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The first report of Agaricales from Camarines Sur, Philippines: Some new records towards mushrooms genetic conservation

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ABSTRACT

Mushrooms are well recognized in their importance in biotechnology such as medicine, enzyme production, and bioremediation. Despite their significance, there is limited work on the collection and proper identification of mushrooms in the country. These kinds of the initiative are recognized as the first activities towards species genetic conservation study as their occurrence was affected by the changing climate condition. In this study, mushrooms under order Agaricales or also known as gilled mushrooms from the Pili Camarines Sur, Philippines, were collected using purposive sampling. The collected samples were photo-documented, hand-picked and molecularly identified using the nrDNA-ITS gene marker. A total of 15 samples were collected and identified, out of this, nine mushroom species namely; the *Leucoagaricus meleagris, Agrocybe retigera, Gymnopilus lepidotus, Gymnopilus dilepis, Crepidotus indicus, Filoboletus manipularis, Clavulinopsis sulcata, Marasmius occultatiformis* and *Gymnopus tamblinganensis* have been suggested to be the first record and report of Agaricales in the Philippines based on nrDNA-ITS gene.

***Key words**: Agaricales, nrDNA-ITS gene, Molecular identification, Phylogenetic analysis*

Introduction

Relatively humid regions are likely to be one of the richest sources of fungal species. In line with this, the Philippines being a tropical country allows the growth of different macro-fungus efficiently in the wild, waiting to be bio-prospected to utilize its potential (Reyes *et al.,* 1997; 1998; Reyes and Abella, 2002; Reyes *et al.,* 2003; Garcia *et al.,* 2004; Tayamen *et al.,* 2004). These organisms can be seen naturally growing on different decomposing materials and are best observed in the middle of the rainy season wherein moisture is abundant for their germination (Dulay *et al.,* 2012).

Mushrooms are essential in the environment as decomposers. It is widely utilized as food and known to have medicinal properties. Particularly these compounds used in medicinal purposes are termed "mushroom nutriceuticals"(Chang and Buswell, 1996) which can be extracted from mushroom mycelium as well as fruiting body and are important to the growing mushroom industry

(Cheung, 2008). Despite their important roles in the environment and their potential, a large number of mushrooms are still unaccounted for, in terms of their identity and diversity and remain insufficiently reported (Mueller *et al*. 2007).

The order Agaricales is one of the most diverse groups under the Basidiomycota, a division of Kingdom Fungi. The Basidiomycota is typically a filamentous fungus composed of hyphae. Most species that belong to this higher Basidiomycota mushroom species reproduce sexually with a club-shaped spore-bearing organ known as basidium that usually produces four sexual spores also known as basidiospores. A wide variety of forms exhibits this order, these ranges from conspicuous wood decaying mushrooms, plant-growth-promoting and mutualistic mycorrhizae, and crop destroying smut and rust fungi, to yeastlike human pathogens (Coelho *et al.*, 2017).

The first report on the collection of Agaricales particularly its identity has been based on its morphological characteristics and the collection has been done based on its economic importance, such as the edible and with medicinal potential (Quimio, 1977; 1986). Sibounnavong *et al.*, (2008) collected and identified at least 2 species of Agaricales; under the Family Tricholomataceae which is *Hobenbuebelia petaloides* (Bull ex Fr.) Schulz and Family Cantharellaceae which is *Cantharellus minor*, from Puncan, Carranglan, Nueva Ecija in the Philippines using morphological characteristics and literature search.

Prior to harnessing the potentials of these materials from the wild, they must be first properly identified. The use of the molecular method for fungal identification is widely recognized. Reports have proven its success in classifying even complex groups of mushroom (Le *et al.*, 2008). Though results from this approach are promising, macroscopic features are still essential to produce presumptive and proper identification.

Materials and Methods

Study site for mushroom collection

Fresh samples of macroscopic fungi were collected from the vicinity of Mt. Isarog located at Pili, Camarines Sur, Philippines (Figure 1).

