Molecular identification and optimization of cultural conditions for mycelial biomass production of wild strain of *Chlorophyllum molybdites* (G.Mey) Massee from the Philippines

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ABSTRACT

Chlorophyllum molybdites is a basidiomycetous fungus that is commonly found growing in cluster on soil with grasses. In this paper, the molecular identification and optimization of cultural conditions for mycelial growth of *C. molybdites* were investigated. The genomic DNA of wild fruiting body was isolated, polymerase chain reaction-amplified using the following internal transcribed spacer (ITS) primer: ITS 1F(F) 5' CTT GGT CAT TTA GAG GAA GTA A 3' and ITS4B R 5' CAG GAG ACT TGT ACA CGG TCC AG 3', blasted in GenBank database, and confirmed the identity using phylogenetic analysis. The optimal nutritional (indigenous media) and physical requirements (temperature, aeration, and illumination) for mycelial biomass production in liquid culture were also established. The results of BLAST and phylogenetic analyses revealed that the genomic DNA isolated showed 99.76% similarity to *C. molybdites* (KP012712.1) and 72% bootstrap support in the phylogenetic tree. Mycelia of *C. molybdites* favorably grew in potato dextrose decoction at pH 5.0–5.5, and when incubated at 24–28°C in an unsealed and either lighted or dark conditions.

1. INTRODUCTION

Mushroom emergence in the Philippines is suitable because of its tropical condition. It allows the proliferation of wild resources of mycological diversity that grows naturally on forest litter, fallen logs, lawns, gardens, and piles of agro-industrial wastes particularly during the rainy season [1]. These wild mushrooms regardless of their edibility can be potential candidates as a source of functional compounds. In the past years, several wild mushrooms were domesticated and cultivated using indigenous solid media that lead to the successful production of their biomass and evaluation of their functional properties. Some of these include *Collybia reinakeana* [2], *Coprinus comatus* [1,3], *Ganoderma lucidum* [4], *Lentinus sajor-caju* [5,6], *Lentinus tigrinus* [6], *Panaeolus antillarum,* and *Panaeolus cyanescens* [7].

The molecular approach of the identification of mushroom is more accurate and reliable than the conventional morphological method, which is oftentimes leading to misidentification of species [8]. Hence, some studies in the past utilized the molecular approach of identification. For instance, four species of wild mushrooms from Mt. Mingan, Gabaldon, Nueva Ecija were molecularly identified as *Stereum hirsutum*, *Micropus xanthopus*, *Pleurotus tuberregium*, and *Trametes elegans* [9], five species of wild mushrooms from Mt. Bankay, Cuyapo, Nueva Ecija were confirmed as *Micropus* sp., *G. lucidum*, *Meripilus giganteus*, *Xylaria papulis*, and *Leucoagaricus cepaestipes* [10], three mushroom species such as *L. tigrinus*, *Lentinus squarrosulus*, and *Polyporus grammocephalus* from the three Aeta tribes were also identified [11]. Therefore, molecular approach is better than morphological method in providing accurate identity of mushrooms.

Chlorophyllum molybdites (G. Mey) Massee, also known as false parasol or green-spored parasol, is a poisonous mushroom that belongs to the family Agaricaceae [12]. It is a saprotrophic and commonly found in humus-rich soil such as farmlands, lawns, garden beds, and parks, throughout the rainy season, and it is also amenable to artificial cultivation [13]. The fruiting body of *C. molybdites* contains compounds that exhibit different biological properties such as anti-plasmodial, antimicrobial, and anti-cancer [13-15]. However, studies about this mushroom are very limited in the Philippines because they are regarded as poisonous by the locals. Therefore, establishing the optimum mycelial growth of *C. molybdites* will lead to the development of its biomass production technology and evaluation of important bioactivities.

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In this paper, we confirmed the identity of the collected wild mushroom using molecular approach and established the optimal cultural conditions for efficient mycelial biomass production of this wild basidiomycetous mushroom.

2. MATERIALS AND METHODS

2.1. Source and Tissue Culture of Mushroom

The fruiting bodies of the wild *C. molybdites* [Figure 1] were collected in Lupao, Nueva Ecija, the Philippines. The collected fruiting bodies were tissue cultured in potato dextrose agar plates and incubated at room temperature to allow mycelial growth. Cultures were properly labeled.

