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Fungal isolates from marine sponge *Chelonaplysilla* sp.: Diversity, antimicrobial and cytotoxic activities

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Abstract. *Handayani D, Artasasta MA, Safira N, Ayuni DF, Tallei TE, Hertiani T. 2020. Fungal isolates from marine sponge* Chelonaplysilla *sp.: Diversity, antimicrobial and cytotoxic activities*. *Biodiversitas 21: 1954-1960.* The purpose of this research was to study the diversity of fungi associated with marine sponges *Chelonaplysilla sp.* and their bioactivities. Fungal isolation was carried out by the multilevel dilution method in *Saboraud Dextrose Agar* (SDA). Twelve fungal isolates were successfully purified, then cultivated using rice for 4-6 weeks at room temperature and subsequently extracted using ethyl acetate. Antimicrobial activities of the fungal extracts were tested against *Staphylococcus aureus, Escherichia coli,* and *Candida albicans* by using the agar diffusion method. The extracts of isolates Ch05 and Ch12 showed a significant antagonistic effect against *S. aureus* and *E. coli* with the diameter that ranged from 15 to 17 mm. Using the brine shrimp lethality test (BSLT), six fungal extracts revealed cytotoxic activity with LC_{50} <100 µg/mL. Isolate Ch10 was the most potential fungus with the strong cytotoxic activity of LC_{50} of 0.90 µg/mL. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted also for six potential fungal extracts against breast cancer cell (T47D). The isolate Ch05 showed moderate cytotoxic activity with IC_{50} of 83.69 µg/mL. The molecular identification was carried out for potential fungi using the ITS marker. The results showed that Ch02 was *Aspergillus oryzae*, Ch05 was *Phomompsis* sp., Ch06 was *Penicillium simplicissimum*, Ch10 was *B. bassiana* and Ch12 was *Aspergillus mellinus*. This study concluded that fungal isolates from marine sponge *Chelonaplysilla* sp. can be explored further for new sources of antimicrobial and anticancer compounds.

Keywords: Antimicrobial, *Chelonaplysilla*, cytotoxic, marine-derived fungi

INTRODUCTION

Bioactive compounds produced by fungi associated with marine sponges have shown potential pharmacological activities and have similar metabolites produced by their hosts (Indraningrat et al. 2016; Youssef et al. 2019). These types of fungi are thought to be the original producer of bioactive compounds in sponges (Proksch et al. 2002). Several new bioactive compounds from marine associated fungi have shown potential new bioactivities, for example, secondary metabolites produced by *Aspergillus similanensis* contained similanpyrone C, similanamide, and pyripyropene (Thomas et al. 2010; Prompanya et al. 2015). Averantin isolated from A. versicolor associated with marine sponge Neopetrosia sp. was proven to have antibacterial and cytotoxic activities (Lee et al. 2010).

Marine sponges which were collected from Mandeh island in west Sumatra-Indonesia were known to be associated with potential fungi which produced antimicrobial and cytotoxic activities (Artasasta et al. 2017; Handayani and Aminah 2017; Handayani and Artasasta 2017; Aminah et al. 2019; Handayani et al. 2019a, 2019b). This study focused to discover the diversity of *Chelonaplysilla* sp. associated fungi and their biological activities against pathogens and breast cancer cells (T47D).

MATERIALS AND METHODS

The extraction process of the pure fungal isolate from marine sponge *Chelonaplysilla* sp.

Marine sponge *Chelonaplysilla* sp. (Fig. 1) from Mandeh island, west Sumatra-Indonesia (1o 6'-1o 13'S, 100o 19'-100o 25'E) (Fig. 2) was used as a fungal source. Identification of the sponge was conducted by Dr. Nicole J. De Voogd, at the Natural Biodiversity Center, Netherland. The fungal isolation was conducted following our previous study using a multi dilution method (Handayani and Artasasta 2017). Pure isolates were cultivated on rice media at room temperature for 4-6 weeks. The extraction process was conducted after the fungal isolates overgrew on the media. Ethyl acetate was used for extracting nonpolar and semi-polar compounds of the fungal isolates with the ratio of 200 mL solvent to 100 mg rice. To complete the extraction process, the fungi were immersed in the ethyl acetate for 3 days. The solvent was evaporated to obtain the dry extracts that were used for antimicrobial and cytotoxic activity as well as phytochemical tests.