The sample collection was carried out in November of 2019. The team together with the person familiar with the area trailed the mountain and diligently looked for any mushroom or macroscopic fungi. These were photo-documented and their morphological characteristics such as color, pileus attachment, presence of gills or pores, and other notable observations were noted for the initial identification. Subsequently, the samples were hand-picked carefully. The knife was used for samples that were hard to detach from the substrate. Garden shovel was used for mushrooms that were growing on soil so that the fruiting body retains its intact. Collected samples were then placed on paper bags and labelled accordingly.

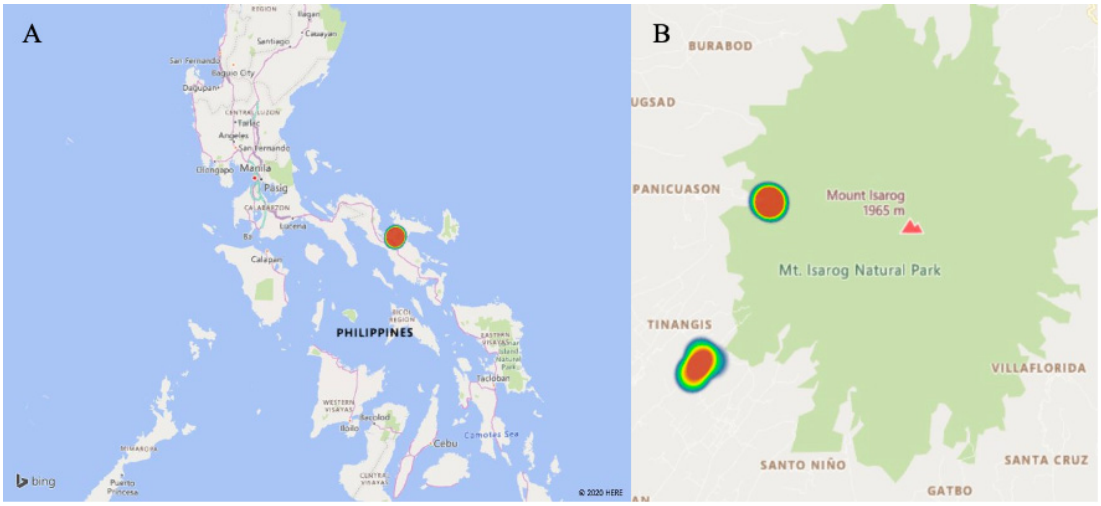

Fig. 1. Map of the Philippines (A) and the vicinity of Mt. Isarog in Camarines Sur where the collection was undertaken (B).

Genomic DNA extraction

Fruiting bodies were subjected for DNA extraction using the cetyltrimethylammonium bromide (CTAB) method according to Murray and Thompson (1980) with minor modification as follows: Seven hundred fifty (750) µl of 2x CTAB buffer prewarmed to 65 °C with 50 µl 20% SDS was added and mixed using vortex and incubated in the dry bath set at 65°C for 45 min, wherein every 5 min the tubes were mixed shortly using a vortex. After incubation, the homogenate was then spun in a centrifuge at 10,000 rpm for 10 min. The supernatant was pipetted in a new 2 ml tube and added with freshly prepared 500µl chloroform: isoamyl alcohol (24:1).

This was then mixed using vortex and then spun in a centrifuge for 30 min at 10,000 rpm. Six hundred (600) µl of ice-cold isopropanol was then added to the mixture, mixed gently and was incubated overnight at -20 °C. The tubes were then spun in a centrifuge for 10 min at 10,000 rpm. The pellet was washed twice with 500 µl of 70% ethanol. The tubes were inverted and placed on top of tissue paper to dry out the ethanol. The DNA pellet was dissolved by adding 50 µl of TE buffer with RNAse (1:100) and incubated at room temperature for 3-4 hours. The quality of the extracted DNA was checked by mixing 1µl of the DNA samples with 1µl loading dye and were dispensed with 1% agarose gel stained with BiotiumGelRed® Nucleic Acid Gel Stain. The DNA sample together with a 1kb universal ladder was run for 30 min in the electrophoresis tank (ENDURO™ GEL XL, Labnet International, Inc.) with 100V. The genomic DNA was visualized in a gel documentation system (ENDURO™ GDS, Labnet International, Inc.)

