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Two new records of Genus *Agaricus* from Western Ghats forests of India

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ABSTRACT

Two species of the genus *Agaricus* L., each from Sect. *Brunneopicti* Heinem. (*A. Chiangmaiensis* Karun., Guinb. and K.D. Hyde) and Sect. *Arvenses* (Konrad and Maub.) Konrad and Maub. (*A. flocculosipes* R.L. Zhao, Desjardin, Guinb. and K.D. Hyde) newly reported from India. The specimens were collected from different parts of Central Western Ghats and studied by morphological, microscopic and molecular phylogenetics using nrITS and nrLSU sequences. The current study presents the descriptions, color photographs, and phylogenetic relationships.

Keywords: *Agaricales*; floccose; ITS; morpho-molecular; taxonomy, Western Ghats.

INTRODUCTION

Agaricus L., a genus of saprophytic fungi, commonly producing fleshy, gilled mushrooms belongs to order *Agaricales* (*Agaricomycetes, Basidiomycota*). The genus is species-rich with more than 500 species worldwide (Callac and Chen, 2018), that are edible, medicinal, and even poisonous. With the advancement in molecular phylogeny, number of investigators (Zhao *et al*., 2011; Chen *et al*., 2017; He *et al*., 2016; Parra *et al*., 2018) has revisited the classification of the genus in the past decade. Presently six subgenera and 24 sections have been recognised under genus *Agaricus* (Callacand Chen, 2018).

The genus *Agaricus* is cosmopolitan in distribution and grows in various habitats,Western Ghats of India is one of the eight global biodiversity hotspots. Bhadra Wildlife Sanctuary falling in this area is quite rich in mushroom population. A study to assess the macrofungal diversity in this area was initiated in the year 2016. Out of the collections studied two interesting species of genus *Agaricus*, namely *A. chiangmaiensis* (sect. *Brunneopicti*) and *A. flocculosipes* (sect. *Arvenses*) were documented which are being described for the first time from India (Upadhyay *et al*., 2017; Saini *et al*., 2018).

MATERIALS AND METHODS

The studied sporocarps are collected from Kakanahosudi (13°45'09.0"N 75°33'07.0"E) and Chibballi (13°43'59.6"N 75°28'33.1"E) of Bhadra Wildlife Sanctuary, Central Western Ghats region of Karnataka during June -September, 2019.

Sampling and morphological characterization: Sporocarps encountered during the survey are collected with field notes on macro-morphological characteristics and habitat conditions were documented on the field key especially designed for the purpose (Atri *et al*., 2017). Collected basidiomata were subsequently dried and utilized for further characterization. Micro-morphological characters are studied using Olympus CH20i binocular light microscope and subsequently identified (Zhao *et al*., 2012; Karunarathna *et al*., 2014).

DNA Extraction and PCR: Freshly collected sporocarps were used for extraction of DNA by the CTAB method (Kantharaja and Krishnappa, 2020). 100 mg of inner tissue of the sporocarp was homogenized directly by micro-pestle (Tarsons) in a micro-centrifuge tube with 500 µL of 2X CTAB extraction buffer at 65°C. Vortexed for few seconds and incubated at 65°C for 1 hour. Slightly cooled and centrifugated at 13000 rpm for 30 minutes. 3 µL of RNase A is mixed with centrifugate and incubated for 10 min at 37°C. 500 µL of PCI (25:24:1) (Himedia) was added to the mixture and centrifuged at 10000 rpm for 10 min at room temperature. To the supernatant 600 µL of ice-cold isopropanol was added and incubated overnight. To pellet the DNA the mixture was centrifuged at 10000 rpm for 10 minutes at 10°C. The pellet was washed twice with 70% ethanol and dissolved in 50 µL of 1X TE buffer. The extracted DNA was analyzed for purity using 0.8% agarose gel electrophoresis and Bio-photometer (Eppendorf India Pvt. Ltd.).

