BJNRD (2017), 4(1): 12-21
Bhutan Journal of Natural Resources & Development

Article

www.bjnrd.org

Open Access

ISSN 2409–2797 (Print)
ISSN 2409–5273 (Online)

https://doi.org/10.17102/cnr.2017.02

Additions to the Knowledge of *Macrolepiota* (Agaricales, Basidiomycota) in Northern Thailand

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Abstract

Macrolepiota is a genus of white spored, gilled mushroom of the family Agaricaceae which are widely distributed in tropical and temperate countries. In this study, five wild edible *Macrolepiota* species were collected from Chiang Rai and Chiang Mai provinces during the rainy season of 2013-2014. All species were characterized up to species level considering macro and micro-morphological characteristics and molecular phylogenetic analyses. Edible *M. dolichaula* and *M. detersa* were identified as new records for Thailand. Further, the ecological parameters which affect their growth and provide clue for their domestication were also explored. The results showed that *Macrolepiota* species prefer to grow in soils rich with organic matters. They can degrade various substrates for their nutrition. This suggests that domestication of *Macrolepiota* species in cheap agricultural substrates could be achieved and this would not only generate additional nutritional food but also provide economic opportunities for farmers in developing countries. Therefore, *Macrolepiota* species are of great interest of study for introducing them as additional commercial mushrooms.

Keywords: Culture collection, macrocharacteristics, microcharacteristics, phylogenetic analyses

Introduction

The white spored-gilled mushroom, *Macrolepiota* belongs to the family Agaricaceae (Krik *et al.,* 2008; Rizal *et al*., 2015). *Macrolepiota* species are mostly found in tropical and temperate regions, having warmer climates (Vellinga 2003; Gierczyk *et al*., 2011). Presently, 62 species of *Macrolepiota* have been described world over (Krik *et al*., 2008). Six *Macrolepiota* species namely *M. detersa* Ge, Yang and Vellinga, *M. dolichaula* (Berk. and Broome) Pegler and Rayner, *M. gracilenta* (Krombh.) Wasser, *M. mastoidea* (Fr.) Singer, *M. procera* (Scop.) Singer, and *M. rachodes* (Vittad.) Singer are identified as edible species from the wild (Ge *et al*., 2010). Caps of *Macrolepiota* are usually large and these are widely consumed in Europe, China, Thailand, and India (Baptista *et al*., 2009; Falandysz *et al*., 2017a and 2017b; Kojta *et al*., 2016; Stefanović *et al*., 2016; Rizal *et al.,* 2016). They can be consumed without loss of their numerous mineral constituents such as Cu, Zn and also their biologically active

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Received May, 2017. Accepted Jul., 2017

organic compounds (Gucia *et al.,* 2012a) and also their biologically active organic compounds.

Macrolepiota species are used as a remedy for indigestion, heart diseases and to treat anemia by people in mushrooms collecting areas in northwest India and Turkey (Kolcuoglu *et al.,* 2007; Kumari *et al.,* 2012; Atri *et al.,* 2014). Alkaloids, amino acids, carotenoids, fatty acids, flavonoids, phenolic, and sterols were isolated in the mushroom extracts of *M. dolichaula*, *M. procera* and *M. rachodes*, while cardiac, glycosides, tannins, and terpenoids were found in mushroom extracts of *M. procera* (Kolcuoglu, 2012; Ilondu, 2013; Kumari and Artri, 2014).

Researchers have shown interest to cultivate some *Macrolepiota* species using different cultivation methods (Manz, 1971; Coetzee *et al*., 1980, Maki and Paccola-Meirelles, 2002; Jones *et al*., 2004; Rizal *et al*., 2014; Rizal *et al.,* 2015; Rizal *et al*., 2016). Rizal *et al*. (2016) found that as saprobe, *M. dolichaula* and *M. deters* have shown the ability to grow in different agar media and degraded agricultural and industrial wastes. Most people in southern China, India, Europe, and Thailand collect these mushroom for consumption however, its acceptance as edible mushroom in other regions of the world has not been reported. Thereby, studying this mushroom for cultivation might make it an additional cultivable mushroom to supplement the diet of people in developing countries.

