, colony reverse color, texture, topography, pattern, exudate drops, radial lines and concentric lines (Habisukan *et al.*, 2021).

Molecular Identification

DNA Extraction

The DNA isolation method in this study followed the procedure of the Jena Bioscience Yeast DNA Preparation – Column Kit. Liquid culture was taken as much as 500 µl, then put into a 1.5 ml microcentrifuge tube, and centrifuged at a power of 8,000 g for 1 minute. Next, the supernatant was discarded, 100 µl of Resuspension buffer and 1 µl of lyticase enzyme were added to the pellet, vortexed for 10 seconds, and then incubated at 37°C for 15 minutes. It was then centrifuged at a power of 10,000g for 1 minute. The supernatant was discarded and 300 µl of Lysis buffer and 2 µl of RNase were added to the pellet, and vortexed for 10 seconds.

Proteinase K was added as much as 8 µl to the lysate, mixed using a pipette, and then incubated for 10 minutes at 60°C, cooled on ice for 5 minutes, and vortexed. Next, it was centrifuged at a power of 10,000 g for 5 minutes. It was inserted into the spin column in a 2 ml collection tube, and 100 µl of Activation buffer was added to the spin column and centrifuged at a power of 10,000 g for 1 minute. The supernatant was pipetted into the prepared spin column, centrifuged at a power of 10,000 g for 1 minute, and the liquid was discarded.

The process was continued by washing the DNA with 500 µl of washing buffer in the spin column, followed by centrifugation at 10,000 g for 30 seconds, and the discarding of the liquid. This was repeated twice, then it was transferred from the spin column to a 1.5 ml Elution tube, where 40 µl of Elution buffer was added to the spin column, incubated for 1 minute, and then centrifuged at 10,000 g for 2 minutes. Then, the DNA was stored at 4°C (Jenabioscience, 2024).

DNA Amplification and Sequencing

The molecular identification of fungal isolates was carried out by through conventional PCR amplification. The PCR amplification process used the Internal Transcribed Spacer (ITS) Forward primer set ITS1 (5'-TCCGTAGGTGAACCT GCGG-3') and Reverse primer ITS4 (5' TCCTCCGCTTATT GATATGC 3') (Tamura *et al.*, 2021). Amplification in the PCR machine from Mix PCR used Taq DNA polymerase, H2O, Forward and Reverse Primers. A 0.2 ml PCR tube was prepared. and the primer sets ITS 1 and ITS 4 were added as much as 2 µl each, 2 µl of DNA template, 12.5 µl of Go Taq® Green Master Mix 2×, and 20 µl of ddH2O. The PCR machine profile settings are shown in the table below.

Table 1. Profile of The Conventional PCR Machine Used in This Study.

Pre-denaturation	Denaturation	Annealing	Extension	Final Extension
95 °C	95 °C	54 °C	72 °C	72 °C
1 cycle	30 cycles	30 cycles	30 cycles	1 cycle
2 minutes	30 seconds	1 minutes	1 minute	5 minutes

Electrophoresis was performed by taking 3 µl of the amplification results from the PCR machine and then loading it on a 1.2% agarose gel, at a voltage of 50 volts for 60 minutes. Making 1% agarose gel was done by dissolving 1 gram of agarose using 100 mL of TAE (Tris Acetate EDTA) 1x, then boiling it using a water bath until the solution turned clear. The agarose solution was then poured into the gel caster and a comb was inserted to make a well hole and then left to solidify. The fungal DNA sample and loading dye solution were then inserted into the well hole on the agarose gel at a ratio of (1:1). The agarose gel containing a TAE buffer was then placed in the electrophoresis chamber, with the well position on the (-) charge. Furthermore, the electrode was connected to the power supply and turned on for up to 50 minutes, at a voltage of 220V. The gel was then soaked in 2 µl/500 ml ethidium bromide solution for 10 minutes, and continued to be soaked in H2O for 10 minutes. The gel was then scanned in a UV transilluminator machine and captured using Gel Doc to observe the results of the amplified DNA bands (Hermansyah *et al.*, 2018).