PCR reactions were carried out using Eppendorf Mastercycler nexus GX2 in 0.2 ml PCR tubes with 50 µL reaction mixture containing, 25 µL double distilled water, 8 µL 10X PCR buffer A (Himedia). 2.5 µL of each primer, 0.5µl of Taq DNA polymerase (3U/µL), 1.5 µL dNTP's mixture (Himedia) and 10 µL of DNA template. The primer pairs ITS 1 and ITS 4 (White *et al*.,1990) for the nrITS region and LROR and LR5 (Vilgalys and Hester, 1990) for nrLSU region were used. The thermal profile for nrITS amplification; 4' 94°C, 32 cycles of 30" 94°C, 1' 52°C, 1' 72°C and a final extension step of 7' 72°C, for nrLSU 5' 94°C, 30 cycles of 30" 94°C, 1' 47°C, 1' 72°C and a final extension step of 7' 72°C. The PCR products were examined on 1% Agarose gel stained with Ethidium Bromide and visualized under Gel image Documentation System (BioRad) followed by clean up and sequencing in Eurofins Genomics India Pvt Ltd.

The obtained sequences were aligned by Clustal W (Madeira *et al*., 2019) in BioEdit sequence alignment editor v. 7.2.5 (Hall, 1999). The consensus sequences are used for the BLAST search on the NCBI GenBank database to identify the sequence similarity and distance tree results. The sequences are deposited to GenBank.

Sequence alignment and phylogenetic analysis: Based on published literature (Kerrigan *et al*., 2005, 2008; Zaho *et al*., 2012; Karunarathna *et al*., 2014,) a total of 38 sequences (23 ITS and 15 LSU) were retrieved from the NCBI GenBank nucleotide database (**Table 1 & 2**) and combined with newly

Table 1: List of species, geographic origin and GenBank Accession numbers of nrITS sequences used in Molecular phylogeny analysis.

Sl. No.	Species	Geographic origin and Year	GenBank Accession No.
1.	***Agaricus flocculosipes***	**India, 2019**	**MN741161**
2.	*Agaricus flocculosipes*	China, 2019	KJ162117
3.	*Agaricus flocculosipes*	Korea, 2015	KP004930
4.	*Agaricus flocculosipes*	China, 2019	KJ162113
5.	*Agaricus flocculosipes*	China, 2019	KJ162116
6.	*Agaricus flocculosipes*	China, 2019	KJ162118
7.	*Agaricus flocculosipes*	China, 2019	KJ162114
8.	***Agaricus chiangmaiensis***	**India, 2019**	**MN741165**
9.	*Agaricus chiangmaiensis*	Belgium, 2011	JF514531
10.	*Agaricus chiangmaiensis*	Thailand, 2011	KC971099
11.	*Agaricus* sp.	Belgium, 2018	JF514518
12.	*Agaricus bresadolanus*	India, 2019	MN744430
13.	*Agaricus romagnesii*	India, 2017	MH862192
14.	*Agaricus sp.*	India, 2015	KR154965
15.	*Agaricuscrocopeplus*	India, 2019	MN907541
16.	*Agaricus crocopeplus*	China, 2019	MN622742
17.	*Agaricus crocopeplus*	China, 2013	KF767446
18.	*Agaricus crocopeplus*	China, 2013	KF767447
19.	*Agaricus subrufescens*	Mexico, 2017	KY704306
20.	*Agaricus arvensis*	South Korea, 2010	HM004552
21.	*Agaricus macrocarpus*	Canada, 2008	MF954620
22.	*Agaricus excellens*	Belgium, 2017	MH861817
23.	*Agaricus augustus*	Netherlands, 2017	MH859051
24.	*Chlorophyllum molybdites*	Sudan, 2019	MK541940
25.	*Chlorophyllum molybdites*	Sudan, 2019	MK541941

generated nrITS and nrLSU sequences separately to conduct phylogenetic analysis. The unaligned sequences datasets used to assess the alignment confidence score for each residue pair under MAFFT (Katoh *et al*., 2019) algorithm of the GUIDANCE (Sela *et al*., 2015) webserver (http://guidance.tau.ac.il). The alignment output is used to conduct maximum likelihood analysis using RAxMLHPC2 on XSEDE with 1000 bootstrap replications and Bayesian analysis was performed with MrBayes on XSEDE for One million generations using the GTR+G+I model as suggested by jModel Test v.2.1.10 (Darriba *et al*., 2012) at CIPRESS Science Gateway (Miller *et al*., 2010). The Bayesian posterior probabilities were calculated and trees are viewed and edited in Fig Tree v.1.4.4 (Rambaut, 2009).