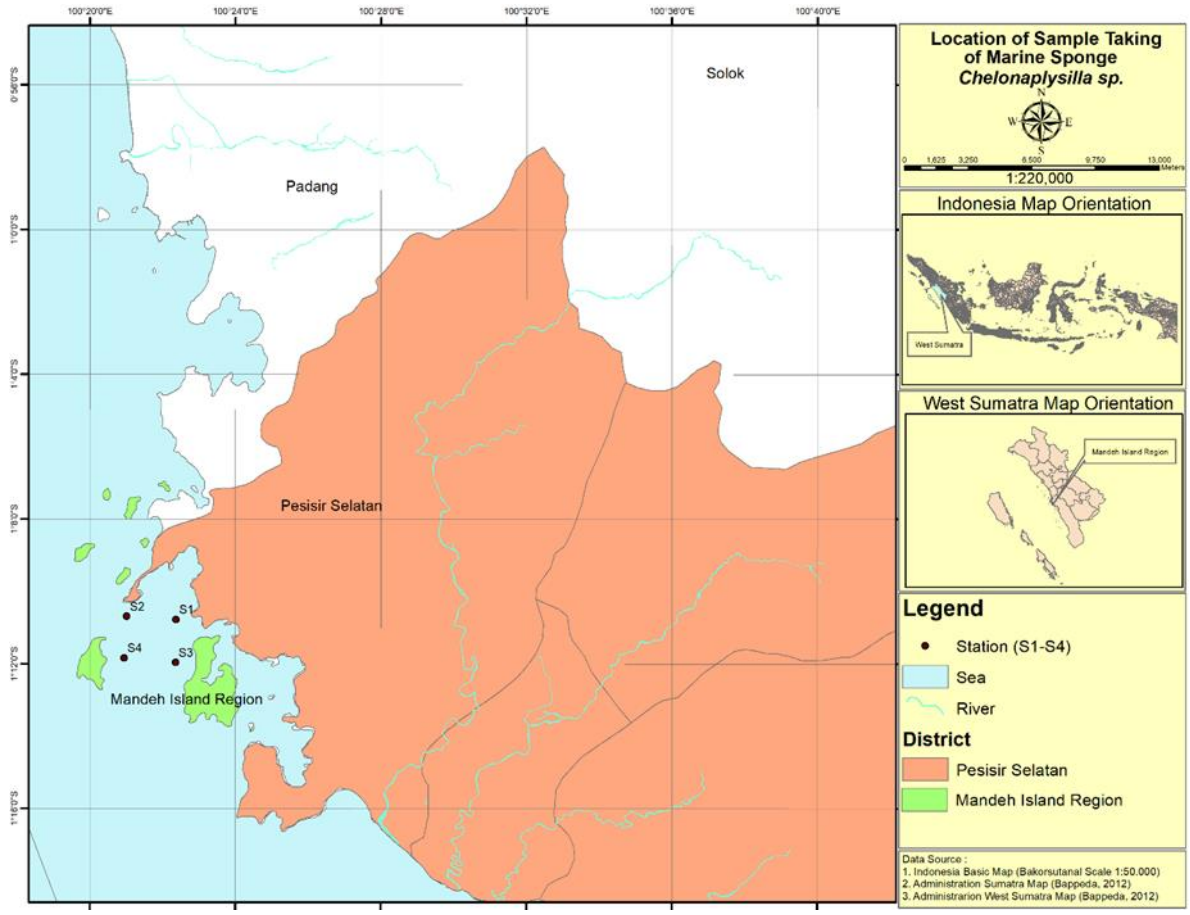

Figure 2. Location of sample taking of marine sponge *Chelonaplysilla* sp. in Mandeh Island, West Sumatra, Indonesia (1º 6'-1º 13'S, 100º 19'-100º 25'E)

Figure 1. Marine sponge *Chelonaplysilla* sp. Bar = 1 cm

Screening of antimicrobial activity

S. aureus ATCC 2592, *E. coli* ATCC 25922, and *C. albicans* were used as the pathogens for antimicrobial screening. This study followed the procedure provided by Balouiri et al (2016) using the agar diffusion method. Sterile paper disk (6 mm) was soaked in each of 5 % fungal extract. Nutrient agar (NA) was used for an antibacterial test, while SDA was used as media for an antifungal test. As positive controls, 30 µg/disc chloramphenicol (antibacterial) and nystatin (antifungal) were used. After incubation for 24 h at room temperature, the diameter of inhibition zones (mm) was measured as an indication of antagonistic effects.

