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Evaluating the mycelial growth of bolete from pine forest in highland Vietnam

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Abstract Ectomycorrhizal fungi were difficultly isolates in artificial culture. The evaluation of mycelial preservation was important for predicting the macrofungal cultivated potential of this group. In this study, 24 bolete sporocarps were collected from *Pinus kesiya* pine forests at Da Nhim Watershed Protection Forest which were successfully isolated. Basing on macroscopic characteristics and molecular phylogenetic retrieved from ITS markers, all sporocarps were identified to be 5 genera as *Suillus, Boletus, Baorangia, Leccinum* and *Tylopilus*. All fungal isolates were evaluated the mycelial growth and then preserved on MMN agar medium at 10 ^{o}C. *Suillus* with the best mycelium growth potential was significantly different from the others. After 1 year and 2 years of storage, 24 fungal isolates were evaluated the mycelial growth by both MMN broth and agar. The results showed that all isolates which belongs to *Suillus* were viable after 2 years preservation except isolate A04101. These are preserved the ectomycorrhizal fungi resources for studies about inoculation, biochemistry and artificial culture.

Keywords: Ectomycorrhizal fungi, Mycelium, *Pinus kesiya*, Bolete

Introduction

Pinus kesiya (Khasi pine, three-needled pine) is the most widely distributed pines in Asia, extends south and east from the Khasi Hills in the northeast Indian state of Meghalaya, to northern Thailand, Philippines, Burma, Cambodia, Laos, southernmost China, and Vietnam (Luu and Thomas, 2004). In Vietnam, *Pinus kesiya* has been recorded in Lam Dong, Ha Giang, Dien Bien, Thua Thien Hue, Quang Nam, Kon Tum, Gia Lai, Dak Lak, Khanh Hoa, Ninh Thuan and Dak Nong Provinces. Especially, Lam Dong Province is the cradle of conifers in the south of Vietnam, the use of *Pinus kesiya* for reforestation is especially important in the current state of shrinking forest land. A useful approach for enhancing the

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performance of out-planted seedlings is controlled inoculation of ectomycorrhizal fungi (EMF).

Ectomycorrhizal fungi (EMF) are fruiting bodies formed by the growth of the extracellular mycelium of the ectomycorrhiza (ECM) under suitable conditions. EMF play important role in forest ecosystems (Smith and Read, 2008), some of which are also high-value food sources. However, the collection still depends on many objective factors and the artificial culture of this fungal group is still limited and not popular. Isolation, preservation, and evaluation the mycelial growth can be used as the indicator for analysing physiology, inoculation on seedlings, biochemistry, ability to cultivation, etc.

Currently, there are two main methods to preserve EMF mycelium as short-term or long-term. The preservation is based on the principle of reducing or arresting the growth and metabolism of fungal mycelium (Smith *et al.,* 2001). However, because of the intrinsic characteristics and not forming conidia as well as sexual structure *in vitro* culture, the preservation of these fungi still faces many difficulties.

Commonly used short-term preservation methods include sub-cultivation, preservation with water, mineral oil, alginate beads, etc. These methods are simple, do not require complicated equipment, be stored at room temperature (close to 25 °C) or at lower temperatures, usually for a short time from 2-5 years. But they have disadvantages such as being labor intensive, do not limit infection with mites or other agents, genetic traits easily affected and being space-consuming (Thomson *et al.*, 1993, Smith and Onions 1994; Houseknecht *et al.,* 2012). Tibbett *et al.* (1999) stored *Hebeloma* isolates by sub-culture method for 3 years at 2 °C. The studies of Richter (2008) showed that the genus *Laccaria* stored in sterile water at 5 ^{o}C is better than *Boletus, Lactarius, Paxillus, Scleroderma*, and *Thelephora*. Hyphae of *Rhizopogon nigrescens* was preserved in calcium alginate gel, the result showed that this genus could survive for 18 months at 8 °C (Paloschi de Oliveira *et al*., 2006).