2.2. Molecular Identification and Phylogenetic Analysis

A 100 mg mycelia were grounded in liquid nitrogen using mortar and pestle. Total DNA was extracted from the mycelia using the cethyl-trimethyl ammonium bromide (CTAB) method based on Murray and Thompson [16] with minor modifications. DNA quality check using electrophoresis was performed using Enduro Gel XL and was viewed in Enduro™ GDS. The genomic DNA was amplified using primer pair internal transcribed spacer (ITS) 1F(F) 5' CTT GGT CAT TTA GAG GAA GTA A 3' and ITS4B R 5' CAG GAG ACT TGT ACA CGG TCC AG 3' from IDT®. Polymerase chain reaction (PCR) was performed with an automated thermal cycler (Applied Biosystems 2720 Thermal cycler). PCR profiles used in this study were made with the following conditions: 35 cycles with an initial denaturation at 95°C for 3 min, final denaturation at 95°C also for 30 s, annealing at 54°C for 30 s, extension at 72°C for 1 min, and final extension for 7 min at 72°C and held at 4°C. The PCR components were made up of 2.5 µl of ×10 PCR buffer, 1.5 µl of 25 mM MgCl$_2$, 1.25 µl of 10 mM DNTP mix, 1.0 µl of ITS1, and 1.0 µl of ITS 4 BR, 0.1 µl of KAPA TaQ standard polymerase, and 16.65 µl of sterilized distilled water (sdH$_2$O) which had a total volume of 25 µl together with 1 µl of extracted genomic DNA. The amplified product was checked using Enduro Gel XL and viewed in Enduro™ GDS. Amplified samples were sent to Apical Scientific Sequencing Laboratory in Malaysia for PCR purification and sequencing procedure. The sample sequence of ITS region was queried on the GenBank on-redundant nucleotide collection using nucleotide BLAST. Default search parameters on the standard nucleotide BLAST (blastn) web interface were used.

Figure 1: Fruiting bodies of *Chlorophyllum molybdites* in their natural habitat

The phylogenetic tree was made using the Neighbor-Joining method on Molecular Evolutionary Genetics Analysis (MEGA X) software [17]. The optimal tree had a branch length of 0.1 nucleotide substitute per site. Percentage of replicate trees in which the associated taxa were clustered together in a bootstrap test (1000 replicates), was shown [18]. The evolutionary distance was computed using the Maximum Composite Likelihood method [19] and is expressed as the units of the number of the base substitution per site. The analysis of the trees involved ten nucleotide sequences in ITS. All positions containing gaps and missing data were eliminated.

2.3. Evaluation of Culture Media

The mycelial biomass production in the different indigenous broth was evaluated. The treatments were designated as follows: Safflower decoction (50 g of Safflower seeds in 1 L of H20), mung bean decoction (50 g of mung bean bran in 1 L of H$_2$O), yellow corn grit decoction (50 g of *Zea mays* cracklings in 1 L of H$_2$O), feed conditioner decoction (50 g of feed conditioner in 1 L of H$_2$O), sorghum decoction (50 g of red sorghum seeds in 1 L of H$_2$O), taro decoction (50 g of Taro corm in 1 L of H$_2$O), potato dextrose decoction (PDD) (200 g of *Solanum tuberosum* tuber + 10 g of dextrose in 1 L of H$_2$O), snap bean decoction (50 g of Snap bean in 1 L of H$_2$O), rice bran decoction (50 g of rice bran in 1 L of H$_2$O), and coconut water (1 L of pure *Cocos nucifera* water). The pH of the liquid media was adjusted to pH 6.0. Media (30 ml) were dispensed in culture bottles and sterilized at 121°C, 15 psi for 30 min. There were seven culture bottles per medium. After cooling, the media were aseptically inoculated with 10 mm mycelial disc of *C. molybdites* and incubated at 24–28°C to allow mycelial growth. After the establishment of the appropriate indigenous culture media, mycelia of *C. molybdites* were grown in the best medium with different pH levels ranging from 4.0 to 9.0 with 0.5 intervals. The pH was adjusted using 1M NaOH and 1M HCl. There were seven culture bottles per pH. The media were sterilized and inoculated with mycelial disks. After 15 days of incubation, the mycelial mats were then harvested, washed with distilled water, and air-dried. Harvested mycelial biomass for each treatment was weighed, best medium was used for the evaluation of the different physical factors such as temperature, aeration, and illumination conditions.