The PCR results obtained were then sequenced using a DNA sequencer. The samples were sent to Makrogen Indonesia to read the nucleotide sequence, then the reading results were downloaded in the form of Qual and Seq files. The sequencing data obtained was then compared with the sequence data contained in GenBank using the Basic Local Alignment Search Tool (BLAST) bioinformatics method (http://www.ncbi.nlm.nlh.gov/) online by matching the fungal DNA sequences available in the National Center for Biotechnology Information (NCBI) Nucleotide database. Analysis of the level of kinship of each fungal sample is described following the construction of a phylogenetic tree using the MEGA11 program (Elita *et al.*, 2022).

RESULT

Macroscopic Identification of the Fungal Contamination

The isolation of fungi on the shells of olive ridley turtle eggs that failed to hatch produced 10 isolates including 4 isolates from Watu Klotok beach (Klungkung Regency), 3 isolates from Serangan beach (Denpasar City), and 3 isolates from Seminyak beach (Badung, Regency). The results of the observations of fungal isolates from the shells of olive ridley turtle eggs that failed to hatch macroscopically are presented in the Table 2.

The results shown in Table 2 and Figure 1 show that the successfully isolated fungal colonies have different macroscopic characteristics. The successfully isolated fungal isolates have white, green and black colony colors with the opposite color of the colony being bone white to yellow with a cottony and granular colony texture. In isolates with codes KL1 and SG1, the colony surface color is white. Isolates with codes KL4, SY2 and SY3 have a black colony surface color, and green is dominated by isolates with codes KL2, KL3, SG2, SG3, SY1. In terms of texture, isolates KL1 and SG1 have a cottony texture and isolates KL2, KL3, KL4, SG2, SG3, SY1, SY2, SY3 have a granular texture. In terms of topography, isolates with codes KL1 and SG1 have a folded topography type.

Isolates with codes KL2, KL3, KL4, SG2, SG3, SY1, SY2, and SY3 have a rugose topography type. Of all the isolates, no exudate droplets were found, which were suspected to be the result of the metabolism of the fungal isolate. In the pattern section, there are two types of colony patterns in the isolates, namely zonation and radial patterns. Isolates with codes KL1 and SG1 have zonation patterns, while isolates KL2, KL3, KL4, SG2, SG3, SY1, SY2, and SY3 have radial patterns. All isolates have radial lines except for isolates with codes KL1 and SG1. Only isolate code KL2 has concentric lines or lines formed within a colony.

Amplification Results Using Conventional Polymerase Chain Reaction (PCR)

Conventional PCR-based molecular identification using 3 isolate samples from different beaches from a total of 10 fungal isolate samples was undertaken. There were several isolate samples with similar macroscopic characteristics on each beach. The isolates are the Seminyak Beach fungal isolate with a black colony surface color, the Serangan Beach fungal isolate with a green colony surface color, and the Watu Klotok Beach fungal isolate with a white colony surface color.

The results of the DNA amplification of fungal isolates on the shells of olive ridley turtle eggs that failed to hatch molecularly using universal primer gene sequences ITS1 and ITS4 are

presented in the Figure 2. From the results of conventional PCR molecular testing using universal primer pairs ITS1 and ITS4, all tested fungal samples were successfully amplified. This can be seen from the appearance of a band parallel to the DNA marker. The size of the DNA fragment produced from the amplification process using universal primer pairs ITS1 and ITS4 is parallel to the DNA marker at a size of ± 550 bp according to the amplification target. This shows that the DNA fragment was successfully amplified and has a size of around ± 550 bp.

BLASTN Analysis Results of the Nucleotide Sequences of Fungal Isolates

The results of the nucleotide sequencing of fungal isolates on the shells of olive ridley turtle eggs that failed to hatch using universal primers ITS1 and ITS4 are presented in the Table 3. Based on the analysis of the results of nucleotide sequence alignment with BLAST software on the NCBI site using the universal primer pair ITS1 and ITS4, it shows a similarity percentage of 91% between the isolates of Seminyak Beach (SY) and Serangan Beach (SG) with the fungal species *Aspergillus niger* strain PHY105. This shows that the fungal isolates of Seminyak Beach and Serangan Beach are the same species as *Aspergillus niger* strain PHY105 with Accession Number KU508400.1. The isolate of Watu Klotok Beach (KL) shows a similarity percentage of 98% with the fungal species *Fusarium solani* strain 87, Accession Number KP137446.1

Table 3. Results of the Fungal Species Analysis in GeneBank.

