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Contributed Paper

## ***Leucoagaricus houaynhangensis* (Agaricaceae), A New Yellowish-green Species from Lao People's Democratic Republic**

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### **ABSTRACT**

*Leucoagaricus houaynhangensis*, a yellowish-green agaric species, is described as a new species from Laos. It is characterised by light yellow to yellowish-green basidioma, covered with dark to dark grayish-brown squamules on pileus, free lamellae, subcylindrical and smooth stipe, membranous and fragile annulus, oblong ovoid and hyaline basidiospore with a germ pore, clavate and 4-spored basidia, clavate to utriform cheilocystidia with slightly a long apical appendage, a hymeniderm pileus covering made up of cylindrical elements, a cutis stipe covering made up of cylindrical elements and absence of clamp-connections in all tissue. Photographs on field and illustrator line drawing of microscopic characters are given. Two known species, *La. flavovirens* J.F. Liang, Zhu L. Yang & J. Xu and *La. viridivavus* (Petch) T.K.A. Kumar & Manim., share some morphological similarities with the newly described taxon here, but they difference by both morphological characters and nrITS DNA.

**Keywords:** Basidiomycota, diversity · distribution, lepiotaceous fungi, phylogeny, taxonomy

### **1. INTRODUCTION**

The genus *Leucoagaricus* (Locq. ex) Singer belongs to the family Agaricaceae, which comprises about 90 species that are widely distributed throughout the world [1-3]. *Leucoagaricus* is a genus of lepiotaceous fungi; several recognized macrocharacters including

pluteoid basidiomata, lack of or rarely sulcate pileus, free lamellae, presence of annulus; and microcharacters like smooth or rough basidiospores with or without a germ pore and dextrinoid, metachromatic, congophilous, cyanophilous, and absence of clamp-

connections in all tissue [2-4]. Locquin [5] placed *Leucoagaricus* in genus *Leucocoprinus* as subgenus *Leucoagaricus* Locquin without Latin description, and Singer [2] later provided a valid Latin diagnosis and gave the genus level for *Leucoagaricus*. However, some mycologist treat it under genus *Lepiota* sensu lato [6-7], and some species of *Leucoagaricus* were placed into genus *Lepiota* (Pers.) Gray and *Leucocoprinus* Pat. in some study such as Adhikari [8-9], Berkeley and Broome [10], Dennis [11], Kumar and Manimohan [12], Manandha and Adhikari [13], Natarajan et al. [14], Pegler [15], and Petch [16, 17]. For accepted classifications of lepiotaceous fungi, *Leucoagaricus* is clearly separated from other genera with independent morphology [4, 18-19]. However, molecular data demonstrate that *Leucoagaricus* is polyphyletic, and *Leucoagaricus* and *Leucocoprinus* are clustered in phylogenetic analysis [4, 20-21].

The colour changes are important for classification in lepiotaceous fungi; for instance, in some species which turn green, blue or red in ammonia vapour have been placed in both *Leucoagaricus* and *Leucocoprinus*, and some species with sericeous pileus covering have placed in genus *Sericeomyces* [3, 21-23]. Colour changes are also useful for classifying at species level; for example, some species turn blue when they are touched or dried such as *La. flavovirens* J.F. Liang, Zhu L. Yang & J. Xu, *La. viriditinctus* (Berk. & Broome) J.F. Liang, Zhu L. Yang & J. Xu and *La. viridiflavoides* Akers & Angels [21, 24-26]. In this study, we would like to provide a new species of *Leucoagaricus* with uniquely yellowish-green colour as new for science.

## 2. MATERIALS AND METHODS

### 2.1 Collecting and Materials Examination

Three specimens of the new species were collected from Houay Nhang Preserved Forest in Xaythany District, Vientiane

Capital (18°05'31.7"N 102°40'34.2"E, 189 m) [27]. Coordinate of locations, forest types, soil and substrate were recorded, and then fresh basidiomata were photographed on fields. Morphological character notes were following Vellinga [3]. Colour annotations of fresh materials were determined based on the colour charts of Kornerup and Wanscher [28]. Specimens were dried in a hot air dryer (30-40 °C) for 24 hours, before being deposited in the Herbarium National of Laos (HNL). Microscopic characters were observed and illustrated from dry specimens using a microscope. Original colour of spores was observed in water and 2.5-10% of KOH; spore wall reaction was observed in melzer's reagent, cotton blue and cresyl blue; and line drawings were observed in Congo red. Each collection was investigated and 25 basidiospores measured per collection. The notation (75, 3, 3) indicated that measurements were made on 75 basidiospores in three samples in three collections, and the size average was given in the description while quotient (Q) of length and width average quotient were also calculated to indicate basidiospore shape. The technical term used for description was following Vellinga [3].