The study was undertaken to characterize wild *Macrolepiota* species in Chiang Mai and Chiang Rai provinces based on their macro and micro-characteristics and molecular data. Furthermore, the habitat characteristics on which these mushrooms grew such as vegetation and the soil physical characteristics i.e., temperature, moisture contents, and pH were explored.

Some researchers in Thailand reported success in the cultivation of *M. gracilenta* (Jones *et al*., 2004) however, there are still many *Macrolepiota* species which have potential for cultivation but remain under studied. Therefore, this study will provide detail information on their ecological characteristics and provide clue for their cultivation as an additional variety of commercial mushrooms (Shim *et al*., 2005; Kumari and Atri, 2014; Thatoi and Singdevsachan, 2014)

Materials and Method

Collecting sites and mushroom sampling

Macrolepiota species were collected during the rainy seasons (May to September) in the year 2013 and 2014, from Chiang Rai and Chiang Mai provinces, Thailand (Table 1).

Ecology of mushrooms

Ecological characteristics of the each species such as habitats and vegetation were recorded respectively. Physical characteristics of soil samples on which mushrooms grew such as organic matter, temperature, pH, and moisture contents were recorded. Approximately 500 g soil samples were collected in plastic bags and taken to laboratory to identify the approximate moisture contents. Samples were weighed and dried at 40 °C for 24 hours. Dried soils were subsequently weighed to find the differences of wet and dried soil.

Table 1: Sampling sites in the study area

Collection No.	Sites	Date of collection
MFLUCC-13-0579	Bandu, Chiang Rai	21/7/2013
MFLUCC-14-0742	Nanglea, Chiang Rai	12/8/2013
MFLUCC-13-0901	Doi Mae Salong, Chaing Rai	18/7/2013
MFLUCC-15-0062	MRC, Chiang Mai	6/7/2014
MFLUCC-15-0063	Doi Mae Salong, Chaing Rai	17/7/2014

Macromorphological and micromorphological characterization

Freshly collected *Macrolepiota* species were brought to Mycology Laboratory, Mae Fah Luang University, for morphological study and photographs. Standard methods for collection, preservation, and description of *Macrolepiota* have been followed, using the terminology of Ge *et al.* (2010). All the mushroom specimens (young and mature) were characterized morphologically based on pileus, lamellae, stipe, annulus, texture, colour, and spore print. Pileus covering was observed on pileus surface and stipe covering on the stipes surface. The smell of the mushrooms was also evaluated.

Micromorphological characteristics were used for preliminary identification of the mushrooms to the species level. To examine the microcharacteristics, sections were cut from dried specimens with a scalpel and mounted on slides with 10% KOH and Congo red. Then the observations and the measurements of basidiospores, basidia, cheilocystidia, structures in squamules, and the locations of clamp connections were made at × 40, × 100 magnifications using a compound microscope (Carl Zeiss™SteREO Discovery.V8 Microscopes, Jena, Germany). Pictures were taken with a digital camera mounted on the microscope.

Culture collection

Mushroom cultures were prepared by taking out an inner tissue from a matured fresh pileus of each sample, which was then isolated aseptically on MEA (Malt Extract Agar) for making starter cultures for the cultivation experiments. Stock cultures were maintained on potato dextrose agar (PDA) and malt extract agar (MEA). Furthermore, distilled water and glycerols were used for preserving the cultures in the slants at 4 ° C. Remaining mushrooms were dried for 24 hours at 40 °C in a mushroom drier to avoid deterioration of tissues and for long term herbarium storage. Dried specimens were sealed in airtight plastic bags with collection numbers and deposited at Mae Fah Luang University Herbarium for further study. The axenic cultures were also deposited in Mae Fah Luang University culture collection (MFLUCC).