No.	Isolates Code	Species Name	Accession Number	Percentage Similarity
1.	SY 2	Aspergillus niger strain PHY105	KU508400.1	91%
2.	SG 3	Aspergillus niger strain PHY105	KU508400.1	91%
3.	KL 1	Fusarium solani strain 87	KP137446.1	98%

Results of the Kinship Relationship of the Fungal Isolates in the Shells of Olive Ridley Turtle Eggs

The DNA sequencing results of the fungal isolates on the shells of olive ridley turtle eggs that failed to hatch were constructed in a phylogenetic tree. The phylogenetic tree was created using the MEGA XI software, using the Neighbor-Joining Tree analysis method, Maximum composite likelihood Parameter model and a bootstrap value of 1,000

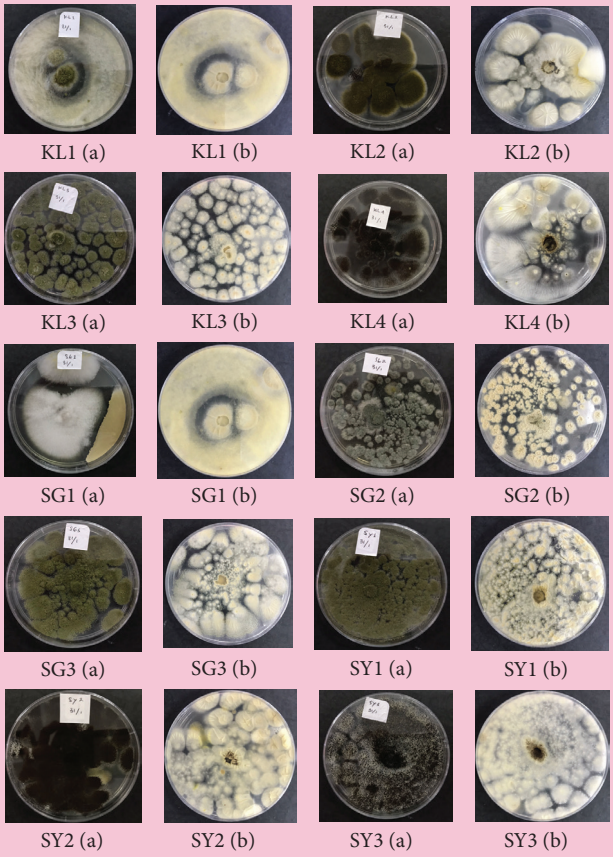

Figure 1. Macroscopic characteristics of the endophytic fungal colonies found on the shells of olive ridley turtle eggs that failed to hatch (KL: isolate code Watu Klotok Klungkung beach, SG: isolate code Serangan Denpasar beach, SY: isolate code Seminyak Badung beach. Description of Petri dishes a: front view, b: reverse of the colony).

replications. The results of the phylogenetic analysis of fungal isolates on the shells of olive ridley turtle eggs are presented in Figures 3 and 4. The results of the cladogram of the kinship relationship of the fungal isolates (Figure 4) show that the fungal isolates from Seminyak Beach (SY) and Serangan Beach (SG) are in one cluster with the fungal sequence *Aspergillus niger* strain PHY105 with a bootstrap value of 64%. The fungal isolates from Seminyak Beach (SY) and Serangan Beach (SG) were identified as *Aspergillus niger* strain PHY105 because the homology value was 91%. The fungal isolates from Watu Klotok Beach (KL) were in one cluster with the

Table 2. Fungi Isolated from Olive Ridley Turtle *(Lepidochelys olivacea L.)* Eggs that Failed to Hatch Macroscopically.