2.4. Evaluation of Physical Conditions

The newly inoculated plate cultures were prepared and incubated in the different physical factors. Three temperature conditions: Room temperature (24–28°C), air-conditioned (18–19°C), and refrigerated (3.4°C) were used in this study. Aeration conditions were designated as follows: Unsealed (with cotton plug) and sealed (cotton plug covered with polypropylene sheet). For illumination conditions, the following factors were used: Continued lighting, alternating light and dark, and total darkness. Under continued lighting conditions, the inoculated culture containers were incubated in a chamber with artificial light (137 lux). For the dark condition, inoculated culture containers were covered with clean black paper. In the alternating light and dark condition, cultures were exposed under natural light during day time, to facilitate lighted conditions and incubated in the dark during night time. Each treatment in all tests was replicated 7 times. After 15 days of incubation, the mycelial mats were harvested, washed with distilled water, air-dried, and weighed.

2.5. Statistical Analysis

The IRRI STAR statistical software was used for statistical analyses of the data. The experiments were laid out in a completely randomized

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design. The analysis of variance was used and treatments were further compared using Tukey's comparison of mean on the influence of culture media, influence of pH, temperature, and illumination conditions. The *t*-test was used for aeration conditions.

3. RESULTS AND DISCUSSION

3.1. Molecular Identification of Mushroom

The modern molecular technique reduces the challenges of inconspicuous nature, inconsistent morphological identification, and indiscrimination among fungal species often associated with the traditional method of nomenclature [20]. In this study, ITS sequence fragment was used to identify the mushroom sample from the GenBank database.

BLAST analysis confirmed that the genomic DNA isolated from the collected mushroom labeled as SAMPLE_K002 was a fungal nrDNA, which showed 99.76% similarity to the GenBank nucleotide sequence of *C. molybdites* (KP012712.1). Phylogenetic analysis further confirmed that among nine related nucleotide sequence, sequence of SAMPLE_K002 was found homologous to *C. molybdites* with 72 % bootstrap support [Figure 2]. Based on the constructed phylogenetic tree, *C. molybdites* is more closely related to *Chlorophyllum hortense* (MK554576.1), *Chlorophyllum agaricoides* (DQ200928.1), and *Chlorophyllum rhacodes* (JQ683124.1) than the other five related species including *Macrolepiota globosa* (AF48282.1), *C. subrhacodes* (MG741975), *Chlorophyllum globosum* (AY243619.1), *Chlorophyllum palaeotropicum* (NR159759.1), and *Leucoagaricus medioflavoides* (GQ329055.1). The optimal tree has the sum of branch length equivalent to 12.91337174.

Similarly, using BLAST sequence analysis of the ITS region, Parnmen *et al.* [21] confirmed the molecular identity of poisonous mushrooms in Thailand with similarities ranging from 88% to 100%. In addition, Reyes *et al.* [8] reported the molecular identity of two newly recorded Termitomyces species in the Philippines, the *T. bulborhizus* and *T. clypeatus* with similarities of 92% and 87%, respectively. Moreover, Adeniyi *et al.* [22] confirmed the identity of *Termitomyces aurantiacus*, *Tricholoma matsutake*, *Tricholoma robustum*, *P. ostreatus*, *Schizophyllum commune,* and *Pleurotus pulmonarius* with similarities between 77% and 100. Nevertheless, in the phylogenetic study of Vellinga *et al.* [23], *Chlorophyllum* was found to belong within Agaricaceae. However, based on similarities in morphology and/or molecular evidence, a few species previously placed in *Macrolepiota* Singer or *Lepiota* (Pers.) Gray, were transferred into *Chlorophyllum* [24].
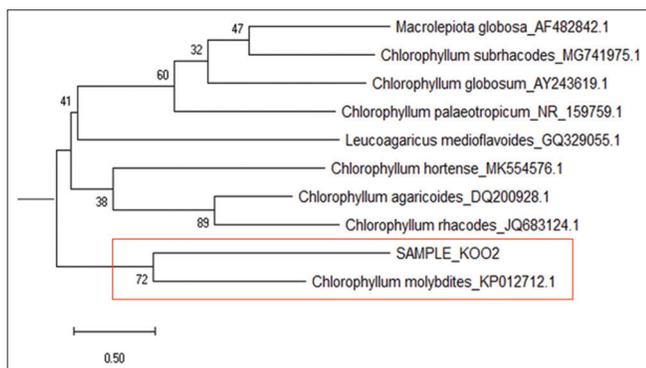