Screening of cytotoxic activity

BSLT

BSLT was used for the evaluation of cytotoxic activity. The eggs of *Artemia salina* were hatched in seawater for 2 days at 37ºC. After hatching, 10 active *nauplii* were put into a test tube containing the fungal extracts which were dissolved in DMSO (dimethyl sulfoxide) up to a concentration of 1000, 100 and 10 µg/mL. Probit analysis was used for determining the value of LC50 (Meyer et al. 1982).

Cytotoxicity assay

MTT assay was carried out to study the cytotoxic activity of fungal extracts against breast cancer cell T47D. These cells were seeded at 1×10^5 cells/mL in 96 well microtiter plates containing DMEM (Dulbecco's modified Eagle's medium), a modification of Basal Medium Eagle

(BME). The cells could attach on the well overnight. Once the cells were confluent, 20 µL of the fungal extract was added to the well with various concentrations (1000, 500, 250, 125, 62.5, and 31.25 µg/mL) then the plates were placed in 5% CO2 incubator at 37oC for 24 h. Thereafter, the media were replaced with PBS. The cells were subsequently treated with 5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltratrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO) and incubated for 4 hours. DMSO was used to dissolve the remaining formazan crystals. Using ELISA reader, the absorbance was measured at 570 nm. The percentage of viability of the cells was determined then converted into the IC50 value (Artasasta et al. 2017).

Fungal identification

Macroscopic and microscopic identification

Macroscopic appearance of the fungi such as color, the diameter of the colony, and colony reverse was observed. Microscopic observation was conducted by using a lactophenol cotton blue solution as a mounting medium and staining agent. The hyphae were put on a slide then mixed with fungal coloring. Conidiophores, vesicles, and conidia were observed under a light microscope (Charya and Garg 2019).

Molecular identification

Molecular identification was performed using ITS1 primer (F5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 primer (R5'-TCC TCC GCT TAT TGA TAT GC-3') with the two-step procedure, DNA extraction, and PCR amplification. DNA extraction was conducted by following Saitoh et al., (2016). The PCR process was conducted by 34 cycles included denaturation at 95oC for 5 min, annealing at 55oC for 1 min, and extension at 72°C for 1 min (Ferrer et al. 2001). The PCR products were sequenced in First Base Malaysia then were trimmed and assembled by using BioEdit V.7.0.5. Furthermore, the sequences were subjected to identification using the BLAST program on NCBI. The Neighbor-Joining (NJ) phylogenetic tree was generated by MEGA 7.0 software (Kumar et al. 2016), using Kimura-2-Parameter with 1,000 bootstrap replications.

Secondary metabolites examination

The standard protocol of Harborne (1984) was followed in this step. Dragendorff reagent for alkaloid, Lieberman Bourchard's reagent for terpenoid and steroid, $FeCl_3$ reagent for phenolic, and Citroborat reagent for flavonoid were used. The fungal extract was spotted on the G60 F254 silica plate then eluted with n-hexane: ethyl acetate eluent (1: 4). Each of the reagents was swapped to each different silica. Each of the secondary metabolites produced different colors based on different reagents. The orange color indicated the presence of alkaloid, pink color for terpenoid, blue or green color for the steroid, purple, red, or pink colors for phenolic, and green color for flavonoid (Harborne 1984).

RESULTS AND DISCUSSION

Antimicrobial activity of marine sponge derived fungi

Twelve fungal isolates were purified from marine sponge *Chelonaplysilla* sp. This sponge was known to exhibit antimicrobial and cytotoxic compounds (Bobzin and Faulkner 1991a, 1991b). Presumably, the bioactive compounds were produced by its associated fungi. Although the active compound produced by the fungus associated with *Chelonaplysilla* sp. has never been reported before, some studies stated the family of this genus was associated with the fungus Streptomyces sp. which has broad-spectrum antimicrobial activity (Selvin et al. 2004; Selvin 2009; Selvin et al. 2009). Antimicrobial activity from twelve fungal extracts showed different results (Table 1). Among all isolates, only three isolates (Ch05, Ch06, and Ch12) performed strong antagonistic activity against both *S. aureus* and *E. coli*. These fungal extracts could be categorized as broad-spectrum because of their abilities to inhibit the growth of both Gram-negative and Gram-positive pathogenic bacteria. Isolate Ch12 was the most potential isolate with a diameter inhibition zone of 16.69 ± 0.51 and 7.96 ± 0.73 mm, respectively. However, there was no fungal extract that showed a strong antagonistic effect against *C. albicans*.