Code	Colony Color	Reverse Color of Colony	Texture	Topography	Pattern	Exudate Droplets	Radial Lines	Concentric Lines
KL1	White	Milky white	Cottony	Folded	Zonasi	-	-	-
KL2	Brownish Green	White	Granular	Rugose	Radial	-	√	√
KL3	Green	Milky white	Granular	Rugose	Radial	-	√	-
KL4	Black	Brownish white	Granular	Rugose	Radial	-	√	-
SG1	White	Yellow	Cottony	Folded	Zonasi	-	-	-
SG2	Green	Yellow	Granular	Rugose	Radial	-	√	-
SG3	Green	Milky white	Granular	Rugose	Radial	-	√	-
SY1	Green	Milky white	Granular	Rugose	Radial	-	√	-
SY2	Black	Milky white	Granular	Rugose	Radial	-	√	-
SY3	Black	Milky white	Granular	Rugose	Zonasi	-	-	-

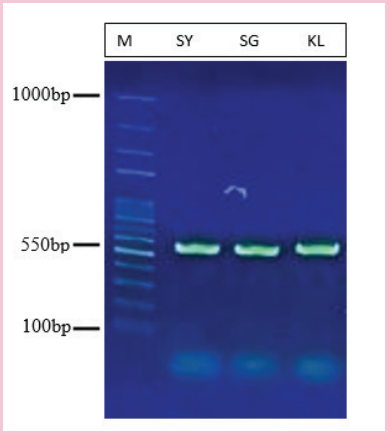

Figure 2. DNA amplification results for the fungal samples on the shells of olive ridley turtle eggs that failed to hatch, detected by electrophoresis. Image caption: M (DNA Marker 1000bp), SY (Seminyak Beach Sample, Badung), SG (Serangan Beach Sample, Denpasar), KL (Watu Klotok Beach Sample, Klungkung).

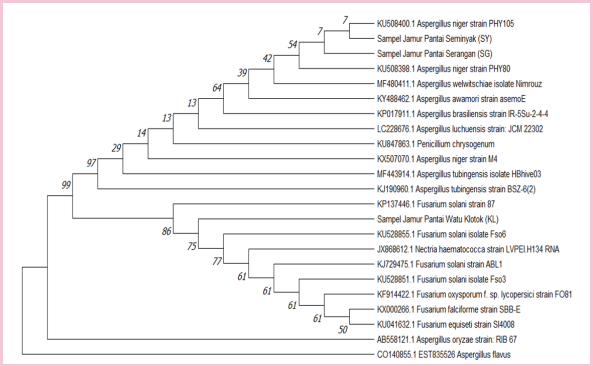

Figure 3. Phylogenetic relationship phylogram of three fungal isolates on the shells of olive ridley turtle eggs that failed to hatch based on ITS markers.

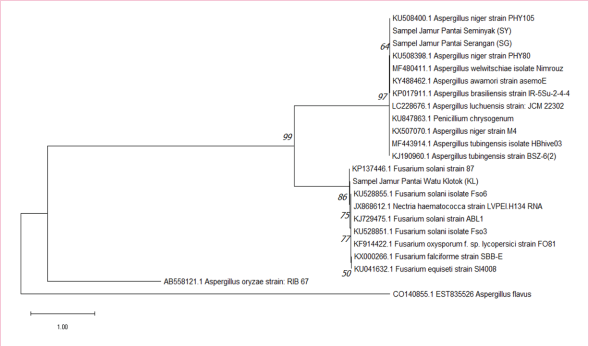

Figure 4. Cladogram of the three fungal isolates in the samples of olive ridley turtle egg shells that failed to hatch based on ITS markers. Neigbor-joining analysis method and Maximum Composite Likelihood model. Bootstrap value 1000

fungal sequence *Fusarium solani* strain 87 with a bootstrap value of 86%. The fungal isolates from Watu Klotok Beach, Klungkung (KL) were identified as *Fusarium solani* strain 87 with a homology value of 98%.