Long-term preservation such as lyophilization and cryopreservation is considered the best preservative for fungi in general. Some advantages of this method are that it is less labor intensive and occupies a small area, allows very long periods of storage and prevents genetic and phenotypic changes (Mazur, 1984). This is a highly technical method and there are differences in cooling rate, cooling temperature and protection agent for each fungal species (Lehto *et al.*, 2008; Dalong *et al.,* 2011). Cryopreservation at ultra-low temperature is considered the most reliable method for most filamentous fungi, however, there is a sensitivity to temperature for filamentous fungi than for fungal spores (Smith, 1998). In the report of Obase *et al.* (2011), 34 fungal strains belonging to 12 ECM genera *Amanita, Cenococcum, Laccaria, Lactarius, Lepista, Paxillus, Pisolithus,*

Rhi-zopogon, Russula, Scleroderma, Suillus and *Tomentella* did not adapt in freeze-drying conditions. Crahay *et al.* (2013) preserved 98 ECM isolates by cryopreservation method, the survival rate after 6 months was 88 % of the isolates. Some isolates belonging to *Suillus luteus, Hebeloma crustuliniforme, Paxillus involutus* and *Thelephora terrestris* could not survive when applying this protocol.

Of the total number of ectomycorrhizal fungi, the group of bolete fungi accounts for about one-quarter (Halling *et al*., 2007) and plays an important role in all forest ecosystems. This group has high food value for example, in the family Boletaceae, a family of fungi of the order Boletales with more than 250 edible species belongs to the genus *Boletus* (62 species), *Leccinum* (19 species), *Xerocomus* (15 species), *Tylopilus* (13 species), or in the family Suillaceae with the genus *Suillus* contributes 30 edible mushroom species (Hall *et al*., 2016). However, only a few fungal strains have been successfully artificially cultivated such as in the genus *Phlebopus* (Sanmee *et al.*, 2010; Le *et al.,* 2017; Chuankid *et al*., 2020; Kumla *et al*., 2022). Therefore, the bolete fungi have great potential for research investigation. The limitations of this study are faced the preliminary identification of successfully isolated fungal samples by molecular biology tools and evaluated *in vitro* growth of some bolete mycelium through the viability of fungal strains after short-term storage.

Materials and methods

Sampling and isolating

All fresh sporocarps were collected from Da Nhim Watershed Protection Forest, Lam Dong Province, in the rain season from Aug to Nov 2019. They were isolated using Modified Melin Norkrans (MMN) agar. The small pieces of inner context (about 2 - 3 mm^3) were cut and placed in the Petri dishes contained 20 mL of MMN agar (added 100 ppm chloramphenicol per liter). Isolated Petri dishes were incubated in the dark at 25 ± 2 ^oC and then sub-culture for collecting the pure isolate.

Determine the colony area of fungal strains

Cut 5 mm diameter mushroom pieces into MMN media plates. Successfully isolated EMF were examined the mycelial growth by measuring the colonial area on MMN medium. All experiments were replicated 3 times. After 21 days of dark incubation at 25 °C, pictures of the fungal plates were taken and measured the area of the colonies using Image J software (Schneider *et al.,* 2012).

Data were analyzed by IBM SPSS Statistics 20 statistical software (IBM Corp, New York, USA).

Identifying by molecular method

Total nuclear DNA was extracted from the mycelium of isolates, purified, precipitated, desiccated and dissolved in TE buffer. The primer pairs ITS1 (5'–TCCGTAGGTGAACCTGCGG–3') and ITS4 (5'–TCCTCCGCTTATTGATATGC–3') (White *et al.,* 1990) were used for amplifying the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA). Amplification was performed following the guide of MyTaq HS Mix (Meridian Bioscience, London, UK). PCR products were purified by ExoSAP IT (Thermo) and sent to 1st BASE Laboratories (Kuala Lumpur, Malaysia) for sequencing. DNA sequences were assembled and edited using AGTC software (Genetyx, Tokyo, Japan). The final sequences were compared with the data in GeneBank (NCBI, USA) by BLAST (NCBI, USA) for general identification. The sequences were submitted to DDBJ (DNA Data Bank of Japan).

Phylogeny tree

The results from the BLAST tool are used for searching reference identical sequences. From the search data combined with the sequence data of the ITS region of all fungal strains, a phylogeny tree was built. Sequence segments were aligned and edited with the MUSCLE tool, the phylogenetic tree was constructed by the Maximum Likelihood method using MEGA version 7 (Kumar *et al*., 2015). The optimal model of datasets based on Akaike criteria (AICs) is 2-parameter Kimura with Gamma distribution. The phylogenetic topology was retrieved by the outgroup of Agaricus sinodeliciosus (Accession No. KM657907)

Preservation of isolated fungal strains

All isolates were preserved by short-term method at cool conditions of 8 - 10 ^{o}C. The ϕ5 mm fungal colonies were transferred from the Petri dishes into inclined MMN agar test-tubes (18 cm with silicosen stoppers (Shinetsu Chemical, Tokyo, Japan)). All tubes were incubated at 25 ^{o}C until the mycelium grew to fill the agar surface. Then, tubes were kept at 10 ± 1 ^{o}C. The viability of fungal strains is checked once a year by transferring mycelium to MMN broth and agar.