Table 1. Antimicrobial activity result of fungal extracts from marine sponge *Chelonaplysilla* sp.

Fungal isolates code	Diameter inhibition zone (mm) ± Standard Deviation (SD)		
	S. aureus	***E. coli***	***C. albicans***
Ch01	-	7.83 ± 0.79	-
Ch02	8.83 ± 1.01	8.54 ± 1.00	-
Ch03	8.25 ± 0.35	-	-
Ch04	8.13 ± 0.76	9.34 ± 2.03	8.13 ± 0.99
Ch05	15.92 ± 0.52	16.33 ± 0.51	7.54 ± 0.19
Ch06	11.96 ± 0.79	12.29 ± 0.72	7.88 ± 0.71
Ch07	-	7.71 ± 0.69	-
Ch08	8.75 ± 0.87	9.54 ± 1.44	-
Ch09	-	-	8.67 ± 0.36
Ch10	-	7.88 ± 076	7.63 ± 1.19
Ch11	-	8.17 ± 1.02	8.56 ± 0.09
Ch12	16.69 ± 0.51	16.88 ± 0.57	7.96 ± 0.73

Note: The value is expressed as the mean ± standard deviation; n=3

Table 2. MTT assay of the extracts of fungi isolated from marine sponge *Chelanoplysilla* sp. against breast cancer cell T47D

Fungal isolates code	IC_{50} (µg/mL)
Ch 01	605.72
Ch 02	743.42
Ch 05	83.96
Ch 09	641.19
Ch 10	670.75
Ch 12	637.24

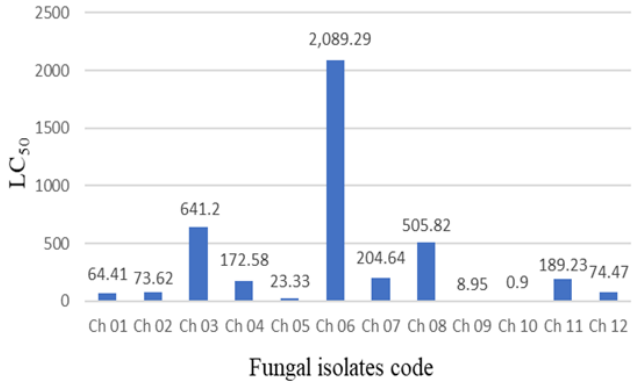

Figure 3. Cytotoxic activity results of the extracts of fungi isolated from marine sponge *Chelanoplysilla* sp. by using BSLT method

Cytotoxic activity of marine sponge derived fungi

BSLT is one of the preliminary toxicity tests for the active compound obtained from the fungal extract. This method is comparable to expensive bioassay methods (Pisutthanan *et al.*, 2004; Wu, 2014). In this study, six fungal isolates (Ch01, Ch02, Ch05, Ch09, Ch10, and Ch12) had LC_{50} value <100 µg/mL (Fig. 3). These isolates could be categorized as having strong cytotoxic activities according to Meyer *et al.*, (1982). Among these isolates, isolate Ch10 had the lowest LC_{50} value (0.90 µg/mL). MTT assay was also conducted for these six potential fungi against breast cancer cell T47D (Table 2). The isolate Ch05 fungal exhibited moderate cytotoxic activity with IC_{50} 83.69 µg/mL. Other isolates did not provide enough cytotoxic activity on these cell lines. Presumably, every cancer cell has its sensitivity against each anticancer agent.

Macroscopic, microscopic, and molecular identification of marine sponge derived fungi

Antimicrobial and cytotoxic assays revealed that isolates Ch02, Ch05, Ch06, Ch10, and Ch12 strains were the most potential to be studied further. Fungal identification of these isolates was conducted macroscopically, microscopically (Fig. 4), and molecularly (Fig. 5 and Tab. 3). From the phylogenetic tree, it was seen the grouping of each isolate following the results of identification.