Amplification of nRDNA-ITS gene fragment using a polymerase chain reaction

The nrDNA ITS region was then amplified using the ITS1-F sequence (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes and Brunes, 1993) and ITS4 sequence (5'-TCCTCCGCTTATTGATAGC-3') (White *et al.*, 1990). The reactions were carried out in 0.2 mL PCR tubes containing 32.80µl of distilled water, 2.5 µl of forward primer (10 µM), 2.5 µl of reverse primer (10µM), 5 µl of dNTPs (25 mM), 5 ng of DNA template, 5 µl 10X buffer and .02µl of Taq (KAPA Taq) polymerase.

The PCR machine was set for 35 cycles with the following temperature set at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec, 55 °C for 45 sec, and 72 °C for 30 sec, with a final extension step of 72 °C for 7 min. One (1) µl PCR products and the 1 kb DNA ladder was subjected for electrophoresis (ENDURO™ GEL XL, Labnet International, Inc.) for 30 minutes at 100 V in a previously prepared 1% agarose gel stained with BiotiumGelRed®Nucleic Acid Gel Stain. The PCR product was visualized in a gel documentation system (ENDURO™ GDS, Labnet International, Inc.)

Sequencing and BLAST analysis

The PCR products were quantified using a fluorometer (Fluoroskan Ascent®, Thermo Scientific) and were sent to the 1st BASE Laboratory at Apical Scientific in Malaysia for PCR purification and sequencing. The results were then subjected to BLAST analysis to identify sequence similarity to sequences available at the NCBI for match query and identification. The nrDNA-ITS sequences of closely similar sequences available on online resources were aligned using the ClustalW provided in the default parameter of MEGA X. Phylogenetic trees were constructed by applying the Neighbor-Joining (NJ) and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model (Tamura & Nei, 1993; Kumar *et al.*, 2018).

Results and Discussion

Mushroom collection and identification

There were 15 Agaricales collected, identified and confirmed. The morphology of the collected samples was confirmed using the nrDNA-ITS gene sequence (Table 1 and Figure 2)

The Agaricales collected mushrooms were classified under 10 Families as suggested by their nrDNA-ITS gene sequence. The four samples belong to Family Strophariaceae, two samples belonged to Pleurotaceae, two samples from Marasmiaceae and one sample each from Family Agaricaceae, Psathyrellaceae, Bolbitiaceae, Pluteaceae, Crepidotaceae, Mycenaceae and Clavariaceae (Table 1).

Importance of collected mushrooms for genetic conservation

The Philippines is considered as one of the 18 megabiodiverse countries containing two-thirds of the

Fig. 2. Agaricales collected from Pili, Camarines Sur, Philippines: A. *Leucoagaricus meleagris* (Family Agaricaceae), B. *Coprinopsis cinerea*, (Family Psathyrellaceae), C-D. *Pleurotus djamor* (Family Pleurotaceae), E. *Agrocybe retigera* (Family Strophariaceae), F. *Gymnopilus lepidotus* (Family Strophariaceae), G-H. *Gymnopilus dilepis* (Family Strophariaceae), I. *Panaeolus foenisecii* (Family Bolbitiaceae), J. *Volvariella volvacea* (Family Pluteaceae), K. *Crepidotus indicus* (Family Crepidotaceae), (L) *Filoboletus manipularis* (Mycenaceae), (M) *Clavulinopsis sulcata* (Clavariaceae), (N) *Marasmius occultatiformis* (Marasmiaceae), O. *Gymnopus tamblinganensis* (Marasmiaceae).

Table 1. Identities of Agaricales mushroom samples using molecular (nrDNA-ITS genes) technique incurred from NCBI-BLAST sequence repository.