### 2.2 Phylogenetic Study

DNA was extracted from dried herbarium collections according to the instructions of the Biospin Fungus Genomic DNA Extraction Kit (Bioer Technology Co., Ltd., Hangzhou, P.R. China). Three collections were investigated for sequences of internal transcribed spacer 1 and 2 (ITS1 & ITS2). The primers ITS1-F and ITS4 were used for PCR [29]. The PCR amplified products were purified and sequenced by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. Sequences were edited and contigs assembled using SeqMan program (DNASTar, Madison, WI, USA) and then all new

sequences were deposited in GenBank. The sequences were performed with Basic Local Alignment Search Tool (BLAST) in National Center for Biotechnology Information (NCBI) of USA database (<http://www.ncbi.nlm.nih.gov/genbank/>).

Thirty-two sequences related to the new taxon were obtained from the GenBank, and a sequence of *Agaricus bisporus* (J.E. Lange) Imbach was used as outgroup (Table 1). A complete data set was aligned using MAFFT version 7.130-win32 [30, 31]. Maximum Likelihood analysis were executed using software MEGA version6 [32, 33], and the settings were: Maximum Likelihood as statistical method, 1000 bootstrap replications, using Hasegawa-Lishino-Yano model, Gamma distributed with Invariable sites (G+I) as rates among sites, and Nearest-Neighbor-Interchange (NNI) as Initial Tree for ML. The evolutionary history was inferred by using the Maximum Likelihood method

based on the Hasegawa-Kishino-Yano model, the tree with the highest log likelihood (-1716.2824) was shown, the percentage of trees in which the associated taxa clustered together was shown next to the branches, initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, a discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4248)), the rate variation model allowed for some sites to be evolutionarily invariable ([+I], 39.6357% sites), the tree was drawn to scale with branch lengths measured in the number of substitutions per site, the analysis involved 35 nucleotide sequences, and all positions containing gaps and missing data were eliminated. There were a total of 371 positions in the final dataset.

**Table 1.** GenBank accession numbers and geographical origins of taxa used in the phylogenetic analysis.

Species	ITS GenBank accession numbers	Country of origin
<i>Agaricus bisporus</i>	FJ223230	Unknown
<i>Leucoagaricus atroazureus</i>	EU416297	China
<i>Leucoagaricus atroazureus</i>	EU416299	China
<i>Leucoagaricus atroazureus</i>	EU416301	China
<i>Leucoagaricus</i> cf. <i>atrodisca</i>	GU903304	USA
<i>Leucoagaricus</i> cf. <i>atrodisca</i>	GU903305	USA
<i>Leucoagaricus cinerascens</i>	AY176408	USA
<i>Leucoagaricus cinerascens</i>	AY176410	USA
<i>Leucoagaricus flavovirens</i>	EU416293	China
<i>Leucoagaricus flavovirens</i>	EU416295	China
<i>Leucoagaricus holosericeus</i>	GQ329058	Italy
<i>Leucoagaricus bouaynhangensis</i>	KX640915	Laos
<i>Leucoagaricus bouaynhangensis</i>	KX640916	Laos
<i>Leucoagaricus leucothites</i>	JQ683082	Israel
<i>Leucoagaricus leucothites</i>	JQ683123	Ukraine

**Table 1.** Continued.

Species	ITS GenBank accession numbers	Country of origin
<i>Leucoagaricus leucothites</i>	KF316477	India
<i>Leucoagaricus leucothites</i>	KT002154	China
<i>Leucoagaricus naucinus</i>	LNU85315	USA
<i>Leucoagaricus naucinus</i>	EU416308	China
<i>Leucoagaricus</i> cf. <i>phaeostica</i>	AF079739	Panama
<i>Leucoagaricus subcretaceus</i>	GQ329052	Italy
<i>Leucoagaricus subcretaceus</i>	GQ329063	Italy
<i>Leucoagaricus subcretaceus</i>	KF410815	India
<i>Leucoagaricus</i> sp.	KR155095	India
<i>Leucoagaricus viridiflavus</i>	GU574745	Unknown
<i>Leucoagaricus viridiflavus</i>	KF963609	Japan
Lepiotaceae	EF527300	Brazil
Lepiotaceae	EF527309	Brazil
Lepiotaceae	EF527314	Brazil
Lepiotaceae	EF527316	Brazil
Lepiotaceae	EF527317	Brazil
Lepiotaceae	EF527326	Panama
Lepiotaceae	EF527333	Panama
Lepiotaceae	EF527342	Panama
<i>Leucocoprinus</i> sp.	KR154960	India