DNA extraction, PCR and sequencing

DNA sequence analyses together with the micro and macro-morpholoical characteristics were used to strengthen the identification. For this, two species *M. dolichaula* strain MFLUCC-13-0579 and *M. detersa* strain MFLUCC-13-0901 were selected as they represented the new record in Thailand. The ITS gene sequence was selected for this purpose as Schoch *et al.* (2012) shared that the ITS could be used as DNA barcoding for fungal identification. For this analysis, the mushroom samples (both dried and mycelium) of MFLUCC-

Table 2: Ecological characteristics of *Macrolepiota* species

Species	Habit	Habitat	Vegetation	Soils	Soil humidity (%)	Soil temp. (ºC)	Soil pH
MFLUCC-13-0579	solitary	leaf litters	banana plants (*Musa* sp.)	loamy	70	25	6.5
MFLUCC-14-0742	gregarious	leaf litter	under (*Litchi chinensis*)	slightly sandy	68	26	7.2
MFLUCC-13-0901	solitary	grassland	deciduous forest	loamy	75	22	7.8
MFLUCC-15-0062	solitary	grassland	deciduous forest dominated by *Castanopsis* and *Lithocarpus echinops*	loamy	85	24	7
MFLUCC-15-0063	solitary	leaf litters	deciduous forest dominated by *Bambusa* sp. and scattered *Castanopsis* sp.	loamy	70	25	6.8

13-0579 and MFLUCC-13-0901 were sent for the extraction of genomic DNA, amplication, and DNA sequencing for the ITS gene of *M. dolichaula* MFLUCC-13-0579 to Biodiversity and Climate Research Centre (BiK-F) Laboratory, Main Frankfurt and *M. detersa* MFLUCC -13-0901 to Helmholtz Centre for Infection Research (HZI), Laboratory, Germany. PCR amplification was performed in 25 µl volumes containing 1.0 µl template DNA, 9.5 µl double distilled water, 1.0 µl of each primer and 12.5 µl of 2 × power Taq PCR Master Mix [A premix and ready to use solution, including 0.1 Units/µlTaq DNA Polymerase, 500 µm dNTP Mixture each (dATP, dCTP, dGTP, dTTP), 20 mM Tris–HCL pH8.3, 100 Mm KCl, 3 mM $MgCl_2$, stabilizer and enhancer. The reaction was carried out with 35 cycles under the following conditions: denaturation (95 ºC, 30 s), annealing (52 ºC, 30 s), extension (72 ºC, 1 min) and final extension (72 ºC, 10 min). The primers used for sequencing the whole ITS regions were ITS5 (forward) and ITS 4 (reverse). Amplified products were confirmed with 1% agarose gel electrophoresis stained with ethidium bromide. The ITS sequences were used for identifying the similarity scores using NCBI nucleotide blast program. Their accession numbers obtained from GenBank were KP859148 for *M. dolichaula* MFLUCC-13-0579 and KR362911 for *M. detersa* MFLUCC-13-0901.

Phylogenetic analyses

DNA sequence fragments of ITS gene were blasted to investigate the closest/exact taxa in the GenBank database. The taxon information and GenBank accession numbers used in the molecular analysis are listed in (Katoh and Standley, 2013) and manually improved with BioEdit v.7.2.5 (Hall, 2004). Phylogenetic analyses were performed using Randomized Accelerated Maximum Likelihood (RAxML). The reconstruction of ML analysis was performed using raxmlGUI v.0.9b2 with the model "GTRGAMMA" (Stamatakis, 2006; Silvestro and Michalak, 2010). Phylogenetic trees were figured in TreeView (Page, 1996).