The surface observation of isolate Ch02 exhibited a white cotton colony and showed a white color in the reverse colony. The diameter of the colony after 5 days was 7 cm (A1). Conidiophore and asexual spore of this isolate were similar to those of genus *Aspergillus*. The surface of the colony of isolate Ch05 was observed to have white threads appearance. The colony diameter after 5 days was 10 cm (B1). This isolate had light blue conidiophore, and asexual spores of this fungus were similar to genus *Phomompsis* (B2). Isolate Ch06 had a dark green surface colony. Its reverse colony was gray color with diameter after 5 day-growth was 5-6 cm (C1). This isolate had gray conidiophore and asexual spores which were similar to genus *Penicillium*. Isolate Ch12 had a white colony with a rough surface after 5 days, the diameter of the colony was around 3-5 cm (E1). This fungus had blue light conidiophore and striated vesicle. These characters place this fungus similar to genus *Aspergillus* (E2). Isolate Ch10 had a white surface colony with a diameter of 8-10 cm after 10 days. This fungus had gray conidiophore (D1). Asexual spores of this fungal were similar to genus *Beauveria* (D2).

The results of molecular identification for isolate Ch02, Ch05, Ch06, Ch10, and Ch12 are presented in Table 3 and Figure 4. The conclusions drawn based on the results of identification using the ITS gene indicated that Ch02 had a 100% identity with *Aspergillus oryzae* and *Aspergillus. flavus*. The 100% identity between these to fungal species resulted in uncertainty regarding the identification method. According to Frisvad et al (2019), *A. oryzae* was the result of domestication of *A. flavus*, thus giving confusion to the naming of those unfamiliar with the morphological characteristics of these two fungi. To distinguish the two, a suggestion will be placed such as the examination of aflatoxins, since *A. oryzae* lost its ability to produce aflatoxin, although phylogenetically it is very closely related to *A. flavus*. *Aspergillus oryzae* produced 4-hydroxy-4-methylpent-2-enyl moiety which was assumed to inhibit the growth of *E. coli* while *A. flavus* produced a compound that showed a strong inhibition activity against *S. aureus* (Xu et al. 2015).

Three portals used to identify fungi molecularly gave different results based on the ITS gene sequence of isolate Ch05. The GenBank identified this isolate as *Phomapsis* sp. (99.8%), Mycobank revealed identity 97.51% with *P. perseae*, while BOLD identified this isolate as *Diaporthe hongkongensis* (99.2%). *Phomopsis* actually is the asexual stage of *Diaporthe* (Gomes et al. 2013). Kobayashi et al (2003), was able to isolate phomopsidin produced by *Phomopsis* sp., a marine-derived fungus. Using GenBank and BOLD, Ch06 was confirmed molecularly as *Penicillium simplicissimum*. Zu et al (2016), reported the presence of three new dihydroisocoumarins produced by *P. simplicissimum* MA-332, a fungus derived from marine mangrove, which showed toxicity activity against brine shrimp and broad-spectrum antimicrobial activities.

Isolate Ch10 was identified by GenBank as *Beauveria bassiana*, and this result was in line with the results obtained from MycoBank and BOLD. Yamazaki et al (2012) reported that they were able to isolate chrysazin and globosuxanthone A from *B. bassiana* TPU942, a marine-derived fungus. Chrysazin and globosuxanthone A were able to inhibit the growth of *C. albicans*, and globosuxanthone A showed cytotoxic activity against two human cancer cell lines, HCT-15 (colon) and Jurkat (T-cell lymphoma). Isolate Ch12 was verified molecularly as *Aspergillus mellinus*. *Aspergillus mellinus* RSPG_204 showed antimicrobial activities against *S. aureus*, *P. aeruginosa* and *C. albicans*, and high cytotoxic activity against MCF7 (breast) cell line (El-Hady et al. 2014). The results from secondary metabolites analysis showed that Ch12 contained terpenoid, while Ch 10 contained saponin.

Terpenoid compounds have been widely isolated from *Aspergillus* genus. Most of the terpenoid compounds also have antimicrobial and cytotoxic activities. Aspergilloxide, a terpenoid compound from the marine-derived fungus

Aspergillus sp. has potent cytotoxic activity toward HCT 116 colon carcinoma (Cueto et al. 2002). Another terpenoid compound from *Aspergillus sp.*, asterpenols A and B, was known also as a potent antimicrobial (Xiao et al. 2013). Less information provided on saponin compounds from *B. bassiana*. Nevertheless, *B. bassiana* has been explored by several researchers. Beauvericin, a major compound from *B. bassiana*, is a potent cytotoxic. This compound also has another bioactivity such as antimicrobial, antiviral and insecticidal (Wang and Xu 2012). Pyridovericin and pyridomacrolidin, other compounds from *B. bassiana*, have been reported to have antimicrobial and cytotoxic activities (Takahashi et al. 1998).