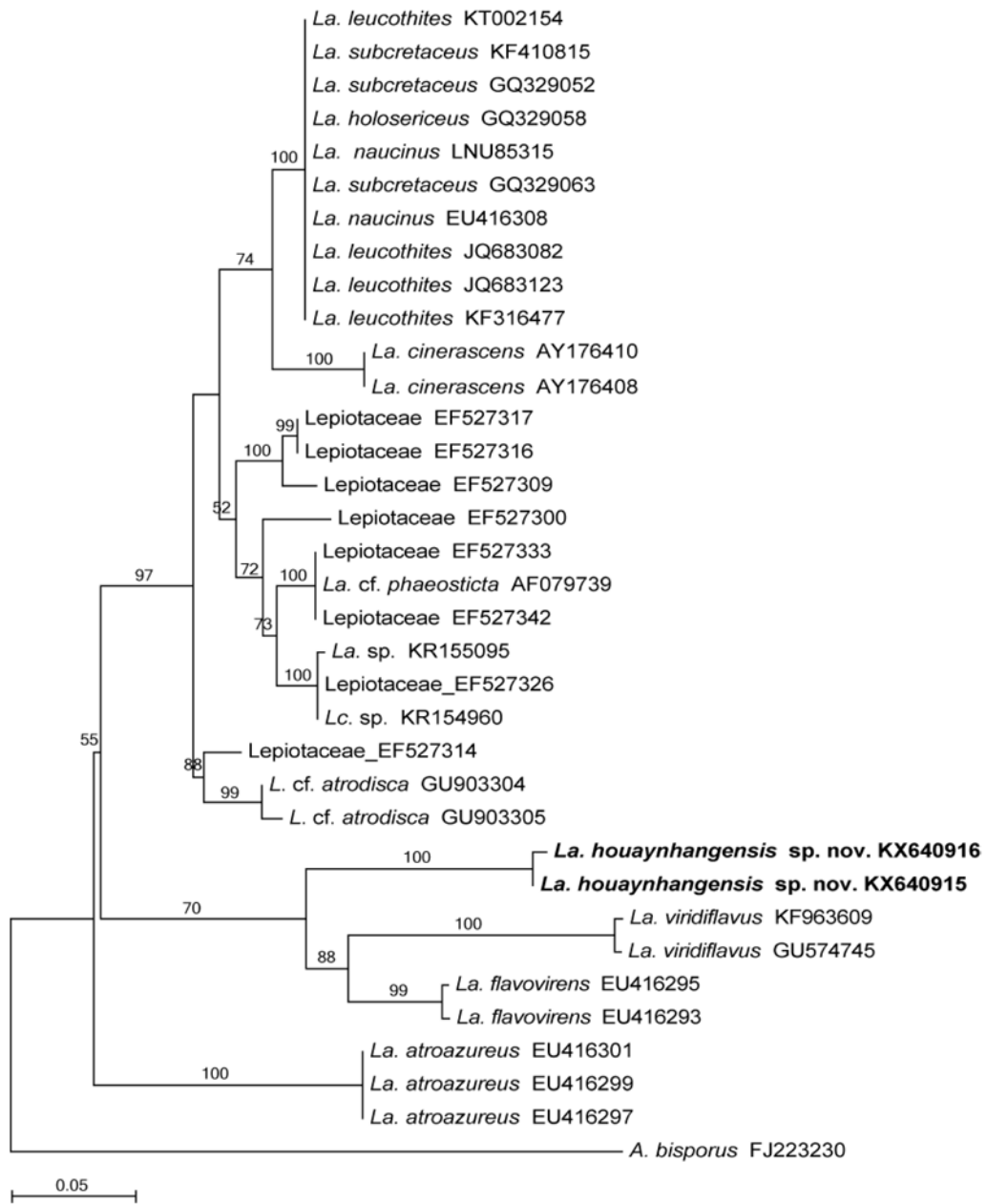
### 3. RESULTS

#### 3.1 Phylogeny Analysis

The BLAST result of two ITS sequences of *La. bouaynhangensis* (holotype, KX640915: 724 bp; paratypes, KX640916: 402 bp) were similar. Some sequences related to new taxa were lower than 89% such as two sequences of *La. flavovirens* J.F. Liang, Zhu L. Yang & J. Xu (EU416295, EU416293), two sequences of *La. viridiflavus* (Petch) T.K.A. Kumar & Manim. (GU574745, KF963609), *La. leucothites*

(Vittad.) Wasser (KF316477, JQ683123).