Figure 1: a. *Macrolepiota dolichaula* MFLUCC-13-0579. b. *Macrolepiota dolichaula* MFLUCC-14-0742. c. *Macrolepiota detersa* MFLUCC-13-0901. d. *Macrolepiota mastoidea* MFLUCC-15-0062. e. *Macrolepiota procera* MFLUCC-15-0063

Results and Discussion

In total, five *Macrolepiota* species were collected in five sites within Chiang Rai and Chiang Mai provinces and the details are shown in Table 1. Mushroom samples collected exhibited somewhat similar

characteristics in terms of habitats, vegetations, and soil characteristics which determine their ecological requirements (Table 2). It was observed that these mushrooms preferred to grow on grasslands, disturbed places like garbage dumping places and on leaf litters in forest floors. Our results are in agreement with Gucia *et al.* (2011) who stated that *Macrolepiota* species are saprobes and grow alone or scattered under trees, at the edges of forests and on grasslands. In general, the soils where *Macrolepiota* species grew were found to be covered with organic litters. The soils were loamy, slightly acidic to neutral (6.5–7.8) with high moisture contents (68–85%) and fructification was seen when the soil temperature was between 22 and 26 °C during rainy seasons.

Table 3: Descriptions of macro and microcharacteristics

Features	**MFLUCC-13-0579**	**MFLUCC-14-0742**	**MFLUCC-13-0901**	**MFLUCC-15-0062**	**MFLUCC-15-0063**
	M. dolichaula	*M. dolichaula*	*M. detersa*	*M. mastoidea*	*M. procera*
Pileus shape	plano-convex	convex	oval	plano-convex	convex
Pileus apex	brownish	brownish	greyish orange	greyish brown	dark brown
Lamellae attachment	white, free with lamellulae	white, free with lamellulae	white, crowded	white, free, with lamellulae	white, free
Pilues diameter (cm)	12 – 8	10 – 12	6 – 9	4 – 8	6 – 10
Squamules on pileus	brownish granular becomes minute and sparse at margin	brownish granular, become sparse and minute at margin	detachable light brown	star shaped, irregularly furfuraceous	small plate, dark brown, closely attached on pileus
Pileus smell	mild	mild	mild	mild	mild
Spore print	white	white	white	white	white
Stipe length (cm)	12	25	12.8	12	18
Stipe shape	long, cylindrical	long, cylindrical	long, subcylindrical	subcylindrical	long, subcylindrical
Internal structure of stipe	fibrous, hollow	fibrous, hollow	fibrous, hollow	fifibrous, hollow	fibrous, hollow
Annulus	ascending,	ascending, membranous, whitish	ascending, membranous,	ascending,membranous, whitish	ascending, membranous, whitish
Squamules on stipe	membranous, whitish, minute squamules forming patterns	minute squamules forming patterns	minute squamules forming Patterns	minute squamules forming patterns	velvet squamules
Basidiospores (µm)	smooth, hyaline 13.5×9.0	smooth, hyaline 12.5×8.0	smooth, hyaline 15.0×11.5	smooth, hyaline 12×8.0	smooth, hyaline 9.0×6.0
Basidia (µm)	clavate, 4- sterigmata 29×13	clavate, 4- sterigmata 28×12	clavate, 4 sterigmata 39×16	clavate, 4 sterigmata 32×12.0	clavate, 4 sterigmata 25×9.5
Cheilocystidia (µm)	clavate, 20×11	clavate, 18×9.0	pyriform, 19×10	subclavate, 15×7	clavate, 14×9.0
Squamules (µm)	sub-cylindrical, 45×16	sub- cylindrical, 45×15	sub- cylindrical, 40×13	sub- cylindrical, 38×12	sub- cylindrical, 65×10
Clamp connection	a few at base of basidia	a few at base of basidia	a few at base of basidia	rare	not observed

Table 4: Taxa information and GenBank accession numbers of *Macrolepiota* specimens used in the molecular phylogenetic analysis