Results

From the bolete sporocarps collected from *Pinus kesiya* forests, 24 fungal strains were successfully isolated. The figures of fresh sporocarps and the colonies of isolates on Petri dishes were shown in Table 1. All successful isolates belonged to genera *Suillus, Boletus, Tylopilus, Baorangia* and *Leccinum*. The results showed that the ratio of successful isolation was the highest in the genus *Suillus* (16/24), next was the genus *Tylopilus* (5/24) (Table 2). Phylogenetic tree based on ITS sequence region were constructed using 24 sequences from isolates and 30 reference sequences from Genbank (NCBI) with accession numbers shown in the tree (Figure 1). It is divided into 2 branches belonging to 2 different families, Suillaceae and Boletaceae. The branch of Suillaceae is divided into two small branches with two groups of *Suillus bovinus* and other species of genus *Suillus.* The branch of Boletaceae represented isolates with at least 4 genera.

The results of the areas of fungal mycelium were statistically processed to rank Turkey ($p<0.5$) some strains of the genus *Suillus* have superiority in mycelium speed compared to other genera, especially strain A04131 (Table 2). The mycelial growth of genera *Boletus, Leccinum* were very low. After 1 year of storage, all strains could grow on the MMN broth, but some of them did not grow on the MMN agar including the strains belonging to genera *Tylopilus, Boletus* and *Leccinum.*

The viability of the fungal strains decreased markedly after 2 years of storage, most strains of the genera *Tylopilus, Boletus, Baorangia* and *Leccinum* were unable to grow on either type of MMN medium. Most strains of the genera *Suillus* could grow on the MMN both, while some strains could not grow on the MMN agar (A04302, A06115, A06202).

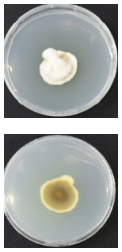
The identification of specimens in this study was precarious due to the high diversity of the bolete group (Nuhn *et al.,* 2013; Wu *et al*., 2014; Wu *et al.,* 2016). Using only a single ITS marker could not be recognized to species. However, the macroscopic characteristics of all specimens were mostly expressed to the focused genera following Halling (2022).

From the list of ectomycorrhizal fungal isolates, two genera of *Suillus* and *Tylopilus* were isolated easily and grown quickly MMN agar. However, after sub-culturing, only some strains of genus *Suillus* retained this feature. The survival of ectomycorrhizal fungi during storage depended on the species or strains. The mycelial growth as well as the survival potential of all isolates belonged to genus *Suillus* was diversified. *Suillus, Boletus, Leccinum* were a part of 15 edible EMF species repleted genera in the world (Boa, 2012). The study of their mycelial growth helped to evaluate the develop further studies. Isolates with fast mycelial growth could be easily cultured for growth characteristics

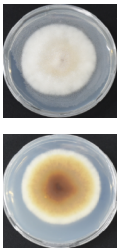
evaluation, mycelium biomass collection mushroom cultivation trialing. Among the 24 isolates, in the genus *Suillus*, two species were identified as *Suillus luteus* and *Suillus bovinus* are edible mushrooms (Hall, 2016; Boa, 2012). *Suillus luteus* were reported artificially cultivated in the garden (Ying *et al.*, 2007). Therefore, the stable mycelial growth should be an indication for further studies. Based on the macroscopic characteristics of sporocarps of the genus *Boletus* (A02101), *Baorangia* (A02107) and *Leccinum* (A02112) were recorded as edible mushrooms according to the local indigenous knowledge. Re-collecting the sporocarps of those genera should be necessary for further studies.