Code	Family	Name	% Identity	Accession
PC02	Agaricaceae	*Leucoagaricus meleagris*	100%	MK412590
PC04	Psathyrellaceae	*Coprinopsis cinerea*	99.09%	KX468975
PC05	Pleurotaceae	*Pleurotus djamor*	100%	KY328723
PC13		*Pleurotus djamor*	99.84%	KY328723
PC07	Strophariaceae	*Agrocybe retigera*	99.75%	MH016951
PC12		*Gymnopilus lepidotus*	98.92%	KX035108
PC10		*Gymnopilus dilepis*	99.53%	AY280980
MG05		*Gymnopilus dilepis*	99.79%	KP074969
PC14	Bolbitiaceae	*Panaeolusfoenisecii*	99.83%	KR867660
PC16	Pluteaceae	*Volvariella volvacea*	100%	MG280838
MG02	Crepidotaceae	*Crepidotus indicus*	84.97%	MK370662
MG06	Mycenaceae	*Filoboletus manipularis*	100.00%	KY243915
MG10	Clavariaceae	*Clavulinopsis sulcata*	88.96%	MK427060
MG15	Marasmiaceae	*Marasmius occultatiformis*	93.16%	MK388150
MG17		*Gymnopus tamblinganensis*	93.41%	AY263450

earth's biodiversity and supports the existence of different kinds of organisms, including mushrooms. The order Agaricales of mushrooms generally have the most familiar types of mushrooms and are well distributed in nature. The order has over 13,000 species already described under 33 extant families and 413 genera (Kirk *et al.,* 2008). The *Leucoagaricus meleagris* (Gray) Singer belong to Family Agaricaceae, some of the mushroom species belong to this family has been reported (Jacob *et al.,* 2017; Reyes *et al.,* 2009; De Leon *et al.,* 2013; Tadiosa *et al.,* 2015; Dulay *et al.,* 2020) however, the *L. meleagris* has not been reported in the Philippines. The *L. meleagris* was a leaf litter degrading mushroom containing cellulase found important for cellulose degradation (Graf *et al.,* 2017) and xylanase that degrades linear polysaccharides xylan into xylose (Boonrung *et al.,* 2014).

The *Coprinopsis cinerea* (Schaeff.) Redhead, Vilgalys and Moncalvo belonged to Psathyrellaceae. The *C. cinerea* is a wood-rotting and edible mushroom that is one of the most common species of mushrooms and found in animal dung and natural substrate. It hasbeen collected in some parts of the Philippines (Torres *et al.,* 2020), it is also known as gray shag and ink cap mushroom because of its long stipe and well-developed cap. The *C. cinerea* however from Camarines Sur has not been reported. Aside from being edible, it is also known as a good decomposer, the *C. cinerea* produced enzyme laccases, which helps in the degradation of lignin in plant fibres (Bouws *et al.,* 2006; You *et al.,* 2014 as cited by; de Mattos-Shipley *et al.,* 2016), and also produces enzymes and secondary metabolites such as lectins, laccases, peroxidases and terpenoids (Kilaru *et al.,* 2006; Courty *et al.,* 2009).

The *Pleurotus djamor* (Rumph. ex Fr.) Boedijn 1959 was under the family Plurotaceae. Like *C. cinerea, P. djamor* was also one of the most common mushrooms found in nature. It is an edible mushroom and has medicinal properties (Pineda-Alegría *et al.,* 2017). It is found out that the *P. djamor* has major compounds including four fatty acids such as pentadecanoic, hexadecanoic, octadecadienoic, octadecanoic acid, and one terpene identified as β-sitosterol (Pineda-Alegría *et al.,* 2017). The *P. djamor* possesses nematicidal metabolites, which could be used as an alternative anthelmintic treatment (Pineda-Alegria *et al.,* 2017; González-Cortázar *et al.,* 2021). The *P. djamor* from Camarines Surwas first reported in this study. The *Agrocybe retigera* (Speg.) Singer was probably a common species and grown in gardens, parks and open areas. However, this species was many times overlooked because this was seen as a common mushroom and its identity was limited using only its morphological characteristics. The *A. retigera* has been reported in Argentina, Brazil, Paraguay and Venezuela (Coimbra, 2015), however, it has not been reported in the Philippines.