The phylogeny tree based on Maximum Likelihood method is given (Figure 1). The two sequences of *La. bouaynhangensis* were clustered with bootstrap support (BS) of 100%, and they were related to sequences of *La. viridiflavus* and *La. flavovirens* with BS of 70%. The species in this clade are characterized yellow to yellowish-green basidiomata and turned blue when touched or dried (Figure 1).



**Figure 1.** Phylogenetic tree based on Maximum Likelihood analysis of nrITS sequences showing relationship among *La. houaynhangensis* and related species. Bootstrap values  $\geq 50\%$  are indicated above the branches. GenBank accession numbers are given for each collection. *Agaricus bisporus* (J.E. Lange) Imbach was used as an outgroup.

### 3.2 Taxonomy

*Leucoagaricus houaynhangensis*

Sysouphanthong **sp. nov.** Figure 2, 3

**Mycobank:** MB817806

**Holotype:** HNL502947

**Etymology:** The name is referred to the first collecting site of this species at Houay Nhang Preserved Forest.

**Diagnosis:** Basidiomata light green to yellowish-green and turning blue when dried, umbonate to plano-convex pileus covered with dark grey to black granulose to squamules and with light green appendiculate margin, free and light green to yellowish-green lamellae, subcylindrical and yellowish-green stipe with light green fibrillose remnants, oblong to ovoid basidiospore with a germ pore,  $5.8-7.2 \times 3.8-5 \mu\text{m}$ , clavate to utriform cheilocystidia with short to long appendages,  $18-32 \times 8-15 \mu\text{m}$ , a hymenodermal pileus covering made up of some cylindrical elements, a cutis stipe covering made up of hyaline hyphae and cylindrical elements, absence of clamp-connections in all tissues.

Pileus 15-35 mm diam, umbonate, expanding via campanulate to applanate to plano-convex, with low umbo, with straight margin; when young granulose, dark grey (1-30F1) to black, soon breaking up into concolourous squamules to squamules fibrillose around granulose umbo toward margin, on light green to yellowish-green (30A5-8) background, attached with light green (30A5) appendiculate margin, fringed and lamellae exceeding when mature. Lamellae free, broadly ventricose, light green to yellowish-green (30A5-6), slightly crowded, with 5 lamellulae series. Stipe  $23-40 \times 3-8.5 \text{ mm}$ , subcylindrical or wider at base zone and slightly tapering to apex, with green to yellowish-green (30A5-6) fibrillose background. Annulus membranous, rarely present or often fragile, with green to

yellowish-green (30A5-6) fibrillose remnants at middle to upper part of stipe. Context in pileus 2.0-3.0 mm wide, concolourous with surface; in stipe hollow, concolourous with surface. Odor and taste not observed. Spore print white.

Basidiospores  $[75, 3, 3] 1 \times w = 5.8-7.2 \times 3.8-5 \mu\text{m}$ ,  $avl \times w = 6.5 \times 4.3 \mu\text{m}$ ,  $Q = 1.4-1.7$ ,  $Q_{av} = 1.5$ , in side-view oblong or ovoid, in frontal view ovoid, oblong, thick-walled, hyaline, with a germ pore, dextrinoid, cogophilous, cyanophilous, metachromatic. Basidia  $12-15 \times 6-8 \mu\text{m}$ , clavate, 4-spored, hyaline. Lamella edge sterile. Cheilocystidia  $18-32 \times 8-15 \mu\text{m}$ , clavate to utriform, with short to long appendages ( $l \times w = 5-20 \times 2.5-3.5 \mu\text{m}$ ), Pleurocystidia absent. Pileus covering a hymenoderm made up of some layers of cylindrical elements, rarely with narrowly clavate elements,  $23-47 \times 4-7 \mu\text{m}$ , hyaline to pale brown-walled, under layer with hyaline hyphae, cylindrical,  $2.5-6.5 \mu\text{m}$  wide. Stipe covering a cutis made up of cylindrical elements,  $1-2.5 \mu\text{m}$  wide, hyaline, with cylindrical hyphae in lower layer,  $1-4 \mu\text{m}$  wide, hyaline. Clamp-connections absent in all tissues.