Figure 2: Phylogenetic tree showing the relationship of SAMPLE_K002 with other related species

3.2. Influence of Culture Media

The luxuriance and rapidity of growth of a certain mushroom partly depend on the appropriate culture medium used in its cultivation in the laboratory [3]. Optimization of culture conditions of *C. molybdites* was carried out on submerged liquid culture conditions. Moreover, the variation of physiochemical and nutritional parameters, such as type of carbon and nitrogen sources, pH, aeration, temperature, illumination, and incubation conditions of mushroom strains can greatly affect the metabolite synthesis [25], hence, also affect the mycelial growth.

It can be inferred from Table 1 that PDD exhibited the highest dry mycelial weight with a mean value of 124.86 mg, while rice bran decoction recorded the lowest mean value of 12.00 mg. Coconut

Table 1: Mycelial biomass yield (dry weight) of *Chlorophyllum molybdites* after 15 days of incubation as affected by different nutritional and physical factors

Nutritional and physical factors	Dry weight of mycelia (mg)
Liquid media	
Safflower decoction	33.57±8.60[bc]
Mung bean decoction	38.29±2.69[bc]
Yellow corn grit decoction	31.00±3.46[bc]
Feed conditioner decoction	27.00±2.00[bc]
Sorghum decoction	31.00±6.00[bc]
Taro decoction	21.00±0.82[bc]
Potato dextrose decoction	124.86±39.57[a]
Snap bean decoction	23.00±2.16[bc]
Rice bran decoction	12.00±3.21[c]
Coconut water decoction	58.00±54.13[b]
pH	
4.0	125.86±43.34[abc]
4.5	140.14±47.60[abc]
5.0	177.29±27.91[a]
5.5	177.86±25.77[a]
6.0	170.14±24.72[ab]
6.5	160.57±28.00[abc]
7.0	156.86±28.44[abc]
7.5	155.86±28.39[abc]
8.0	151.86±25.73[abc]
8.5	108.57±45.91[c]
9.0	118.43±34.92[bc]
Temperature	
24–28°C	173.57±26.21[a]
18–19°C	69.57±8.22[b]
3.4°C	7.43±0.53[c]
Aeration	
Unsealed	171.14±10.76[a]
Sealed	146.43±42.12[b]
Illumination	
Continued lighting	174.86±16.96[a]
Alternating dark and light	151.29±33.79[a]
Dark	144.29±30.84[a]

Values are mean±SD of seven replicates. Means with the same letter are not significantly different from each other using Tukeys' honest significant difference and *t*-test

water recorded the second-highest dry mycelial weight of 58.00 mg. Statistical analysis revealed that there is a significant difference on the mycelial biomass yield of *C. molybdites* grown between PDD, coconut water, and rice bran decoction. However, the last two liquid media were not significantly different in terms of mycelial biomass yield from sorghum decoction, mung bean decoction, yellow corn grit, feed conditioner decoction, sorghum decoction, taro decoction, and snap bean decoction.

Based on the results of our study, PDD was the most suitable medium for the production of mycelial biomass. The luxuriant growth of *C. molybdites* on PDD can be attributed to the nutrient composition of the medium. Potato tuber is rich in carbohydrate, dietary fiber, ascorbic acid, potassium, total carotenoids, and antioxidant phenols such as chlorogenic acid and its polymers, and anti-nutrients such as α-solanine, and protein, amino acids, other minerals, and vitamins [26]. In addition, according to Kaal *et al.* [27], glucose supplementation promotes the growth and rapid establishment of the mushroom and it offers additional easily metabolizable carbon sources to the substrates.