Table 2: List of species, geographic origin and GenBank Accession numbers of nrLSU sequences used in Molecular phylogeny analysis.

Sl. No.	Species	Geographic origin and Year	GenBank Accession No.
1.	***Agaricus flocculosipes***	**India, 2019**	**MN741164**
2.	*Agaricus flocculosipes*	China, 2015	KT951463
3.	*Agaricus macrocarpus*	Ukraine, 2017	MH874113
4.	*Agaricus essettei*	China, 2015	KT951514
5.	*Agaricus arvensis*	Netherlands, 2017	MH872779
6.	***Agaricus chiangmaiensis***	**India, 2019**	**MN741166**
7.	*Agaricus sp.*	Malaysia, 2015	KT951482
8.	*Agaricus bresadolanus*	India, 2019	MN744432
9.	*Agaricus romagnesii*	India, 2017	MH873880
10.	*Agaricus subrutilescens*	China, 2015	KT951522
11.	*Agaricus litoralis*	Spain, 2015	KT951483
12.	*Agaricus litoralis*	Hungary, 2018	MK277488
13.	*Agaricus crocopeplus*	India, 2019	MN907636
14.	*Agaricus crocopeplus*	China, 2013	KF767449
15.	*Agaricus crocopeplus*	China, 2013	KF767448
16.	*Leucoagaricus crystallifer*	Hungary, 2018	MK278287
17.	*Leucoagaricus crystallifer*	Germany, 2002	AY176412

RESULTS

During our survey, two species of genus *Agaricus* L.,were identified by morphological characters and molecular phylogenetic studies based on nrITS and nrLSU sequences. The initial BLAST searches of both the regions show homology with *A. Chiangmaiensis* and *A.flocculosipes*. The consensus sequences are deposited to the NCBI GenBank Submission portal (https://submit.ncbi.nlm.nih.gov/subs/ genbank/).

***Phylogenetic analysis*:** The phylogenetic study using nrITS and nrLSU sequences was conducted separately including 235 (nrITS) and 133 (nrLSU) distinct alignment patterns with 24.71% (nrITS) and 30.54% (nrLSU) proportion of gaps and undetermined characters in the aligned datasets. The final RAxML trees are shown in **Fig. 1** and **Fig. 2** with the final ML optimization likelihood values of -2312.66 (nrITS) and -2466.07 (nrLSU). RAxML and Bayesian analysis of both datasets resulted in phylogenetic reconstructions with highly similar topologies. The newly recorded *Agaricus* spp. were formed well-supported clades with >60 bootstrap values.

Taxonomy

Agaricus chiangmaiensis Karun., Guinb. & K.D. Hyde, Chiang Mai Journal of Science **41** (4): 773 (2014)

MycoBank number: 800272

GenBank accession numbers: MN741165 (ITS), MN741166 (LSU).

Pileus 80-160 mm in diameter, hemispherical to convex, white to yellowish-white, dry surface with small grey to pinkish brown triangular scales (**Fig. 3-A**). Context 5-10 mm thick at the attachment, white, soft. Lamellae free, crowded, 3-6 mm wide, white or light grey when young becoming dark brown to chocolate brown at maturity (**Fig. 3-B**). Annulus superior, prominent, two-layered, upper membranous, lower floccose, split into coarse scales forming wheel-like appearance (**Fig. 3-C**). Stipe 55-180 ×10-18 mm, obclavate, central, hollow or fistulose, context white. Basidiospores 7.0-9.2 × 3.0-3.5 µm, (n = 20, Lm =7.52µm, Wm = 3.81µm, Q = 1.62 – 2.31, Qm=1.97) chocolate brown, present in mass, oblong, rarely ellipsoid (**Fig. 3-D, Fig. 4-A**), germ pore absent, smooth, thick-walled. Basidia 20-25 × 6.5-9.5 µm, clavate (**Fig. 4-B**), tetrasporate. Cheilocystidia 16-18 × 7-10 µm, hyaline, smooth, pear-shaped (**Fig. 4-C**) with small cylindrical base. Pleurocystidia absent.

