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FIRST RECORD OF *CANTHARELLUS MINOR* IN VIETNAM

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SUMMARY

This species of mushroom with orange fruiting bodies and yellow flesh grows in clumps on the forest land in the coordinates 11°56'34.45" N, 108°28'33.56" E in the pine (*Pinus kesiya*) forest, Da Lat City, Lam Dong Province, Vietnam. The results of analysis on the morphology, both macro- and micro-morphological characteristics of this mushroom showed that, pileus: 5-15 mm wide, infundibuliform; yellowish to orange; margin incurred, wavy-liked margin, non-striate; Pileus surface: smooth, scaleless, yellowish; Lamellae: distant, decurrent, not intervenose, concolorous to pileus; Context: concolourous to the pileus, slight sweetness, aromatic flavor; Stipe: cylindrical shape, surface smooth, concolourous to pileus, 1-2 mm diameter, 20-50 mm length; Basidiospores: 6-11.5 x 4-6.5 µm, ovoid-ellipsoid with smooth surface; Basidia: 65 x 10 µm, cornuted 4-6 spores per basidium. Phylogenetic analysis of *nrLSU* sequence yielded consistent topology in different taxa of *Cantharellus*. The phylogenetic position of XC02 was obtained and accepted at sub-generic level: subgenus *Parvocantharellus*. This clade was suggested to be monophyletic, and separated from other sub-generic levels. Morphologically phylogenetically distinct from the other species of clade 4, such as *C. appalachiensis*, *C. tabernensis*, *C. aff. Congolensis*. The highly supported monophyletic group with referent *Cantharellus minor* was obtained with the bootstrap value of 99, indicated that XC02 was significant closely to *Cantharellus minor*. Phylogenetic of *nrLSU* analysis revealed clades with statistical support corresponding to morphological observation, thus, XC2 was concluded as *Cantharellus minor*.

Keywords: *Cantharellus, Cantharellus minor, nrLSU, funnel-shaped fungus,taxonomy*

INTRODUCTION

Cantharellus minor Peck Rep. (Annual) New York State Mus. Nat. Hist. 23. 1872. was originally described by Peck (1872). This species, a popularly known and commercialized fungus, belongs to the genus *Cantharellus* Adans.: Fr. *C. minor* has been reported as one of the smallest of the *Cantharellus*, found on soil, forming ectomycorrhizal association with the tree of *Cedrus deodara*, *Quercus dilatata*, etc. The yellowish lamellae are described as very narrow, distant, sparing branched, decurrent, concolourous, and fade to yellowish white in maturity. The pileus range from 0.5 cm to 3.0 cm wide, thin, convex, expanded and depressed, becoming funnel-shaped in some. The stipe is less than 4 cm, base attenuated, central, solid, concolours to the pileus, surface glabrous. They fruit in the summer and fall (Peck, 1872; Kuo, 2006). This species is native to Eastern North America, Canada, Western Ghats, Kerala, India, Japan, etc., however, it was not recorded in Vietnam. During the survey of fungi in Lam Dong (11°56'34.45" N, 108°28'33.56" E), a province located in the Central highlands (namely Tay Nguyen) region of Vietnam, specimens belonging to *Cantharellus* were collected. In this study, these Vietnamese specimens were identified based on morphology, molecular phylogenetic analysis and compatibility between our collected specimens and other strains in many countries of the world.

MATERIALS AND METHODS

Fungal collection

Basidiomata, coded XC02, was collected in a Pine forest (*Pinus kesiya* Royle ex Gordon), a fast-growing, has natural distribution in South-East Asia, ca. 1502m altitude, at Xuan Tho Commune, Da Lat City, Lam Dong Province, Vietnam. General information on collection: Height: 1.502 m; Humidity: 87%; Temperature: 20^{o}C; Light intensity: 3.012 lux; Coordinates: 11^{o}56'34.45" N, 108^{o}28'33.56" E. The specimens were pick up by digging them out carefully and preserved by immediately wrapped in the wax paper and placed in the collection bags. Attempts were made to collect all different developing stages of the basidiocraps to have an idea of size, color and shapes. In the laboratory, specimens were exposed to room temperature, and then, 1% Mercury (II) chloride was impregnated for 5 – 10 minutes, finally, dried at 60^{o}C and stored for further analysis.

