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First record of *Cantharellus minor* from Vietnam with identification support from a combination of *nrLSU* and *nrSSU* phylogenetic analysis

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Abstract

Background: A previously identified sample XC02, which was collected from a pine forest (*Pinus kesiya* Royle ex Gordon), in Xuan Tho Commune, Da Lat, Lam Dong Province, Vietnam, was identified as *Cantharellus minor* based on morphology and *nrLSU* phylogeny analysis. Sequence analysis of multiple genes are becoming more and more common for phylogenetic analysis of mushrooms.

Method: Total DNA was isolated from sample XC02. The primer NS1, NS4 were applied to amplify the target gene *the nuclear ribosomal small subunit DNA* (*nrSSU*). For phylogenetic analysis, individual and concatenated datasets (*nrSSU* and *nrLSU-nrSSU*) were constructed. Phylogenetic tree was constructed with MEGA 6.0 with a 1000 replicate bootstrap based on the neighbor joining, maximum likelihood, maximum parsimony method.

Results: A concatenated dataset containing a total of 14 sequences from *Cantharellus*, *Craterellus (Cantharellaceae, Cantharellales)* and *Hydnum* (*Hydnaceae*, *Cantharellales*) were constructed. For the specimen XC02, the phylogenies based on the first, second, and third datasets (*nrLSU*, *nrSSU*, and *nrLSU-nrSSU*) and the morphological analysis, reported in our previous study, strongly confirmed the identity of XC02 as *Cantharellus minor*.

Conclusion: The combination between the morphological analysis and phylogenetic analysis is confirmed as the best approach for the identification of *Cantharellus* and other mushroom species that we collected in the Central Highlands, Vietnam.

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Introduction

Cantharellus minor Peck (Mycobank# 179458), a popular edible and commercialized mushroom of the *Cantharellus* genus, annual Report on the New York State Museum of Natural History 23, 1872, was first identified in 1872 by Peck [1]. *C. minor* grows on soil, forming ectomycorrhizal association with *Cedrus deodara*, *Quercus dilatata* [2]. The distribution of *C. minor* was previously reported to be restricted only to the northern part of South America. However, current reports show that this species are present in the Western Ghats, Kerala, India, etc. [2]. Of note, *C. minor* has not been recorded in Vietnam. During our expedition to validate the fungal diversity in the 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, we collected sample XC02 which belong to the *Cantharellus* genus (*Cantharellaceae*) [1]. According to our previous report, the specimen was yellow, aromatic in flavor with smooth cylindrical stipe (1 – 2 mm in diameter and 20 – 50 mm in length). Pileus surface was smooth, scaleless, and yellowish (5 – 15 mm wide) with incurred, non-striated and wavy-like margin and infundibuliform yellowish to orange. Lamellae was similar to pileus in color, distant, decurrent without intervenose. Basidiospores were ovoid-ellipsoid with smooth surface (6 – 11.5 x 4 – 6.5 µm with each basidium containing 4 – 5 cornuted spores [1]. *nrLSU* phylogenetic analysis showed that XC02 formed a highly supported monophyletic group with referent sequence from *Cantharellus minor*, and separated this group from other referent taxa. Therefore, based on the above morphological description and molecular analysis of *nrLSU*, the specimen XC02 was identified as *Cantharellus minor* (Figure 1). Notably, this record was the first record of *C. minor* from Pine forest of Lam Dong, Vietnam.

Cantharellus and the closely related *Craterellus* genus, which can be recognized by their lack of division into cap, stipe and rudimentary, have recently been investigated using molecular phylogenetic analysis, including *nrLSU*, *nrSSU*, *Rpb2* [2, 3]. Based on the phylogenetic analysis of *nrLSU*, *nrSSU*, *mtSSU*, and *Rpb2* data, *Cantharellus* and *Craterellus* consistently formed monophyletic and sister groups thereby elucidating the phylogeny of species in *Cantharellales* order [4]. Moreover, the dataset of the publication is also the largest up to now on Genbank (NCBI).

In this current study, with the aim of strengthen the identification of XC02 as *Cantharellus minor* as well as and a wider scope to apply this data set onto the further analysis of samples that we collected in the Central Highlands, we conducted the molecular phylogenetic analysis of the combined set of *nrLSU* and *nrSSU* genes.

Methods

Fungus collection

The specimen XC02 was collected in a pine forest (*Pinus kesiya* Royle ex Gordon) near Xuan Tho Commune, Da Lat, Lam Dong Province, Vietnam (Figure 1). General pick up information: Height: 1.502 m; Humidity: 87%; Temperature: 20°C; Light intensity: 3.012 lux; Coordinates: 11°56'34.45" N, 108°28'33.56" E. In the laboratory, specimens were conditioned to room temperature, submerged in 1% Mercury (II) chloride for 5 – 10 minutes, dried at 60°C, and stored for further analysis.