**Habitat and distribution:** solitary, saprotrophic, on humus soil; widespread in deciduous forests of Houat Yang Preserved Forest, Xaythany District, Vientianr Capital, Lao PDR.

**Material examined:** Laos, Vientiane Capital, Xaythany District, Houay Nhang Preserved Forest, collected date 12 July 20014, P. Sysouphanthong (PS2014-1448, HNL502947, holotype); *ibidem* collected date 20 August 20014, P. Sysouphanthong (PS2014-1463, HNL502962, paratypes); *ibidem* collected date 2 September 20014, P. Sysouphanthong (PS2014-1483, HNL 502982, paratypes).



**Figure 2.** Fresh basidiomata of *La. bouaynhangensis*. a-c (HNL502947), d (HNL502962), e-f (HNL502982).

#### 4. DISCUSSIONS

*La. bouaynhangensis* is commonly recognized by light green to yellowish-green whole basidiomata with turning blue when dried, umbonate pileus covered with dark grey to black granulate to squamules with light green appendiculate margin, free lamellae, subcylindrical stipe with light green fibrillose remnants, oblong ovoid basidiospore with a germ pore, clavate to utriform cheilocystidia with short to long appendages, a hymeniderm pileus covering, a cutis stipe covering, and absence of clamp-connections in all tissues.

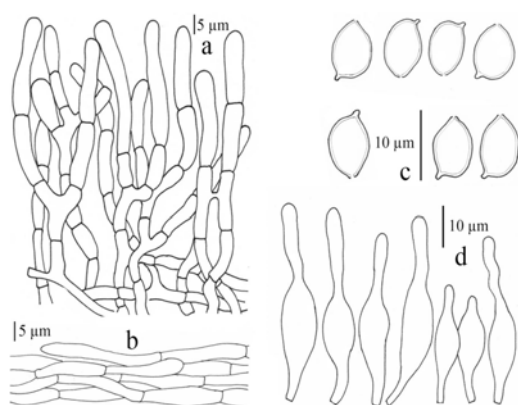
Since some species of *Leucoagaricus* with yellow to yellowish-green basidiomata come to close to *La. bouaynhangensis*, and they are often confused on macromorphology. Firstly, *La. flavovirens*, a yellow species with dark bluish green, is the most similar to *La. bouaynhangensis* on field by dark grey to blackish on pilial surface with purplish-green to grayish green to radially fibrillose squamules, free and greenish-yellow lamellae, greenish yellow stipe and concolourous on context of pileus and stipe; however, it differs from *La. bouaynhangensis* by lack of a germ pore on basidiospore, shorter appendage on

cheilocystidia, and a cutis pileus covering [19]; and The BLAST result of nrITS sequence showed that *La. flavovirens* was only 83% related to *La. bouaynhangensis*, and the ML tree also showed that *La. bouaynhangensis* was clearly separated from *La. flavovirens* (Figure 1).

The second yellowish-green species, *La. viridiflavus*, are close to *La. bouaynhangensis* with the same olive green basidiomata with blushing blue, shape and size of basidiospore with a germ pore, similar shape and size of cheilocystidia with a long appendage; but differs at glabrous pileus without grey or black squamules and a cutis pileus covering structure, and with amorphous contents in cheilocystidia and covered with exudates towards the apex of appendage which is not found in *La. bouaynhangensis* [12]. Two nrITS sequences of *La. bouaynhangensis* were identical with 100% BS, and there were only sequences of *La. viridiflavus* and *La. Flavovirens* come to relate with low BS (Figure 1).

*La. sulphurellus* (Pegler) B.P. Akers *La. viridiflavoides* B.P. Akers & Angels are distinguished from *La. bouaynhangensis* by having cutis pileus covering structures and presence of pleurocystidia [25, 34].

There were some studies of basidiomycota in Southeast Asia, numerous yellow-green species of lepiotaceous fungi are distinguished from *La. bouaynbangensis*. For example, *Lepiota citrophylla* (Berk. & Broome) Sacc. has spurred basidiospores and a trichodermal pileus covering [35] and *Verrucospora vulgaris* Pegler has angular or verrucose basidiospores (36-38).



**Figure 3.** Micromorphology of *La. bouaynbangensis* with scale bars (HNL502947). a= hymeniderm structure of pileus covering, b= a cutis structure of stipe covering, c= oblong ovoid basidiospores, d= clavate to utriform cheilocystidia with appendages.

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