These results are congruent with the findings of several studies on the optimization of various mushroom species. For instance, Dulay *et al.* [28] reported that potato decoction was the most suitable medium that favored the mycelial growth of *P. antillarium*. Luangharn *et al.* [29] observed that *Laetiporus sulphureus* produced the largest colony diameter and mycelial density in potato dextrose agar among six culture medium tested. Kalaw *et al.* [30] used potato sucrose gulaman on two strains of *V. volvacea* which showed the largest colony diameter and shortest incubation. The observation of Soytong and Asue [31] in *Pleurotus giganteus* suggested that although the mycelial growth produced in PDD was slower than the other media, the mycelia were thick and heavy.

Rice bran which has the lowest biomass production, on the other hand, is good sources of organic nitrogen (N_2) that is necessary to the growth of the mycelial biomass; however, it can interfere in productiveness and biological efficiency of the fungus [32]. According to Silva *et al.* [33], the low nitrogen level can stimulate ligninolytic enzyme production, however, a nitrogen level higher than the mushroom required can represses it, thus inhibit the mycelial growth of *C. molybdites* in this study.

The pH of the culture medium is a very important factor for mycelial growth of fungi [34]. The mean dry mycelial weight at different pH levels varied significantly from each other such that pH range of 5–5.5 was the optimum, this optimum was comparable in a wide pH range recorded the highest mean value of 177.29 and 177.86 mg, respectively, while pH 8.5 exhibited the lowest mean value of 108.57 mg. This suggested that this mushroom can grow in a wide range of pH. In general, mushrooms can grow in a wide range of pH of the medium [30]. Interestingly, Jayasinghe *et al.* [35] confirmed it on his report that *G. lucidum* strains could grow on potato dextrose agar at a broad pH range, such as pH 5.0–9.0. Enzyme activity is greatly affected by pH and mushroom species have evolved the means to function under specific environment [4]. The previous studies showed that various mushroom species grew best from slightly acidic conditions to neutral pH which coincides with the results obtained in the present study, that is, pH 5.0 to 5.5. Peksen *et al.* [36] reported that the best suitable pH for mycelial growth of *Hydnum repandum* was found to be at 5.5 pH on potato dextrose yeast agar. Reyes *et al.* [3] revealed that *C. comatus* grown on coconut water gelatin with pH 6.5 produced very dense mycelial growth 6 days after incubation. Lai *et al.* [37] reported that the optimum pH for radial growth of *Lignosus*

rhinocerus mycelia on potato dextrose agar was between pH 6.0-7.0. The best pH value for the mycelial growth of *T. terum* was 4.5–6.0 on potato dextrose agar [38].

3.3. Influence of Physical Factors

Since PDD and pH 6.0 were determined as the most appropriate medium for mycelial growth, this liquid medium was also used in the evaluation of optimum temperature, aeration conditions, and illumination conditions.

Temperature is an important factor that affects microbial growth, production of metabolic products, and sporulation of mushroom. The *C. molybdites* incubated at room temperature produced the heaviest dry mycelial weight of 173.57 mg. As expected, no mycelial growth was observed in the refrigerated condition. Comparison among means revealed that there was a significant difference between room temperature and air-conditioned temperature both in terms of mean dry mycelial weight. This result suggested that *C. molybdites* can be incubated at a temperature between 24 and 28°C to attain the optimum mycelial biomass production. Similarly, Shim *et al.* [39] reported that the mycelial growth of *Paecilomyces fumosoroseus* had been expedited gradually in proportion to the rise of temperature and the growth was most suitable at 25°C. Kim *et al.* [40] revealed that the temperature suitable for the mycelial growth of *Oudemansiella radicata* was at 25°C. The growth of mycelia of *G. lucidum* was faster at room temperature (32°C) [4].