In summary, fungal isolate Ch12 and Ch10, from marine sponge *Chelanoplysilla* sp., showed potential antimicrobial and cytotoxic activities, respectively. Molecular identification revealed that Ch 12 was *A. mellinus* and Ch 10 was *B. bassiana*. Isolate Ch10 and Ch 12 have already submitted to NCBI with accession number of MT000965 and MT003976, respectively. Further study is important to conduct due to the presence of secondary metabolites which can be investigated further for the possibility of being developed as antimicrobial and anticancer agents.

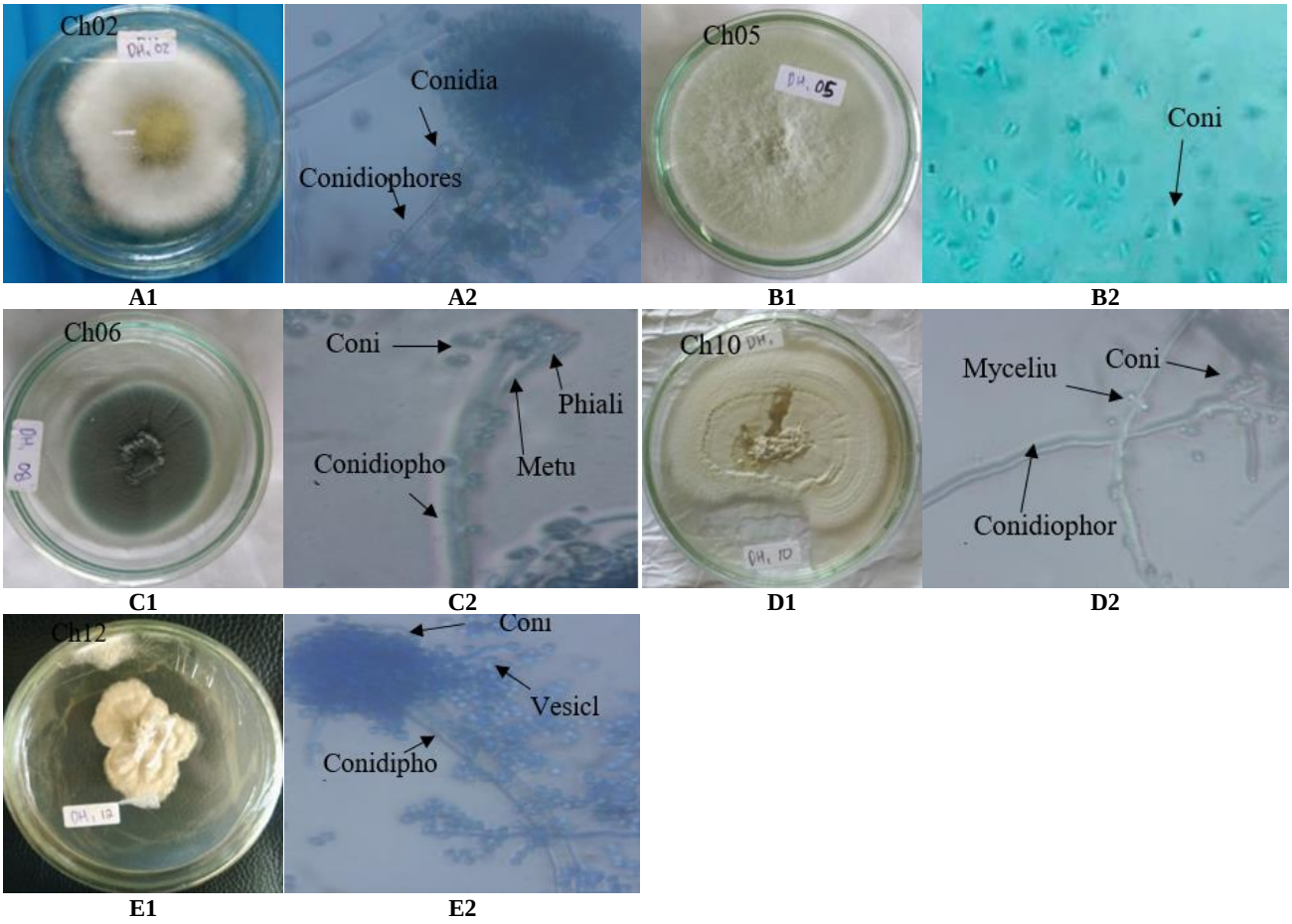

Figure 4. Macroscopic and microscopic identification of fungal isolates Ch02, Ch05, Ch06, Ch10, and Ch12