DISCUSSION

According to the macroscopic analysis, *Aspergillus sp.* is suspected of being the fungal isolates with green and black surface colonies, a velvety or granular texture, and dry colony consistency. Research by Cyrilla *et al.*, (2018) indicates that filamentous colonies of the *Aspergillus niger* species have a black colony surface color, velvety texture, and dry consistency, while filamentous colonies of the *Aspergillus flavus* species have a green colony surface color, velvety or granular texture, and dry consistency. These macroscopic characteristics are in line with the findings of the study. *Aspergillus sp.*'s macromorphological traits include a white colony edge that changes to a brownish-black color by the time the colony reaches seven days of age. The colony has a circular form, and the spores are tiny (Izzatinnisa *et al.*, 2020).

Fusarium sp. is a type of fungal isolate with white surface colonies that resemble cottony-textured threads with a dry consistency. According to research by Afriani and Heviyanti (2018), *Fusarium sp.* fungus has macroscopic characteristics that include concentric colonies, smooth colony texture, a loose density, and white colony surfaces that are yellowish white on the reverse of the colony.

Fusarium spp. and *Aspergillus spp.* dispersed throughout the densely-packed sand nest circumstances have the potential to damage healthy eggs and infect eggshells while the eggs are being incubated in the nest. Due to fungal infection, rotting and damaged turtle eggs might harm other healthy eggs in the same nest, lowering the likelihood that the eggs will hatch (Praja *et al.*, 2018). The softened shape of the eggs and the presence of yellow patches on the eggshells of olive ridley turtles indicate the presence of fungal contamination. In addition to olive ridley turtle eggshells, olive ridley turtle hatchlings in the care of the Turtle Conservation and Education Center Bali (TCEC) have been found to be susceptible to fungal infections on their bodies, specifically in the carapace, neck, eyes, and fins (Ayuningtyas *et al.*, 2019).

The outcomes of traditional PCR molecular testing indicate the existence of a brilliant DNA band or band parallel to the ±550 bp-long DNA marker. Primers and the appropriate annealing temperature are two critical parameters that impact the quality of molecular detection results using traditional PCR in the molecular observation results shown in Figure 2. This is evident from the DNA amplicon results, which show a thick, brilliant DNA band, indicating a high concentration and degree of purity in the sample (Hermansyah *et al.*, 2018).

According to research by Hermansyah *et al.*, (2018), high-quality product bands that are parallel to one another and appear thick and brilliant are indicative of successful amplification outcomes and will result in a single DNA band pattern. This suggests that the amplification process's primers and annealing temperatures were appropriate. According to a study by Sholihah *et al.*, (2019), a universal primer set used in the PCR method will result in DNA bands with a molecular

weight of 550–570 bp when used for fungal analysis. After that, the next segment will be sequenced in order to identify the fungus species based on how similar it is to previously discovered species.

Aspergillus niger strain PHY105 and *Fusarium solani* strain 87 were the types of fungus isolates found on the shells of olive ridley turtle eggs that did not hatch, according to the sequencing data. This is consistent with the findings of the tests that were done on fungal isolates under a microscope. *Aspergillus sp.* fungi are responsible for fungal isolates with green and black surface colonies that have a velvety or granular texture and a dry colony consistency. *Fusarium sp.* fungus species are responsible for fungal isolates with white surface colonies that resemble cottony textured threads and have a dry consistency. According to research by Cyrilla *et al.,* (2018), the macroscopic features of the *Aspergillus niger* fungus species include black colonies, filamentous, velvety texture, and a dry consistency. Furthermore, the fungus *Fusarium sp.* exhibits macroscopic features such as concentric colonies, a smooth colony texture, loose density, and white colony surfaces, with a yellowish white colony on the reverse (Afriani and Heviyanti, 2018).

The phylogenetic tree reconstruction in this study produced four clusters, one *Aspergillus sp.* cluster, one *Fusarium sp.* cluster, one *Aspergillus oryzae* cluster and one *Aspergillus flavus* cluster. The third and fourth clusters consisted of one isolate, namely *Aspergillus oryzae* and *Aspergillus flavus*, which formed their own clusters separate from other Aspergillus fungal isolates. The *Fusarium* and *Aspergillus genera* are the sources of two sizable groupings. The bootstrap values obtained for the fungal isolates from Watu Klotok beach (KL), Serangan beach (SG), and Seminyak beach (SY) indicate a reasonably high degree of confidence in the clusters that were established. The degree of trust in the reconstructed tree increases with the bootstrap value. The phylogenetic tree and branching will remain unchanged if the bootstrap values fall between 70 and 100 (Susilowati *et al.*, 2020).