Aeration is another important physical factor to be considered for efficient mycelial growth [4]. As shown in Table 1, the mean dry mycelial weight was higher in unsealed (171.14 mg) compared to sealed (146.43 mg). This implied that the mycelial growth in unsealed was better compared to sealed based on higher yield in mycelial biomass. Furthermore, massive aerial mycelia in sealed condition suggested that the mycelia were growing toward the top of the culture bottle, where the air seems to be present, which clearly observed as shown in Figure 3. The results of this study conform with the findings of De Leon *et al.* [5] who reported that *L. sajor-caju* in unsealed Petri plates produced larger mycelial diameter, while the sealed Petri plates produced smaller mycelial diameter after 4 days of incubation. Similarly, Bustillos *et al.* [7] reported that *Panaeolus antillarium* and *P. cyanescens*, when cultured in unsealed plates, produced thicker
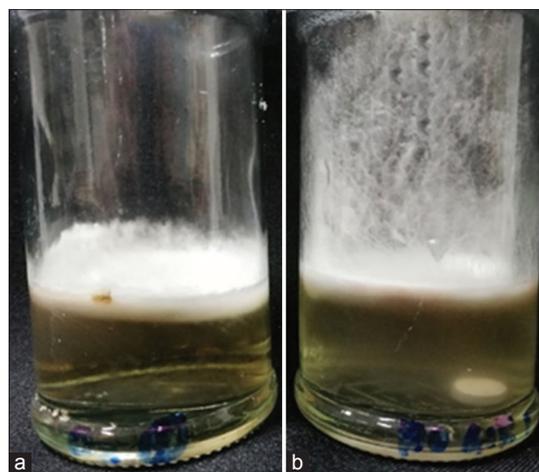

Figure 3: Mycelia of *Chlorophyllum molybdites* G. Mey (Masee) produced from the two aeration conditions: unsealed (with cotton plug, a) and sealed (cotton plug covered with polypropylene sheet, b)

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mycelia compared to the sealed plates 8 days after incubation. On the other hand, findings of Reyes *et al*. [3] showed that mycelial growth of *C. comatus* was promoted in sealed plates.

Statistical analysis revealed that there was no significant effect of various illumination conditions (lighted, alternating light and dark, and total darkness) on dry mycelial weights. Although in terms of dry mycelial weight, the lighted condition (174.86 mg) was higher in both light and dark as well as total darkness condition, with means of 151.29 mg and 144.29 mg, respectively. Nevertheless, these results indicate that *C. molybdites* could be incubated in either lighted, alternating light and dark (every 12 h), and total darkness conditions. According to Chang and Miles [41], the growth of most mushrooms was not sensitive to light, although strong light may inhibit or even kill the mycelia. This conform with the responses of *C. comatus* and *C. reinakeana* to light [3,42]. In addition, findings of Liu *et al*. [43] revealed that illumination does not affect the mycelial growth of *Isaria farinosa* on PDD. Furthermore, Sung *et al*. [44] observed no obvious difference in the colony diameter of *Ophiocordyceps longissima* between light and dark conditions on solid media. In contrary, Kalaw *et al*. [30] revealed that illumination significantly influenced the mycelial growth of five mushroom species on solid medium: *L. sajor-caju* and *S. commune* (CLSU strain)*,* whereas dark condition showed maximum growth of *G. lucidum* (strain B), *L. tigrinus* (CLSU strain), *G. lucidum* (strain A), and *C. cinerea* (Sto. Domingo strain). Wu *et al*. [45], findings on the other hand, revealed that red and yellow light showed stimulatory effects on *P. eryngii* mycelial growth on solid medium; however, total darkness was the best condition for biomass production on submerged condition.

4. CONCLUSION

BLAST analysis revealed that the mushroom SAMPLE K002 is identified as *C. molybdites* (KP012712.1) with 99.76% identity. Phylogeny analysis shows that it has 72% bootstrap support KP012712.1. The optimum liquid medium for mycelial growth of *C. molybdites* (G. Mey) Massee is PDD at pH range 5–5.5, at room temperature and unsealed (with cotton plug). Illumination does not affect the mycelial biomass production on liquid media. The mycelia of this mushroom are a highly potential source of functional and bioactive compounds; however, the toxicity and functional properties of its mycelia of this mushroom need to be established which will be our next study.

5. ACKNOWLEDGMENTS

6. CONFLICTS OF INTEREST

Authors declared that there are no conflicts of interest.

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