Table 1. The fresh sporocarps and the colonies of fungal isolates on MMN agar after 21 days

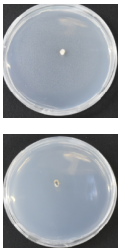
A06115 *Suillus bovinus*	**A02101 *Boletus* sp.**	**A02103 *Tylopilus* sp.**
		
A02106 *Tylopilus* sp.	**A02107 *Baorangia* sp.**	**A02110 *Suillus* sp.**
		
A02112 *Leccinum* sp.	**A02205 *Tylopilus* sp.**	**A02206 *Suillus bovinus***
		
A04101 *Suillus luteus*	**A04131 *Suillus luteus***	**A04302 *Suillus* sp.**

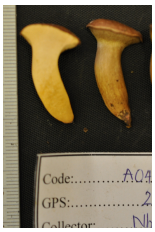
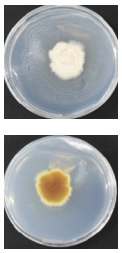
A04127 *Suillus bovinus*

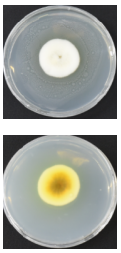
A06112 *Suillus luteus*

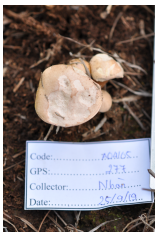
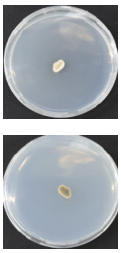
A04105 *Suillus* sp.

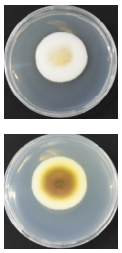
A05120 *Suillus* sp.

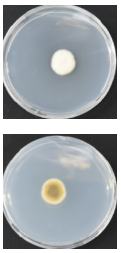
A05113 *Suillus* sp.

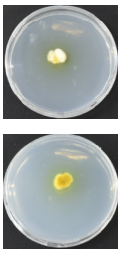
A06105 *Tylopilus* sp.

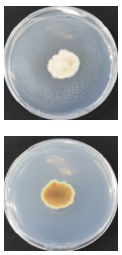
A06116 *Suillus* sp.

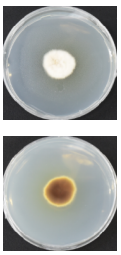
A06202 *Suillus* sp.

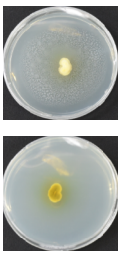
A06301 *Tylopilus* sp.

A07301 *Suillus bovinus*

A08201 *Suillus bovinus*

A09108 *Suillus bovinus*

Table 2. List of 24 EMF strains isolated from sporocarps collected from *Pinus kesiya* forest and the viability of fungal strains after 2 years of storage

EMF Strain	Accession number	Genus	The area of fungal mycelia (mm^2)	Viability after 1 year		Viability after 2 years	
				MMN agar	MMN liquid	MMN agar	MMN liquid
A06115	LC651050	*Suillus bovinus*	$630.67^{de} \pm 11.53$	+	+	-	+
A02101	LC651028	*Boletus* sp.	$94.45^{mn} \pm 14.22$	-	+	-	-
A02103	LC651029	*Tylopilus* sp.	$231.3^{kl} \pm 135.41$	-	+	-	-
A02106	LC651030	*Tylopilus* sp.	$173.79^{klm} \pm 13.4$	-	+	-	-
A02107	LC651031	*Baorangia* sp.	$163.75^{lm} \pm 11.17$	-	+	-	-
A02110	LC651032	*Suillus luteus*	$759.34^{cd} \pm 19.73$	+	+	+	+
A02112	LC651033	*Leccinum* sp.	$15.12^{n} \pm 1.75$	-	+	-	-
A02205	LC651034	*Tylopilus* sp.	$287.51^{kl} \pm 16.37$	-	+	-	-
A02206	LC651035	*Suillus bovinus*	$272.68^{kl} \pm 21.96$	+	+	-	+
A04101	LC651037	*Suillus luteus*	$574.51^{efg} \pm 10.21$	-	+	-	-
A04105	LC651039	*Suillus* sp.	$627.93^{de} \pm 18.51$	+	+	+	+
A04127	LC651040	*Suillus bovinus*	$594.48^{ef} \pm 13.28$	+	+	+	+
A04131	LC651041	*Suillus luteus*	$2820.35^{a} \pm 55.06$	+	+	+	+
A04302	LC651042	*Suillus bovinus*	$19.91^{n} \pm 1.22$	+	+	-	+
A05113	LC651044	*Suillus* sp.	$300.05^{ik} \pm 10.66$	+	+	+	+
A05120	LC651045	*Suillus* sp.	$1290.63^{b} \pm 70.76$	+	+	+	+
A06105	LC651047	*Tylopilus* sp.	$163.52^{lm} \pm 11.55$	-	+	-	-
A06112	LC651049	*Suillus luteus*	$824.84^{c} \pm 15.69$	+	+	+	+
A06116	LC651051	*Suillus bovinus*	$429.68^{hi} \pm 21.66$	+	+	+	+
A06202	LC651052	*Suillus bovinus*	$454.12^{gh} \pm 15.62$	+	+	-	+
A06301	LC651053	*Tylopilus* sp.	$91.01^{mn} \pm 9.67$	-	+	-	-
A07301	LC724024	*Suillus bovinus*	$233.05^{kl} \pm 13.1$	+	+	+	+
A08201	LC724025	*Suillus bovinus*	$463.99^{fgh} \pm 10.56$	+	+	+	+
A09108	LC724026	*Suillus bovinus*	$243.67^{kl} \pm 111.1$	+	+	+	+