Table 3. The results of Molecular identification of fungal isolates Ch02, Ch05, Ch06, Ch10, and Ch12

Fungal isolates code	GenBank	Identity (%)	MycoBank	Identity (%)	BOLD	Identity (%)
Ch02	*Aspergillus oryzae*	100%	*A. flavus*	100%	*A. oryzae, A. flavus*	100%
Ch05	*Phomapsis* sp.	99.80	*P. perseae*	97.51	*Diaporthe hongkongensis*	99.2
Ch06	*P. simplicissimum*	100	*P. rolfsii*	97.461	*P.simplicissimum*	100
Ch10	*Beauveria bassiana*	100	*B. bassiana*	99.184	*B. bassiana*	100
Ch12	*A. mellinus*	100	*A. mellinus*	100	*Aspergillus mellinus*	100

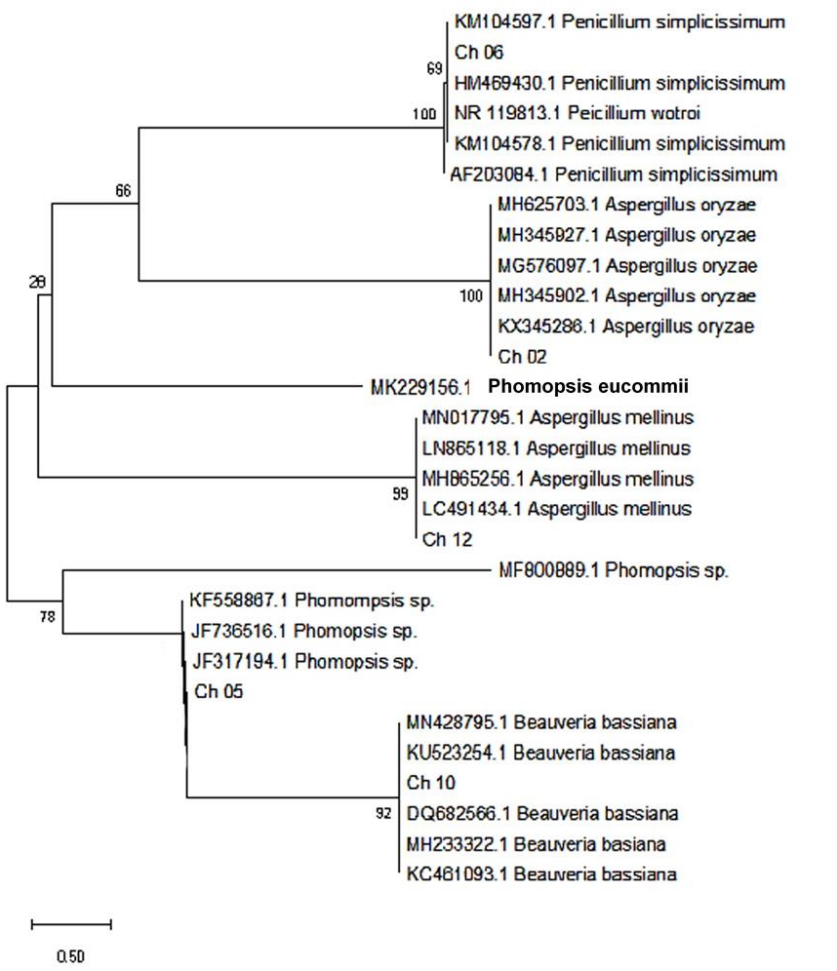

Figure 5. The phylogenetic tree of ITS sequences of fungal isolate Ch02, Ch05, Ch06, Ch10, and Ch12 inferred using the Neighbor-Joining method

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