Name of the species	Voucher	Locality	GenBank accession number (ITS, 5.8S)
Macrolepiota sp.	-	Australia	JF495077
M. turbinata	MalajczukH0219	Perth, Australia	NG042584
M. velosa	HKAS 29487 ITS	-	NR119459
M. gasteroidea	H0052	Australia	JF495031
M. orientiexcoriata	HKAS 45863 ITS region	China	NR119833
M. detersa	HKAS 55306 ITS region	China	NR119832
M. detersa		China	NR119832
M. detersa	MFLUCC130901	Northern Thailand	KR362911
M. detersa	HKAS58250	China	JN180324
M. detersa	HKAS58245	southwest China	JN180323
M. detersa	MFLU121784	Northern Thailand	KJ524560
M. sp.	P87	Australia	JF495078
M. procera	Huijser	Italy	HQ423291
M. rhodosperma	Lou-fungi 18650	Italy	HQ423284
M. eucharis	-	-	AF482854
M. excoriata	H.A. Huijser 6264	Belgium	HM488870
M. mastoidea	H.A. Huijser 6268	-	HM488865
M. konradii		-	JQ683125
M. dolichaula	MFLUCC:13-0579	Northern Thailand	KP859148
M. dolichaula	MFLU121782	Northern Thailand	KJ524565
M. dolichaula	HKAS50457	China	HM125523
M. dolichaula	MFLU121869	Northern Thailand	KJ524567
M. dolichaula	MFLU121771	Northern Thailand	KJ524562
M. dolichaula	MFLU121820	-	KJ524564
M. dolichaula	MFLU121764	-	KJ524568
M. dolichaula	-	Australia	AY083193
M. dolichaula	FM22	Pakistan	KJ643334
M. dolichaula	-	-	AF482839
M. dolichaula	Su 11	Pakistan	KT725783
M. dolichaula	MFLU121816	Northern Thailand	KJ524566
M. dolichaula	HAI-37	Isreal	JQ683120
M. dolichaula	MFLU121776	Northern Thailand	KJ5254563
M. dolichaula	PUN4336	northwest India	JQ928939
M. orientiexcoriata	HKAS45863	China	HM125528
M. zeyheri	LE-9887	-	JQ683126
M. gracilenta	LE-9866	Isreal	JQ683122
M. puellaris	YSU-1	Isreal	JQ683121
M. psammophila	HAI-4	Isreal	JQ683117
M. subsquarrosa	HAI-13	Isreal	JQ683116
M. rickenii	HAI-SP-80	Isreal	JQ683114
M. affinis	HAI-5	Isreal	JQ683111
M. fuligineosquarrosa	HAI-331	Isreal	JQ683105

Table 4 cont ...

M. prominens	HAI-554	Isreal	JQ683102
M. permixta	TO AFM12	Italy	HM246503
M. fuliginosa	TO AFM11	Italy	HM246502
M. olivieri		Berkeley	AF482846
M. neomastoidea		Berkeley	AF482845
M. fuligineosquarrosa	HAI-331	-	KC884716
M. clelandii		Berkeley	AF482838
M. colombiana	NVE 439	Colombian Amazonia	KF937346
Leucoagaricus barssii	-	North California	AF482845
L. meleagris	-	Netherlands	AF482867

Macromorphological and micromorphological characterization

Macrocharacteristics observed on fruiting bodies of *Macrolepiota* were compared with literature reported by Lebel and Syme (2012) and Muhammad *et al*. (2014) who worked on *Macrolepiota* species descriptions and documentation. Based on the macrocharacteristics (Table 3) two mushroom strains MFLUCC-13-0579 (Figure 1a) and MFLUCC-14-0742 (Figure 1b) were identified as *M. dolichaula,* strain MFLUCC-13-0901 as *M. detersa* (Figure 1c), MFLUCC-15-0062 as *M. mastoidea* (Figure 1d) and MFLUCC-15-0063 as *M. procera* (Figure 1e). Morphological characteristics of each mushroom are shown in Table 3.