Note: Data with different letters within the same column indicate a significant difference at $p < 0.05$ according to Turkey's multiple range test. + (alive); - (do not grow)

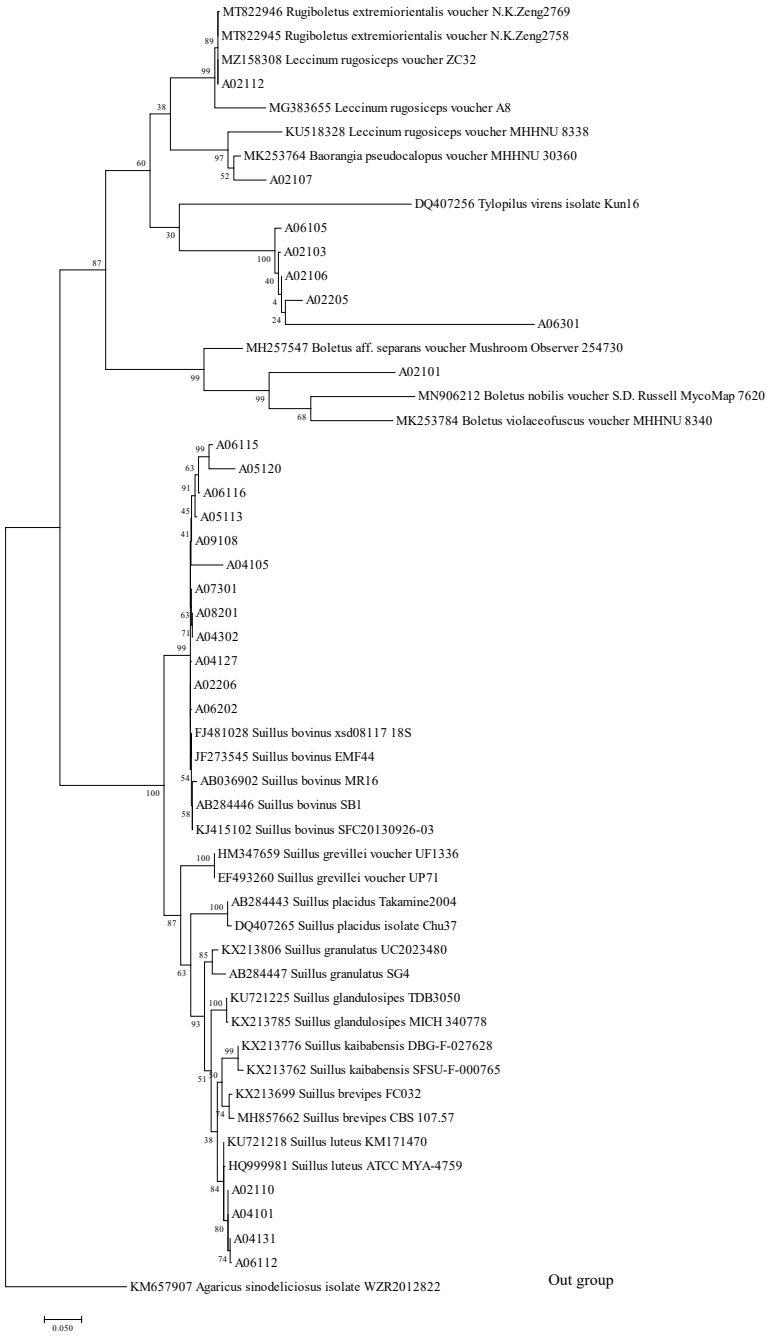


Figure 1. Molecular Phylogenetic analysis by Maximum Likelihood method

Discussion

The methods of preserving filamentous fungi were differentiated depending on the available equipments. Each method had its own advantages and disadvantages. In general, the popular sub-culture preservation method used distilled water, mineral oil in cool temperature or freeze-drying (Obase *et al.,* 2011), or freezing by cryopreservation (Kitamoto *et al*., 2002; Crahay *et al.,* 2013). The preservation effect was different for different EMF species. For example, freezing at -196 °C or -80 °C showed that *Laccaria bicolor* could be preserved without loss of viability but *Paxillus involutus* or *Pisolithus tinctoriu*s did not survive and the mycelium was damaged (Corbery and Tacon, 1997). Table 2 shows that cool temperature storage on slant agar was only suitable for *Suillus* genus, and the best time for sub-culture is less than 1 year, especially for genera such as *Tylopilus, Boletus, Baorangia, Leccinum.* It is necessary to study on different preservation methods for these objects to choose an appropriate method.

In summary, the bolete group had different mycelial growth and survival potential in cool temperature preservation. The genus *Suillus* had better mycelial growth and survival potential than other genera (*Boletus, Baorangia, Tylopilus* and *Leccinum*) in this study. The sub-culture should be repeated at least once a year.

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References

Boa, E. (2012). Local Communities and Edible Ectomycorrhizal Mushrooms. In book: Edible Ectomycorrhizal Mushrooms (pp.307-315) Chapter: 17 Publisher: Springer Editors: Zambonelli A, Bonito GM.