The genus *Gymnopilus* P. Karst. includes more than 200 saprobic species, moreover, Campi *et al.,* (2021) mentioned that many species of *Gymnopilus* were cited in Argentina, Bolivia, Brazil, Chile, Colombia, Ecuador and Uruguay. The *Gymnopilus*

lepidotus was first characterized and reported as new records of fungi from Iguazu National Park from Argentina (Lechner *et al.,* 2006) and then further characterized by Campi *et al.,* (2021). Nonetheless, reports of this species in the Philippines are scarce. Gymnopilus can be recognized by its bright, golden to rust-brown color as described by Rees *et al.,* (2004).

The *Gymnopilus lepidotus* is a subtropical and tropical species under Agaricales Family, mostly thrived at fallen logs and was found to have enzymes for lignin degradation. On the other hand, in 2017 a new record of *G. dilepis* was reported in Thailand (Suwannarach *et al.,* 2017). The *G. dilepis* was also reported in Australia, India, Indonesia, Japan, Malaysia, Papua New Guinea, Sri Lanka and the United Kingdom (Benkert, 1987; Treu, 1998, Guzman-Davalos, 2003; Guzman Davalos *et al.,* 2003; Thomas *et al.,* 2003; Rees *et al.,* 2004, Kasuya *et al.,* 2016). This is probably the first report of *G. dilepis* in the Philippines based on its morphology and as supported by the nrDNA-ITS gene fragment. The *G. dilepis* is a saprophyte and is commonly found in solitary or in clusters in decaying logs or debris and decaying woods. The *G. dilepis* are hallucinogenic, psilocybin-producing mushrooms (Reynolds *et al.,* 2017), an indole alkylamine and tryptamine compound promising for depression (Carhart-Harris *et al.,* 2016).

The *Panaeolus foenisecii* (Fries) Kuhner was first reported and molecularly identified using the ITS gene region in Sri Lanka (Ediriweera *et al.,* 2015) and first reported in the Philippines (Lopez *et al.,* 2016). Similar to *G. dilepis*, the *P. foenisecii* also produces psilocybin (Stijve *et al.,* 1983; Hadley, 1980; Dewhurst, 1980).

The *Volvariella volvacea* is an edible mushroom and also known as paddy mushroom or straw mushrooms. The *V. volvacea* is most commonly found and utilized as food in the Philippines (Tantengco and Ragrario, 2018; Lazo *et al.,* 2015), molecular identification using the random amplified polymorphic DNA (RAPD) also have been done to discriminate this species (Abella *et al.,* 2014). The *V. volvacea* has important amino acid components such as glutamic acid and valine. It also contains carbohydrate, sugar, protein, ash, fats, vitamins and organic acids and potassium as the most abundant mineral and has Vitamin B3 (Eguchi *et al.,* 2015). This species has now undergone domesticationfor commercialization as foods and condiments.

The *Crepidotus indicus* A. M. Kumar and C. K. Pradeep was characterized and first reported as a new collection in India, and used nrLSU rDNA for molecular identification (Kumar *et al.,* 2018). There has been no report of this species in the Philippines so far. The potential and significance of this mushroom have not been explored so far. However, some species under this Genus were found to have ligninolytic enzymes potential for dye degradation (Mtui, 2007).

The *Filoboletus manipularis* (Berck.) Singeris a bioluminescent fungus also observed as a poroid mushroom. It has been first documented in Malaysia (Chew *et al.,* 2015), Southern Vietnam (Vydrakova *et al.,* 2014) and the Island of Hawaii (Hemmes *et al.,* 2019). In a particular study, adaptation to high-temperature stress of this species results in the accumulation of 9(11)-dehydroergosterol and ergosterol peroxide and increased thermal plasticity correlate with high storage lipid (triglycerides) content, accumulation of phosphatidic acid in the membrane (Senik *et al.,* 2019). The dynamics of sterols and sphingolipids and their molecular mechanisms under temperature stress may provide information on the role of lipid rafts in the thermal plasticity of basidial fungi.