According to the microcharacteristics observed (Table 3), basidia in all species had four sterigmata containing four basidiospores. A few clamp connections were seen at the base of basidia of two strains of *M. dolichaula* (MFLUCC-13-0579 and MFLUCC-14-0742) and *M. detersa* MFLUCC-13-0901 however, clamp connections were extremely rare in *M. mastoidea* (MFLUCC-15-0062) and not observed in *M. procera* (MFLUCC-15-0063). After comparison of the description made on the species collected in China by Ge *et al*. (2010) and in Africa by Mbaluto *et al.* (2014) first two similar strains MFLUCC-13-0579 and MFLUCC-14-0742 showed similar characteristics of *M. dolichaula* MFLUCC-13-0901 as *M. detersa*, MFLUCC-15-0062 as *M. mastoidea* and MFLUCC-15-0063 as *M. procera*.

Phylogenetic analysis

The sequencing data were obtained for *M. dolichaula* (MFLUCC-13-0579) and *M. detersa* (MFLUCC-15-0901) which were identified as the new record to Thailand. The NCBI blast using nucleotide sequences showed *M. dolichaula* (MFLUCC-13-0579) and *M. detersa* (MFLUCC-13-0901) having 100% similarity score with *M. dolichaula* and *M. detersa* respectively. Phylogenetic analyses were performed to confirm whether the strains are *M. dolichaula* or *M. detersa*. The dataset comprised 52 taxa including newly obtained strains of *M. dolichaula* and *M. detersa* with *L. barssii* and *L. meleagris* as the out group taxa (Table 4). The ITS sequence data were analyzed for reconstruction of ML analysis with the ML final score is -13608.608834. The phylogeny showed that the strain MFLUCC-13-0901 is clustered with other *M. dolichaula* retrieved from GenBank, with the bootstrap support of 100% (Figure 2) and the strain MFLUCC-13-0901 is claded with the *M. detersa* obtained from GenBank with the bootstrap support of 100% (Figure 2). *Macrolepiota mastoidea* (MFLUCC-15-0062) and *M. procera* (MFLUCC-15-0063) were identified based on macrocharacteristics and microcharacteristics.