Habit and Habitat: Growing in groups on soil with humus-rich conditions in the dry deciduous forest.

Specimen examined: India, Karnataka, Chikkamagaluru, NR Pura, Kakanahosudi (13°45'09.0"N 75°33'07.0"E); Kantharaja R & Krishnappa M - 10 Sep 2019 (KUBOTMK-KR-92).

Agaricus flocculosipes R.L. Zhao, Desjardin, J. Guinberteau& K.D. Hyde, *Mycoscience* **53**: 302 (2012).

MycoBank number: 561690

GenBank accession numbers: MN741161 (ITS), MN741164 (LSU).

Pileus 60-150 mm in diameter, convex or Plano-convex, cream-colored, smooth to some what rough in some cases, greyish brown to brown scales present all over the surface (**Fig. 5-A**), margin appendiculate with scattered partial veil remnants. Context 8-12 mm thick, white when young, becoming ochraceous to brown in age at maturity. Lamellae free, 4-12 mm wide, white to creamy pink when young and reddish-brown to dark brown at maturity. Stipe 100-140 × 8-15 mm, hollow, yellowish-white, cylindrical with bulbous

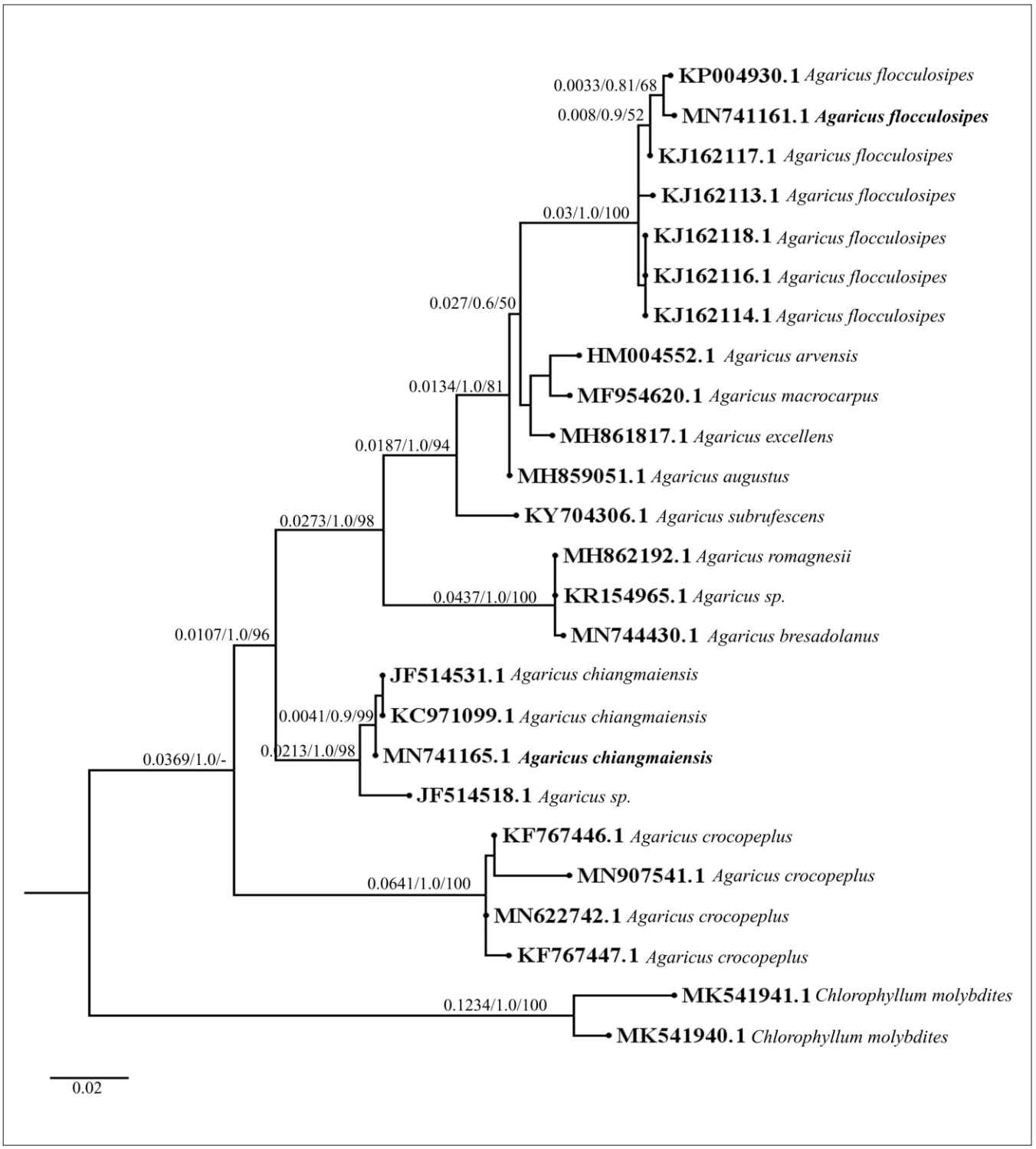

Fig. 1: RAxML tree of *Agaricus* spp. based on maximum likelihood analysis of nrITS sequences by GTR+G+I model with *Chlorophyllum molybdites* as outgroup showing Branch Length (BL), Bayesian posterior probability (PP) values (>0.5) and Bootstrap Support (BS) values (>50%). (BL/PP/BS).

base, surface covered with white flaky-floccose, erect scales below the annulus. Annulus persistent (**Fig. 5-B**), lower surface floccose, concolorous with stipe surface. Basidiospores 5.2-8.7 × 3.0-4.9 µm (n = 20, Lm = 5.8µm, Wm = 3.6µm, Q = 1.51 – 1.72, Qm = 1.61), smooth, ellipsoid, reddish-brown (**Fig. 5-C, Fig. 6-A**), no germ pore. Basidia 12-18 × 6-9 µm, cylindrical to clavate (**Fig. 6-B**), tetrasporic. Cheilocystidia occasionally present, 15-25 × 10-15 µm,