Chuankid, B., Schrey, H., Thongbai, B., Raspe, O., Arnold, N., Hyde, K. D. and Stadler, M. (2020). Secondary metabolites of Phlebopus species from Northern Thailand. Mycological Progress, 19:1525-1536.

Corbery, Y. and Tacon, F. L. (1997). Storage of ectomycorrhizal fungi by freezing. Annales des sciences forestières, INRA/EDP Sciences, 54:211-217.

Crahay, C., Declerck, S., Colpaert, J. V., Pigeon, M. and Munaut, F. (2013). Viability of ectomycorrhizal fungi following cryopreservation. Fungal Biology, 117:103-111.

Dalong, D. M., Guoting, G. Y., Liqiang, L. M. and Li, C. (2011). Tolerance of ectomycorrhizal fungus mycelium to low temperature and freezing-thawing. Canadian Journal Microbiology, 57:328-332

Hall, I. R., Lyon, T., Wang, Y. and Buchanan, P. (2016). A list of putative edible or medicinal ectomycorrhizal mushrooms. Retried from https://www.researchgate.net/publication/302319041_A_list_of_putative_edible_or_medicinal_ectomycorrhizal_mushrooms.

Halling, R. E. (2022). Boletales – Boletaceae s.l. Retried from http://sweetgum.nybg.org/science/projects/boletineae/wpcontent/uploads/sites/6/2022/03/synopsis_list.pdf

Halling, R. E., Chan, H. T. and và Lee, S. S. (2007). Basidiomycetes: Boletaceae. In: E.B.G. Jones, K.D. Hyde, S. Vikineswary (eds.) Malaysian Fungal Diversity. Mushroom Research Centre, University of Malaya and Ministry of Natural Resources and Environment, Malaysia, 41-53.

Houseknecht. J. L., Sung-Oui, S. and Jianlong, J. Z. (2012). Viability of fastidious Phytophthora following different cryopreservation treatments. Fungal Biology, 116:1081-1089.

Kitamoto, Y., Suzuki, A., Shimada, S. and Yamanaka, K. (2002). A new method for the preservation of fungus stock cultures by deep-freezing. Mycoscience, 43:0143-0149.

Kumar, S., Stecher, G. and Takuma, K. (2015). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular biology and Evolution.