Macro- and micromorphology analysis

Morphological observations were studied and recorded by using guidelines according to Lodge *et al.,* (2004). Macroscopic characters were carefully recorded in the field keys specially designed for the purpose and photographed using digital camera. The following macroscopic characters including pileus, lamellae, context, etc., were also noted. The color notation was noted from Kornerup, Wanscher (1981). Micromorphological features of specimens were examined and observed with Olympus B51 (Tokyo, Japan) microscopes with light and phase contrast optics. The following microscopic characters were found particularly for the identification and confirmation of Cantharellaceae, including spore morphology, basidiospores, hyphae, basidium structure.

Molecular studies: DNA extraction, PCR and DNA sequencing

For molecular characterization, genomic DNA was isolated from the dried fruit bodies of collected specimens of Cantharellaceae. The dried fruit body's powder of specimen was added in a lysis buffer containing 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 150 mM NaCl, 2% SDS, 0.1 mg/mL Proteinase K). During incubation at 65^{o}C for overnight, the cell suspension was mixed thoroughly by inverting the tube several times. Then, the supernatant was collected by centrifugation. The solution of 700 µL of PCI (Phenol/Chloroform/Isoamylalcohol with ratio of 25:24:1) was added and centrifuged. The upper solution was collected, precipitated with absolute ethanol, and washed with 70 % ethanol. DNA concentration was identified by using OD_{260}. The DNA was purified by elution through the manufacturer's instructions of Wizard®DNA clean-up system in order to remove the contaminants. Finally, isolated genomic DNA were kept in TE buffer at -20°C for further studies.

Primer pairs: LR0R (Forward primer): 5'-GTACCCGCTGAACTTAAGC-3' and LR5 (Reversed primer): 5'-ATCCTGAGGGAAACTTC-3' (Vilgalys, Hester, 1990) were used to amplify a portion of *nrLSU* (nuclear ribosomal large subunit) gene. The final volume for PCR was 15 µL with a specified program: 1 cycle of 95°C for 5 mins; 40 cycles of 95 °C in 30 s, 55°C in 30 s. 72°C in 2 mins; 1 cycle of 72°C in 5 mins. Aliquots of amplification products (5 µL) were electrophoresed on 2.0% agarose gel and visualized on UV transilluminator. The amplified product was sequenced at Nam Khoa (Vietnam) Company with the same primers.

Sequence proofreading and phylogenetic analysis

DNA sequences were proofread to remove ambiguous signals at both ends. The software, including SeaView 4.2.12, Chromas Lite 2.1.1, were used for proofreading. The data set of *nrLSU* was established by sequences downloaded from GenBank. Phylogenetic tree was constructed with MEGA 6.0 with a 1000 replicate bootstrap based on the maximum parsimony method (Dunham *et al*., 2003).

RESULTS

Taxonomy

Cantharellus minor Peck Rep. (Annual) New York State Mus. Nat. Hist. 23. 1872.

[Description based on Vietnamese specimens, Fig. 1]. **Pileus**: 5-15 mm wide, infundibuliform; yellowish to orange; margin incurred, wavy-liked margin, non-striate. **Pileus surface**: smooth, scaleless, yellowish; Lamellae: distant, decurrent, not intervenose, concolorous to pileus; **Context**: concolourous to the pileus, slight sweetness, aromatic flavor. **Stipe**: cylindrical shape, surface smooth, concolourous to pileus, 1-2 mm diameter, 20-50 mm length. **Basidiospores**: 6-11.5 x 4-6.5 µm, ovoid-ellipsoid with smooth surface. **Basidia**: 65 x 10 µm, cornuted 4-6 spores per basidium.

Figure 1. A. *Cantharellus* minor collected in Pine forest; B. Lamellae; C. Fruit body; D. Basidia; E-F: Hyphae; G: Basiospore

Amplification of *nrLSU* gene, phylogenetic analysis of *nrLSU* data set

DNA after extraction and purification was amplified with primer LR0R and LR5, then, electrophoresis on 2.0% agarose gel showed a significant and clear band of 950 bps (Fig. 2A). The PCR products was sequenced, as the results, the signals of peaks in both strands were significant and good for reading (Fig. 2B). According to BLAST results, XC02 sequence was similar to *C. minor*, strain BB 07.002 (Accession number: KF294625) with total score = max score = 1544, Ident = 99%, E-value = 0.0).