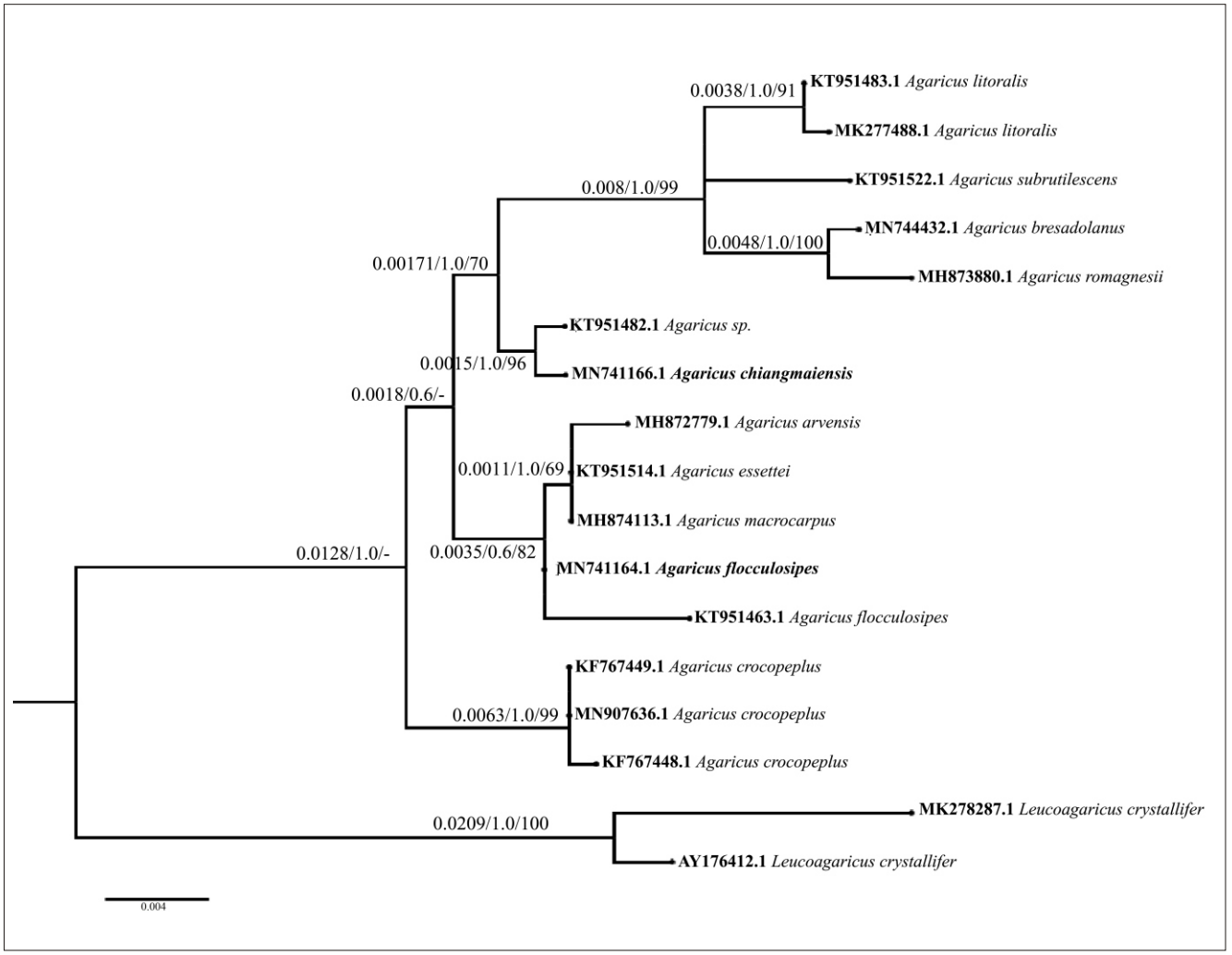

Fig. 2: RAxML tree of *Agaricus* spp. based on maximum likelihood analysis of nrLSU sequences by GTR+G+I model with *Leucoagaricus crystallifer* as out group showing Branch Length (BL), Bayesian posterior probability (PP) values (>0.5) and Bootstrap Support (BS) values (>50%). (BL/PP/BS).

ellipsoid to clavate (**Fig. 6-C**), smooth, Pleurocystidia absent. Pileipellis 5-10 µm wide, hyphae cylindrical, straight or sometimes curved.

Habit and Habitat: Growing scattered on decaying leaf litter in the dry deciduous forest.

Specimen examined: India, Karnataka, Chikkamagaluru, NR Pura, Chibballi (13°43'59.6"N 75°28'33.1"E); Kantharaja R & Krishnappa M – 15 Sep 2019 (KUBOTMK-KR-121).

DISCUSSION

Agaricus chiangmaiensis was first described from the Chiang Mai province of Thailand. The species is characterized by triangular dot-like scales on the surface of the pileus, dual-layered annulus forming a wheel-like appearance and granular scales on the stipe (Karunarathna *et al*., 2014). The taxonomic details of the Indian material generally confirms with the original description of the species except for the size of the stipe.The Indian collection possesses a slightly thin and lengthy stipe in comparison to the description of the stipe provided in the original description (Karunarathna *et al*., 2014). Such differences are usually due to the variation in the ecological and nutritional factors. In the phylogenetic study using ITS sequences the newly generated sequences did cluster with the original sequences from Thailand (KC971099) and a sample submitted to the National Botanic Garden of Belgium (JF514531). The alignment shows only two nucleotide differences between Thai and Indian sequences.

Agaricus flocculosipes is a species with a large geographical distribution range (Gui *et al*., 2014) and a potentially cultivable species from Thailand. Three species of section *Arvenses* are characterized by the nature of annulus having a colored cap; *A. subrufescens, A. augustus,* and *A. flocculosipes* (Zhao *et al*., 2012). The species can easily be distinguished from *A. arvensis* by light brown to brownish-orange grain like tiny scales. The taxonomic details of the Indian sample are in agreement with the original description provided by Zhao *et al*., 2012.The phylogenetic study shows newly generated nuclear ribosomal ITS sequences forming a