DNA extraction, PCR assay and target gene sequencing

The phenol/Chloroform method (pH= 8) was applied to isolate genomic DNA. The fruit body was added to a lysis buffer (2.0% SDS, Tris-HCl pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.1 mg/ml Proteinase K). During incubation at 65°C for overnight, it was mixed thoroughly by inverting the tube several times. Then, the supernatant was collected by centrifuged. 700 µL of PCI (Phenol/Chloroform/Isoamylalcohol with ratio of 25:24:1) solution was added and centrifuged. The upper solution was collected, precipitated with absolute ethanol, and washed with 70% ethanol. DNA concentration was identified by using OD260. Finally, isolated genomic DNA were stored in TE buffer at -20°C for further studies.

Figure 1: (A) Specimen XC02, identified as *Cantharellus minor*, collected in Pine forest; (B) Fruit body [1].

The primer pairs, used to amplify *nrSSU* region, are shown in Table 1. The final volume for PCR was 15 µL with a specified program: 1 cycle of 95°C for 5 min; 40 cycles of 95°C in 30 s, 42.2°C in 30 s, 72°C in 2 min; 1 cycle of 72°C in 5 min. 5 µl aliquots of amplification products were electrophoresed on a 2.0% agarose gel and visualized in a UV transilluminator. The amplified product was sequenced at Nam Khoa (Vietnam) Company with the same primers.

Target gene	Primer	Sequences (5' – 3')
nrSSU	NS1 (F)	GTAGTCATATGCTTGTCTC
	NS4 (R)	CTTCCGTCAATTCCTTTAAG

Table 1: The primers' sequences used in current study [5].

Taxa and sequences collection, DNA proofreading and phylogeny analysis

The data set of *nrLSU*, *nrSSU* were established by sequences downloaded from Genbank. The nrLSU, nrSSU were noted with strain, accession number, name of taxon and locality. The multiple gene data used in current study was established based on the combination of *nrLSU* and *nrSSU* data. *Craterellus cornucopioides*, *Hydnum repandum* and *Craterellus tubaeformis* were used as the outgroup for analysis. The amplified DNA sequences were proofread to remove ambiguous signals at both ends. Software, including SeaView 4.2.12, Chromas Lite 2.1.1, were used for proofreading. The phylogenetic tree was constructed, based on the neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) methods, by using Molecular Evolutionary Genetics Analysis (MEGA) version 6.0. Additionally, the best evolution model was predicted by using jModelTest [6].

Results

The systematic concatenated *nrLSU, nrSSU* dataset

The *nrLSU* dataset included 59 sequences belonged to *Cantharellus*, and 1 sequence of *Craterellus*. The *nrSSU* dataset included 11 sequences belonged to *Cantharellus*, 2 sequence of *Craterellus*, and 1 sequence of *Hydnum*. The concatenated dataset was established based on individual data sets of *nrLSU* and *nrSSU*. As a result, the *nrLSU-nrSSU* dataset, which comprised of a total of 14 sequences representing 8 species of *Cantharellus* (*Cantharellales, Cantharellaceae*), 2 species of *Craterellus* (*Cantharellales, Cantharellaceae*), and 1 species of *Hydnum* (*Cantharellales, Hydnaceae*), was collected from Genbank (NCBI) and listed in Table 2.

nrSSU amplification and the *nrSSU* phylogeny analysis

In current study, extracted and purified DNA was amplified with NS1 and NS4 primers. Electrophoresis on 2.0% agarose gel showed a significant and unique band of 1,102 bp. The PCR product was sequenced. Sequencing signals of both strands were similar and good for reading (Figure 2). *nrLSU* amplification and alignment (the first dataset, containing 59 sequences) were reported in our previous study [3]. The evolution model that was most fit with the nrLSU dataset was Kimura 2-parameter. As a result, XC02 formed a highly supported monophyletic group with referent sequences from *Cantharellus minor* (Accession number: KF294625, KF294632) [1].

For the *nrSSU* dataset (the second dataset, containing 14 sequences and an XC02 sequence), phylogeny analysis was conducted based on the topology constructed by Maximum parsimony (MP), Neighbour Joining (NJ), and Maximum likelihood (ML) with 1000 bootstrap replication. The topology analysis showed that XC02 formed a monophyletic group with Clade 4 referent sequences. The XC02 sequence, within this clade, was strongly supported on the monophyletic group with referent sequence: *Cantharellus minor* (Accession: DQ898672), based on the observed bootstrap values of 98, 96, 97 (for ML, NJ, MP methods, respectively) (Figure 3).