Fungal isolates from Seminyak Beach (SY) and Serangan Beach (SG) were found in one cluster with sequences of *Aspergillus niger* strain PHY105, *Aspergillus niger* strain PHY80, *Aspergillus welwitschiae* isolate Nimrouz, *Aspergillus awamori* strain asemoE, *Aspergillus brasiliensis* strain IR-5Su-2-4-4, *Aspergillus luchuensis* strain JCM 22302, *Penicillium chrysogenum*, *Aspergillus niger* strain M4, *Aspergillus tubungensis* strain BSZ-6(2) and *Aspergillus tubungensis* isolate Hbhive03. Fungal isolates from Seminyak Beach (SY) and Serangan Beach (SG) were determined to be closely related to *Aspergillus niger* strain PHY80 with a bootstrap value of 64%. Isolates from Seminyak Beach (SY) and Serangan Beach (SG) were identified as the *Aspergillus niger* strain PHY105 fungal species with a similarity percentage of 91%. This is in accordance with the research conducted by El-Naggar *et al.* (2019), namely the *Aspergillus niger* fungal species is in one cluster together with the *Aspergillus welwitschiae, Aspergillus awamori, Aspergillus brasiliensis, Aspergillus luchuensis, Aspergillus tubungensis,* and *Aspergillus foetidus* fungal species.

In the Watu Klotok (KL), the coastal fungus isolates are in one cluster with the sequences of *Fusarium solani* strain 87, *Fusarium solani* isolate Fso6, *Fusarium solani* strain ABL1, *Fusarium solani* isolate Fso3, *Fusarium oxysporum* strain FO81, *Fusarium falciforme* strain SBB-E, *Fusarium equisenti* strain SI4008 and *Netria haematococca* strain LVPEI.H134. Here, the Watu Klotok (KL) coastal fungus isolate has a close kinship with Fusarium solani isolate Fso6 with a bootstrap value of 86%. The Watu Klotok (KL) coastal isolate was identified as the *Fusarium solani* strain 87 fungal species with a similarity percentage of 98%. This is in accordance with the research conducted by Šišić *et al.,* (2018), which stated that *Fusarium solani* was found in one cluster along with *Fusarium oxysporum, Fusarium falciforme, Fusarium equisenti*, and *Netria haematococca.*

The results of this study only focus on three beaches in the eastern part of Bali Province, which still requires the further exploration of olive ridley turtle nests in other coastal areas in Bali Province. Given the high tourism activity and domestic waste entering the waters of Bali, more comprehensive surveillance is still needed, especially in preventing the fungal contamination of olive ridley turtle eggs.

CONCLUSION

A total of 10 fungal isolates were successfully isolated with 3 fungal colonies showing the same macroscopic morphological characteristics. The three isolates were *Aspergillus niger* strain PHY105 (isolates SY and SG) and *Fusarium solani* strain 87 (isolate KL). Phylogenetic tree analysis showed that the Seminyak beach (SY2) and Serangan beach (SG3) fungal isolates were closely related to the Aspergillus niger strain PHY105. The Watu Klotok beach (KL1) fungal isolate was closely related to the *Fusarium solani* strain 87 fungal species. Relocation to the turtle egg hatching incubator can also be considered to maintain environmental quality.

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CONFLICT OF INTEREST

The author declares there to be no conflict of interest.

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AUTHOR'S CONTRIBUTION

Conception and design of the study: PAW, IWR, and NMWA. Conduction of the study, and the analysis and/or interpretation of the data: PAW and NMWA. Drafting of the manuscript: PAW, MKJK, and IGW. Critical review and revisions: PAW, IWR, MKJK, and IGW.

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