The *Clavulinopsis sulcata* Overeem 1923 belongs to Family Clavariaceae. The *C. sulcata* grows in a leaf-littered environment. It appears to have very long, slender, cylindrical pinkish to orange fruiting bodies. It was first described as *Clavariaminiata* and collected from South Africa (Berkeley, 1843). The potential of this mushroom has not been fully done. However, its niche suggests that this mushroom has the ability to degrade lignocellulosic materials. The *Marasmius occultatiformis* Antonín, Ryoo & H.D. Shin, 2012 belongs to Family Marasmiaceae. It was first reported as new records from Russian Far East, specifically in the Primorsky Territory (Kiyashko *et al.,* 2014). It was found solitary or in small groups in a leaf-littered environment. This is the first report on this species in the Philippines. Its potential and importance to the environment have not been explored so far. Another species under the Family of Marasmiaceae is the *Gymnopus tamblinganensis* A. W. Wison, Desjardin and E. Horak 2004. It was first reported in Indonesia (Wilson *et al.,* 2004) and previously described by Retnowati (2018). The *G. tamblinganensis* was found in leaf-littered environment, it appears brown with concolorous radial striations extending to a lighter brown margin. Like

the *M. occultatiformis*, the potential of *G. tamblinganensis* has not been explored.

Phylogenetic analysis

Phylogenetic analysis was employed to prove the relatedness of the amplified sequence from the NCBI sequence repository and clarify the relationship of the different mushrooms collected (Figure 2). The phylogenetic tree of the identified Agaricales samples using the nrDNA-ITS fragment suggests 3 major clades. The first clade (red), includes *G. tamblingensis*, *F. manipularis* and *C. sulcata* belonging to Family Marasmiaceae, Clavariaceae and Mycenaceae, respectively. The next major clade (green) also shows a low bootstrap value (42) explaining that *M. occultatiformis* is far related to *P. jamor*. Another interesting result here is that sample PC13 and PC05 shows 100 bootstrap values proving they are the same species which is *P. djamor*. The last clade (blue) proves the closeness of identified samples *A. retigera*, *G. lepidotus* and *G. dilepis* as previous reports are taxonomically classified under the Family Strophariaceae. Looking at the said clade, PC10 and MG05 are grouped together verifying that they are of the same species which is *G. dilepis.*

The remaining five samples which were *P. foenisecii*, *L. meleagris*, *V. volvacea*, *C. cinerea*, and *C. indicus* did not form any group for them to be classified as a major clade. It is also important to take note that comparing the tree to Table 1., *M. occulatiformis* and *G. tamblinganensis* should have fallen on the same clade as they are taxonomically classified to fall under the same Family Marasmiaceae. Instead, the former was grouped to the second clade and the latter to the first major clade. Hence, the study claims that *M. occulatiformis* and *G. tamblinganensis* possess low genetic relatedness thus, this rearrangement of its classification