The concatenated dataset of *nrLSU-nrSSU* analysis

The best-fit model of DNA evolution for the analyses, for the concatenated dataset (the third dataset), was obtained using the jModelTest2 [6]. The GTR + G model was the most-fit model employed across sites for *nrLSU-nrSSU* dataset. Phylogenetic analysis was presented in Fig. 4. The *Cantharellus* in the concatenated dataset again formed a strong monophyletic group and separated from the outgroup. All the species in our dataset formed 3 clades that were previously reported as the sub-generic groups of *Cantharellus*, including the subgenus *Cantharellus* (Clade 1), *Cinnabarinus* (Clade 3), *Parvocantharellus* (Clade 4). Notably, the subgenus *Rubrinus* (Clade 2) was

STT	Taxon	Strain	*nrLSU*	*nrSSU*
1	*Cantharellus appalachiensis*	GRSM 77088	DQ898690	DQ898668
2	*Cantharellus cascadensis*	OSC 75985	AY041162	DQ898688
3	*Cantharellus cascadensis*	OSC 75975	AY041163	DQ898689
4	*Cantharellus cibarius*	OSC 75940	AY041157	DQ898684
5	*Cantharellus cibarius*	GRSM 77029	DQ898693	DQ898670
6	*Cantharellus cibarius var. roseocanus*	OSC 67712	AY041152	DQ898685
7	*Cantharellus cinnabarinus*	GRSM 77031	DQ898692	DQ898669
8	*Cantharellus formosus*	OSC 76054	AY041165	DQ898686
9	*Cantharellus lateritius*	GRSM 77030	DQ898694	DQ898671
10	*Cantharellus minor*	MA 40172	DQ898691	DQ898672
11	*Cantharellus subalbidus*	OSC 75937	AY041149	DQ898687
12*	*Craterellus cornucopioides*	UPSF 11800	AF105298	AF184191
13*	*Hydnum repandum*	TM 0268	DQ898741	AF026641
14*	*Craterellus tubaeformis*	KHL 8552	AF347095	AF026636

Table 2: The concatenated dataset of nrLSU-nrSSU gene was used for construction of phylogenetic trees
Note: * out group

Morphology*	*nrLSU**	*nrSSU*	*nrLSU-nrSSU*	Final identification
C. minor	*C. minor* (KF294632, KF294625)	*C. minor* (DQ898672)	*C. minor* (DQ898691, DQ898672)	*C. minor*

Table 3: The results based on the morphological analysis and individual, concatenated dataset phylogenetic analysis
Note: * based on our previous report [1]

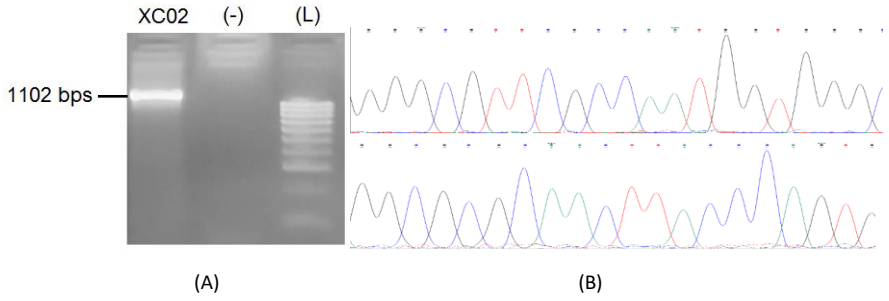

Figure 2: (A) Electrophoresis of PCR product of nrSSU. (L) 100 bps ladder; (-) negative control; (B) A part of the sequences from the forward primer (upper) and reverse primer (lower)

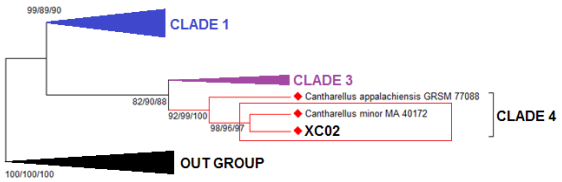

Figure 3: Phylogenetic relationships among XC02 and nrSSU dataset. The bootstrap value located on the branches were according to ML, NJ, and MP methods.