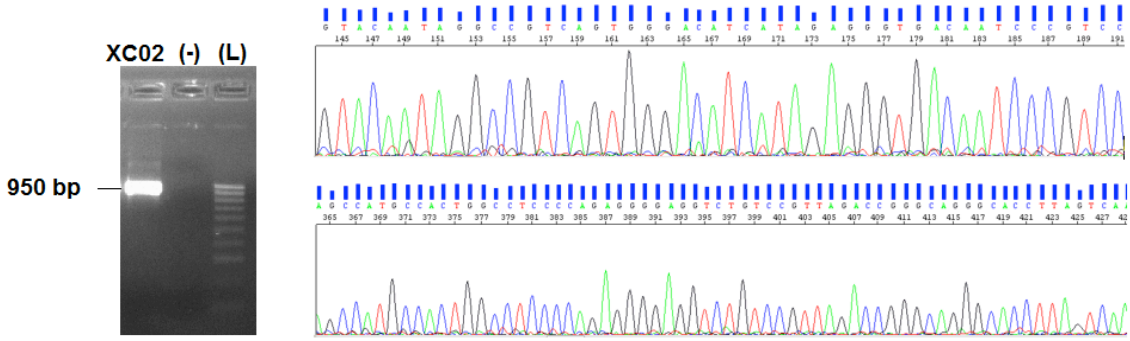

Figure 2. (A) Electrophoresis of PCR product of *nrLSU*. (L) 100 bp Ladder, (-) negative control, and XC002; (B) A part of the sequences from the forward primer (upper) and reverse primer (lower).

The final data set of *nrLSU* consisted of 61 sequences of 633 characters, including 58 sequences belonging to *Cantharellus* (reference data), 1 sequence belonged to *Craterellus* (served as outgroup), and a XC02 sequence. The molecular phylogenetic analysis based on the topology constructed by maximum parsimony, showed the formation outgroup (*Craterellus*) and six clades that are here obtained and accepted at sub-generic levels, including subgenus *Cantharellus* (Clade 1), subgenus *Rubrinus* (Clade 2), subgenus *Cinnabarinus* (Clade 3), subgenus *Parvocantharellus* (Clade 4), subgenus *Pseudocantharellus* (Clade 5), and subgenus *Afrocantharellus* (Clade 6).

According to XC02, formed the monophyletic group with several Clade 4b (belonging to subgenus *Parvocantharellus*) referent sequences, including *C. appalachiensis*, *C. tabernensis* and *C. minor*. Notably, within this clade, XC02 formed a higly supported monophyletic group (Bootstrap = 99) with two sequences: *C. minor* (Accession numbers: KF294625, KF294632) (Fig. 3), and separated this group from other referent taxon in clade 4. Combining with morphological identification, the molecular identification showed similar result as *C. minor*. Therefore, we concluded that XC02 is *C. minor*.