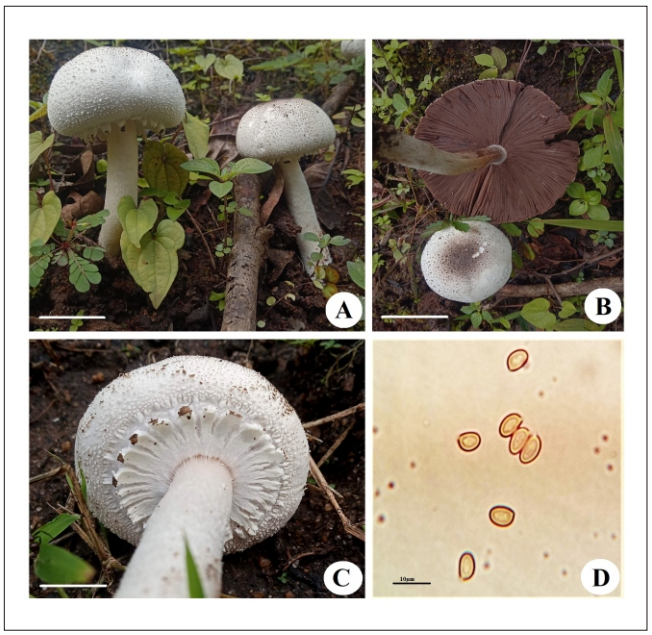

Fig. 3: ***Agaricus chiangmaiensis***; A-B Sporacarp in habitat, C. Wheel like annulus, D. Basiodiospores. Scale Bar; A-C= 5cm, D= 10µm.

Fig. 5: ***Agaricus flocculosipes***; A-B Sporacarp in habitat, C. Basiodiospores. Scale Bar; A-B= 5cm, C= 10µm.

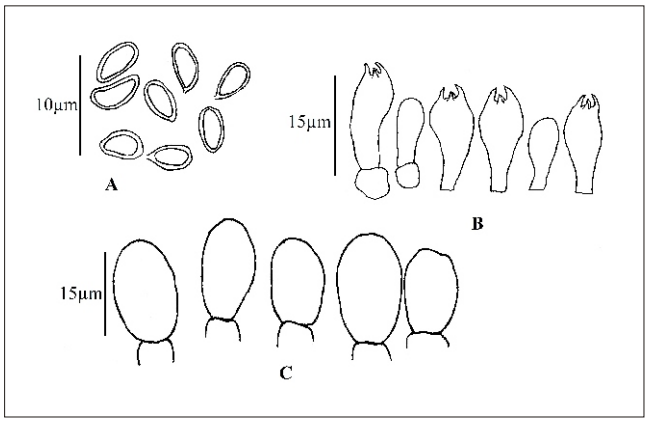

Fig. 6: ***Agaricus flocculosipes***; A. Basiodiospores, B Basidia, C. Cheilocystidia

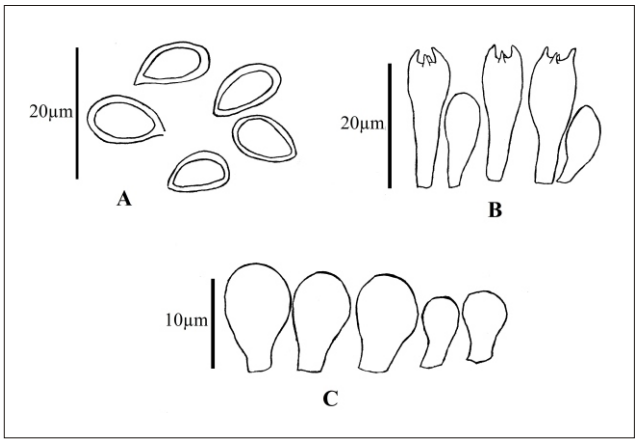

Fig. 4: ***Agaricus chiangmaiensis***; A. Basiodiospores, B. Basidia, C. Cheilocystidia.

cluster with original sequences from Thailand with more than 60% bootstrap support and high Bayesian PP value (>0.8). Hence, based on the conclusions drawn from morphological and molecular studies, the Indian sample is identified as *A. flocculosipes*.

Phylogenetic analysis by nuclear ribosomal RNA large subunit gene shows grouping in respective sections (sect. *Brunneopicti* for *A. Chiangmaiensis* and sect. *Arvenses* for *A. flocculosipes*) this further confirms the identity of the species.

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