Kumla, J., Suwannarach, N. and Lumyong, S. (2022). Cultivation of edible tropical bolete, *Phlebopus spongiosus*, in Thailand and yield improvement by high-voltage pulsed stimulation. Agronomy, 12:115.

Le, T. T. N., Tran, C. H., Phan, M. H., Bui, V. T., Pham, N. D. H. and Ho, B. T. Q. (2017). Mycelial cultivation of *Phlepobus spongiosus*, an edible ectomycorrhizal mushroom in southern Vietnam. Journal Science Ho Chi Minh City Open University, 7:14-21.

Lehto, T., Brosinsky, A., Heinonen-Tanski, H. and Repo, T. (2008). Freezing tolerance of ectomycorrhizal fungi in pure culture. Mycorrhiza, 18:385-392.

Luu, N. D. T. and Thomas, P. I. (2004). Conifers of Vietnam. Darwin Initiative, UK.

Mazur, P. (1984). Freezing of living cells: mechanisms and implications. American Journal Physiology Cell Physiology, 16:125-142.

Nuhn, M., Binder, M., Taylor, A., Halling, R. E. and Hibbett, D. S. (2013). Phylogenetic overview of the Boletineae, Fungal Biology, 117:479-511.

Obase, K., Lee, S. Y., Chun, K. W. and Lee, J. K. (2011). Regeneration of ectomycorrhizal fungal isolates following deep freezer storage. Mycobiology, 39:133-136.

Paloschi de Oliveira, L., Rossi, M. J., Furigo Junior, A., Nunes Silva Filho, G. and Lopes de Oliveira, V. (2006). Viability and infectivity of an ectomycorrhizal inoculum produced in an airlift bioreactor and immobilized in calcium alginate. Brazil Journal of Microbiology, 37:251-255.

Richter, D. L. (2008). Revival of saprotrophic and mycorrhizal basidiomycete cultures after 20 years in cold storage sterile water. Canadian Journal of Microbiology, 54:595-599.

Sanmee, R., Lumyong, S., Lumyong, P. and Dell, B. (2010). *In vitro* cultivation and fruit body formation of the blackbolete, *Phlebopus portentosus*, a popular edible ectomycorrhizal fungus in Thailand. Mycoscience, 51:15-22.

Schneider, C., Rasband, W. and Eliceiri, K. (2012). NIH Image to ImageJ: 25 years of image analysis. Nature Methods, 9:671-675.

Smith, D. (1998). The use of cryopreservation in the ex-situ conservation of fungi. CryoLetters, 19:79-90.

Smith, D. and Onions, A. H. S. (1994). IMI Technical Handbooks 2: the preservation and maintenance of living fungi, 2nd edn. CAB International, Wallingford.

Smith, D., Ryan, M. J. and Day, J. G. (2001). The UK national culture collection biological resource: properties, maintenance and management. UK National Culture Collection, Egham.

Smith, E. and Read, D. J. (2008). Mycorrhizal symbiosis, 3rd edn. Academic Press in an Imprint of Elsevier, Great Britain, pp.787.
Thomson, B. D., Malajczuk, N., Grove, T. S. and Hardy, G. E. S. J. (1993). Improving the colonization capacity and effectiveness of ectomycorrhizal fungal cultures by association with a host plant and re-isolation. Mycological Research, 97:839-844.
Tibbett, M., Sanders, F. E., Cairney, J. W. G. and Leake, J. R. (1999). Temperature regulation of extracellular proteases in ectomycorrhizal fungi (*Hebeloma* spp.) grown in axenic culture. Mycological Research, 102:707-714.
White, T. J., Bruns, T. D., Lee, S. and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols, a guide to methods and applications. Academic Press, San Diego, pp.315-322.
Wu, G., Feng, B., Xu, J., Zhu, X. T., Li, Y. C., Zeng, N. K., Hosen, M. I. and Yang, Z. L. (2014). Molecular phylogenetic analyses redefine seven major clades and reveal 22 new generic clades in the fungal family Boletaceae. Fungal Diversity, 69:93-115.
Wu, G., Li, Y. C., Zhu, X. T., Zhao, K., Han, L. H., Cui, Y. Y., Li, F., Xu. J. P. and Yang, Z. L. (2016). One hundred noteworthy boletes from China. Fungal Diversity, 81:25-188.
Ying, G., Lu, M. and Li, L. (2007). Artificial cultivation method for *Suillus Luteus*. CN101204131A. China.

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