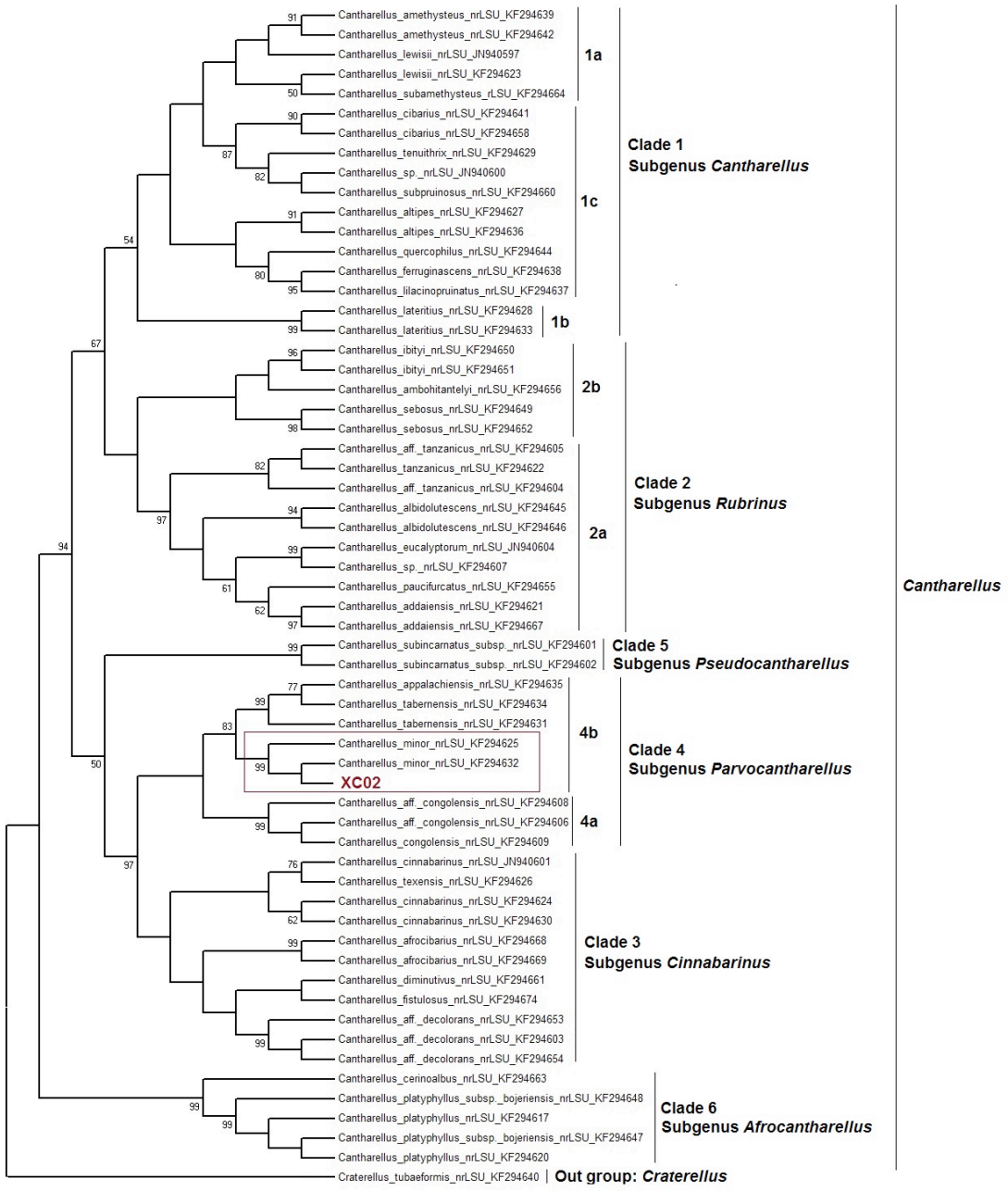

Figure 3. Molecular phylogenetic analysis of *nrLSU* by maximum parsimony for 61 sequences with a 1000 replicate bootstrap proportions.

Discussion

The traditional taxonomy of fungi emphasizes the morphology, including marco-, and mircro-morphology features to delimit a taxon. Its taxonomy relies on the size, shape of fruiting structures, lamellae, basidium, spore morphology, coloration and habitat to define taxa. In this study, the description of the morphology of XC02 with numerous striking features showed the similar to the morphological description of *Cantharellus minor* Peck Rep. (Annual) New York State Mus. Nat. Hist. 23. 1872. which was originally described by Peck (1872).According to Mitchell *et al.,* (1995), they suggested that molecular phylogenetic approaches to fungal evolution have proved valuable information towards the goal of understanding the relationship among specific fungal group. In this study, *nrLSU* (the nuclear ribosomal large subunits), the most popular locus for DNA-based mycological studies for taxon identification, was used. The *nrLSU* is a part of the rDNA gene for the nuclear genome, has been widely used as the potential marker for fungal species identification in recent years. Noteworthy taxonomic works on the nrLSU phylogenetics analysis on Cantharellaceae include those of Dahlman *et al.,* (1993), Feibelman *et al.,* (1997), Moncalvo *et al.,* (2006), Arora and Dunham, (2008). According to phylogenetic analysis, phylogenetic analysis of *nrLSU* sequence yielded consistent topology in different taxa of *Cantharellus*. The phylogenetic position of XC02 was here obtained and accepted at sub-generic level: subgenus *Parvocantharellus*. This clade was suggested to be monophyletic, and separated from other sub-generic levels. Morphologically phylogenetically distinct from the other species of clade 4, such as *C. appalachiensis*, *C. tabernensis*, *C. aff. Congolensis*. The highly supported monophyletic group with referent *C. minor* was obtained with the bootstrap value of 99, indicated that XC02 was significant closely to *C. minor*. In conclusion, phylogenetic of *nrLSU* analysis revealed clades with statistical support corresponding to morphological observation, thus, XC2 was concluded as *C. minor*.

CONCLUSION

We have successfully applied the morphological characterization in combination with phylogenetic analysis of *nrLSU* to delimit sample XC02, which collected in a Pine forest (*Pinus kesiya* Royle ex Gordon), ca. 1500 altitude, at Xuan Tho Commune, Da Lat City, Lam Dong Province, Vietnam and identified as *Cantharellus minor* Peck. This is the first record of *Cantharellus minor* in Vietnam.

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