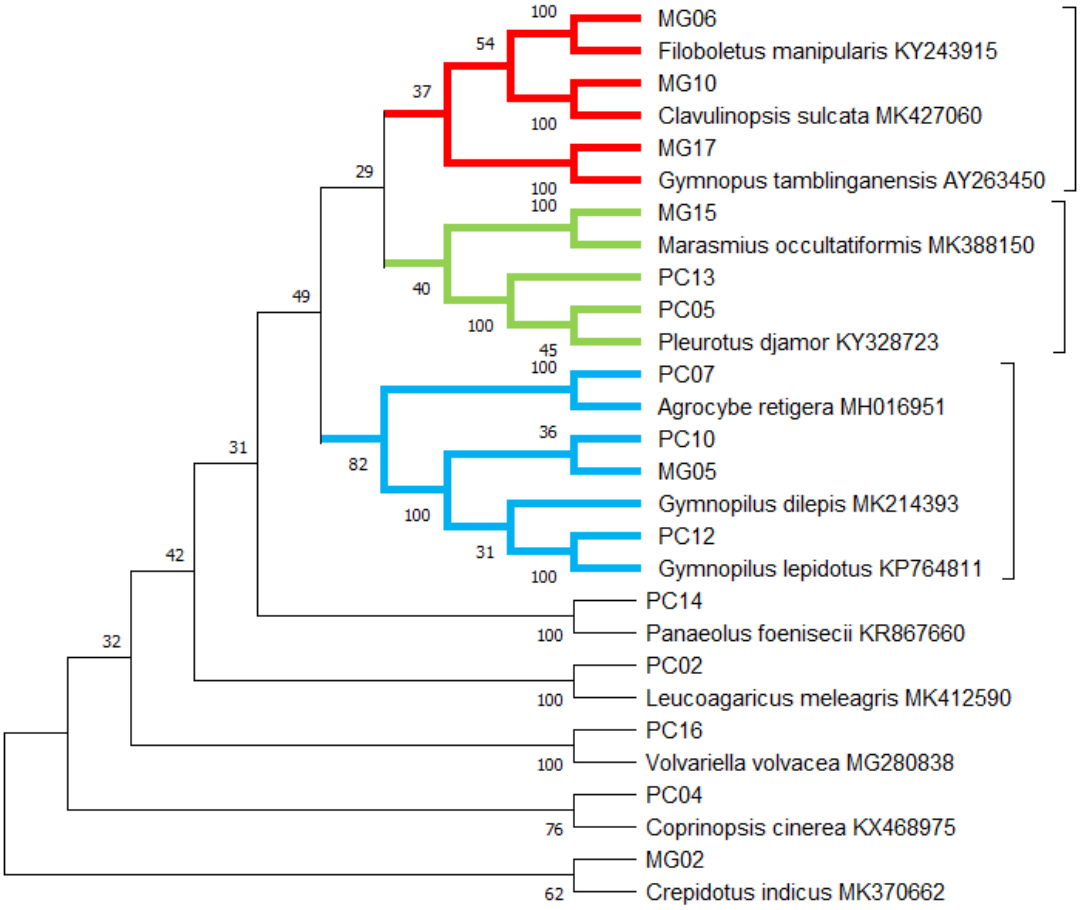

Fig. 3. Phylogenetic tree based on partial rDNA gene sequence of Agaricales samples collected, showing the relationships between related sequences from the NCBI-BLAST. Bootstrap values are shown at the nodes acquired from 100 resampled data sets.

must be done. Another interesting result in the analysis is the low genetic relatedness of the amplified sequence PC04, and MG02 to *C. cinerea*, and *C. indicus.* Though PC04 and MG02 have 99.09% and 84.97% sequence similarity, respectively.

From a phylogenetic point of view, most identities inquired from percent identity from BLAST is supported by the results from the tree. However, there are some results that do not corroborate the tree. Looking at the study of Khan *et al.,* (2011), it was claimed that morphological and RAPD markers showed different patterns of genetic diversity among different *Pleurotus* species. This proves that data from morphologic identification and molecular identification may be some time contrasting which is why these both fields must be used in identification. Adding to this, using molecular techniques, particularly phylogenetic analysis, may help fix the previous mushroom taxonomy which was solely based on morphology.

A previous report by Wen *et al.,* (2017) used this technique to prove Cordycepts gunniiis Meta Cordyceps neogunniis sp. and Wu *et al.,* (2014) redefined and produced new generic clades in family Boletaceae using phylogenetic analysis. In line with this, in some cases, morphology alone cannot prove an identity specifically on those mushrooms which share almost identical physical characteristics. Molecular techniques such as DNA isolation and the culture-based assay can detect overlooked lineages that were based on typical visual observations (Shirouzu *et al.*,2020).

Conclusion

Fifteen mushrooms belonging to 10 Families under the order Agaricales, were collected and identified. From our perspective, nine of these samples were considered the first record and reports of Agaricales in the Philippines, the following species are; *Leucoagaricus meleagris, Gymnopilus lepidotus, Gymnopilus dilepis, Crepidotus indicus, Filoboletus manipularis*, *Agrocybe retigera*, *Clavulinopsis sulcata, Marasmius occultatiformis* and *Gymnopus tamblinganensis.*

Germplasm collection and preservation initiatives

The mycelia and sterilized portion of the fruiting bodies of collected mushrooms were preserved in a cryovial with 10% glycerol and stored in -80 deep freezer in the Cryogenic Laboratory of the Central Luzon State University, Science City of Munoz, Nueva Ecija, Philippines. In addition, information of the mushroom collected were deposited in database (iCollect) of organisms at the Biotechnology and Analytical Laboratory of the Central Luzon State University, Science City of Munoz, Nueva Ecija, Philippines

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