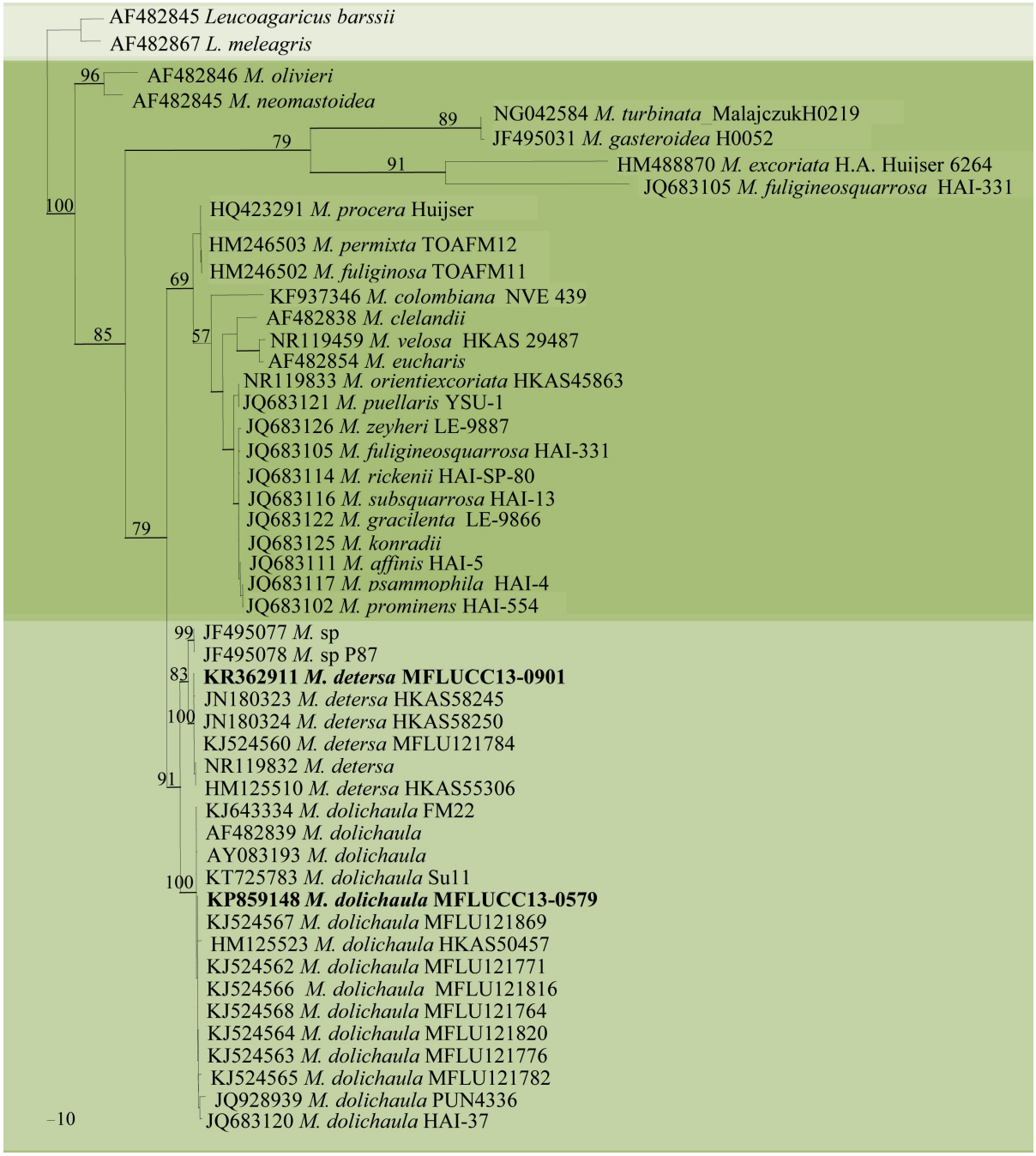

Figure 2: Phylogenetic tree obtained from RAxML analysis showing the phylogenetic positions of *M. dolichaula* (MFLUCC-13-0579) and *M. detersa* (MFLUCC-15-0901) with some selected *Macrolepiota* species from GenBank based on ITS rDNA sequences. Bootstrap support values for maximum likelihood (ML) higher than 50% are provided at the nodes. The tree is rooted with *L. barssii* and *L. meleagris.*

Conclusion

Five *Macrolepiota* species namely *M. dolichaula* (MFLUCC-13-0579; MFLUCC-14-0742), *M. detersa* (MFLUCC-13-0901), *M. mastoidea* (MFLUCC-15-0062) and *M. procera* (MFLUCC-15-0063) were collected from Chiang Mai and Chiang Rai provinces in northern Thailand. *Macrolepiota dolichaula* (MFLUCC-13-0579) and *M. detersa* (MFLUCC-15-0901), identified based on both morphological characteristics and phylogenetic analyses,

are new records to Thailand.

In general, *Macrolepiota* species prefer to grow in soils rich with organic matters which were confirmed by our study. It is advised not to eat edible *Macrolepiota* grown on pesticides, insecticides or heavy metal contaminated habitats because these mushroom mycelia take up could be characterized by elevated content of Cd, Hg, Pb in edible caps of the fruiting bodies and frequent eating of these mushrooms should be avoided (Falandysz *et al*., 2017b).

Taxonomic and phylogenetic analyses of *Macrolepiota* are important for identification and, ecological characteristics are important for further experiments on mushroom domestication. The ecology of *Macrolepiota* presented here may thus help the mushroom cultivators to identify the cultivation protocols i.e. optimizing mycelial conditions and cultivation methods to domesticate these mushrooms in local and commercial farms in Thailand.

Acknowledgements

This work was financed by Mae Fah Luang University and Thailand Research Fund Grant. Hiran A. Ariyawansa, Jian-Kui Liu, and Miss Benjarong Thongbai are also thanked for their help in DNA extraction, PCR, and sequencing.

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