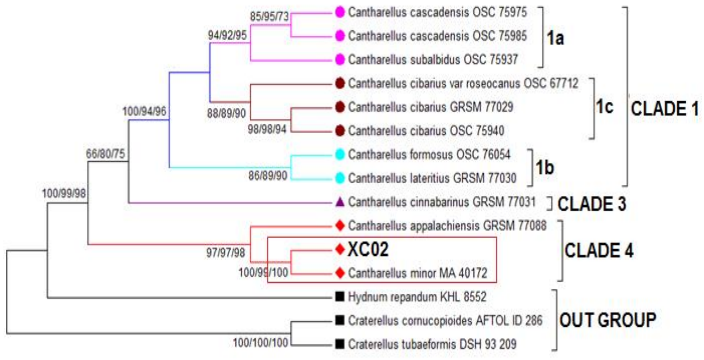

Figure 4: Phylogenetic relationships among XC02 and 14 concatenated sequences based on the analysis of ML, NJ, MP method. The tree was rooted using Craterellus group. The support values associated with each internal branch correspond to ML, NJ, and MP method.

not observed due to the missing *nrSSU* database in Genbank (NCBI). XC02 formed a monophyletic group in the subgenus *Parvocantharellus*, including *Cantharellus appalachiensis* and *Cantharellus minor*. Notably, within this clade, the XC02 formed a highly supported monophyletic group with referent sequence: *Cantharellus minor* with very high bootstrap values for ML, NJ, MP, respectively, and separated this group from the other referent taxon.

In the phylogenies based on the first, second, and third datasets (*nrLSU*, *nrSSU*, and *nrLSU-nrSSU*) and the morphological analysis, reported in our previous study, XC02 shared the same result as *Cantharellus minor* (Table 3). Therefore, the molecular analysis strengthened the identification of XC02 as *Cantharellus minor*. Moreover, the construction of the three datasets will facilitate further analyses of other samples collected in the Central Highlands.

Discussion

The pine forest (*Pinus kesiya* Royle ex Gordon), ca. 1502 m altitude, at Xuan Tho Commune, Da Lat, Lam Dong Province, Vietnam is located in the Central Highlands, which is a unique area that is a hotspot mushroom biodiversity [7]. Therefore, the natural composition of mushroom species needs to be studied, developed and preserved. In the trip to validate the fungal diversity in the Central Highlands of Vietnam, the sample XC02, identified as *C. minor* based on the morphological analysis and *nrLSU* dataset phylogenetic analysis, was collected. *C. minor* is the native fungus to the Eastern North America [8]. Recently, *C. minor* has been reported to be discovered in evergreen forests in the Western Ghats, Kerala, India, Malaysia [9].

In previous study, the records of *Cantharellus* species from the northwestern Himalayas of India were reported [2]. In their report, they provided the key to identify all the recognized species based on the morphology and the molecular database of *ITS* and *nrLSU* sequence. Among those species, *C. minor* formed the monophyletic group with *C. cinnabarius* based on the *nrLSU*, and formed the group with *C. elongatipes*, *C. appalachiensis*, and *C. cinnabarius* based on the *ITS*. However, *C. minor* differed mainly based on pileus size and colour. Notably, although *Cantharellus* species were recorded from over the world, only few molecular databases are available. Therefore, in addition to its phylogenetic analysis to the study of *Cantharellus* taxanomy, the combination of the species having many sequences, such as ITS, nrLSU, nrSSU, etc. may enhance the higher phylogenetic analysis power. Therefore, in this study, to strengthen the identification of XC02 and toward a wider scope to apply this data set onto the further analyses of our samples, we reconstructed the phylogenetic trees using these sequences of *nrLSU* and *nrSSU* further analysis. Therefore, in addition to the first dataset (*nrLSU*), the second and third dataset (*nrSSU*, *nrLSU-nrSSU*) were created and analyzed by MEGA [10]. Both datasets showed the strongly supported species group subclades in all the dataset for phylogeny of *Cantharellus*. For the specimen XC02, as a result, there were no conflicts in the identification of evolution relationship between referent sequences in all dataset. The highly supported monophyletic group with referent *C. minor* was obtained with high bootstrap value (> 95), indicated that XC02 is closely related to *C. minor*. Phylogenetic analyses based on individual and concatenated datasets revealed clades with statistical and strongly support corresponding to morphological observation. Thus, XC2 was concluded as *C. minor*. Although *Cantharellus* species are recored and reported from all over the world, only few species have nrSSU sequences in public database (Genbank, NCBI), indicating the poor coverage of these species. However, in summary, combining the morphological and molecular data is the clearly the best approach to make the progress in the further studies of samples that we collected in the Central Highlands region. We have successfully applied the phylogenetic analyses based on individual (*nrLSU*, *nrSSU*) and concatenated datasets (*nrLSU-nrSSU*) to strengthen the identification of XC02 as *Cantharellus minor*. Additionally, the combination between the morphological analysis and phylogenetic analysis is confirmed as the best approach to apply this onto the further analysis of *Cantharellus* and other mushroom samples that we collected in the Central Highlands, Vietnam.

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Conflict of Interest Statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

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