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Ophiocordyceps xuansonae A NOVEL SPECIES OF GENUS *Ophiocordyceps* IN VIETNAM

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ABSTRACT

The *Ophiocordyceps* is an important globally distributed genus that includes over 239-recorded species. Many species of the genus are famous for their significance in ecology, agriculture and pharmaceuticals. There have been 12 species of genus *Ophiocordyceps* found in Vietnam. In this study, the samples of *Ophiocordyceps* parasitizing the larva of order Lepidoptera at Xuan Son National Park in Phu Tho province were collected and characterized using both morphology and molecular analyses. The morphology comparison and ITS sequence analysis revealed that the fungus belongs to a new species of *Ophiocordyceps*. The paper presents the morphological and molecular description of the species.

Keywords: Ophiocordycipitaceae, *Ophiocordyceps xuansonae*, New species, Xuan Son, medicinal fungi.

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INTRODUCTION

The insect parasitizing fungi have been widely used for different applications in agriculture, pharmaceuticals, and genetic studies. However, its taxonomy had not yet been stable until the emergence of molecular and bioinformatic tools. Fungi in the Ophiocordycipitaceae family (Ascomycota) parasitize a range of insects with more than 469 recorded species worldwide (Catalogue of life, 2022; Hyde et al., 2020). Initially, Ophiocordycipitaceae species were classified under the Clavicipitaceae family due to their morphologies, cylindrical asci with a prominent apical cap, and filiform ascospores with part-spores. Sung et al. (2007) used *nrSSU, nrLSU, tef1, rpb1, rpb2, β-tubulin (tub)* and *atp6* to analyse the phylogeny of Clavicipitaceae species. The molecular analysis separated the studied species into three different clades representing three families of Clavicipitaceae, Cordycipitaceae and Ophiocordycipitaceae (Sung et al., 2007). The previously accepted *Ophiocordyceps* species were moved to Ophiocordycipitaceae (Hyde et al., 2020; Sung et al., 2007).

Vietnam has tropical climate conditions that are suitable for diverse fungal, plant and animal communities. However, research on ascomycetes in general and on insect parasitizing fungi, in particular, was limited. There have been 12 species of *Ophiocordyceps* listed in the checklist of fungi in Vietnam but there were only one species found at Lang Biang (*Ophiocordyceps langbianensis)* with a detailed morphological description (Thuan et al., 2021). The other eleven species were *Ophiocordyceps formicarum, O. myrmecophila, Ophiocordyceps formosana, O. geniculata, O. nutans, O. oxycephala, O. sphecocephala, Ophiocordyceps brunneipunctata, O. irangiensis, O. pseudolloydii,* and *O unilateralis*) (Kiet, 2014). The lack of detailed descriptions drives doubt about the precision of the listed names and difficulties of identification.

In this study, the samples were collected from March 2016 to December 2018 at Xuan Son National Park, Phu Tho province and the Copia Nature Reserve, Son La province in both anamorphic and teleomorphic stages. The samples were characterized morphologically and genetically and a novel species was described.

MATERIALS AND METHODS

Materials

The sample XS154 was a parasite in larva Lepidoptera, collected at Xuan Son National Park at 21^{o}13. 835^{o}N – 104^{o} 93. 791^{o} E; 1,200 m latitude.

Methods

Fungal isolation

The single spore isolation method was applied on both PDA (200 g potato, 20 g dextrose, 10 g agar, 1000 ml distilled water) and SDAY (40 g dextrose, 10 g peptone, 10 g yeast extract, 17 g agar) media, supplemented with 100 mg/L chloramphenicol. Spore germination was checked after 24−48 hours of incubation, using the stereo microscope Olympus SZ61. The pure fungal colonies were then moved to tubes containing new PDA and SDAY media for further incubation for 4–5 days. The cultures then were kept at 4 ^{o}C and inoculated for further uses (Choi et al., 1999; Noman et al., 2018).

Morphological descriptions

Morphological characteristics are important in fungal identification at genus and species levels. The characteristics of the host, stroma, perithecia, asci, ascospore, and anamorphic characteristics (growth media, colonies, mycelia, conidiophores, conidia…) were observed under an Olympus SZ61 stereo microscope and a light microscope Olympus CX31 (Kobayasi, 1982; Luangsa-ard et al., 2007, 2008, 2018; Samson et al., 1988).

DNA extraction

300 mg of fungal biomass of each sample was crushed in an Eppendorf tube containing 500 µl 2X CTAB and incubated at 65 ^{o}C for 60 minutes. A volume of 500 ml of Chloroform: Isoamyl alcohol (24:1) was added into the tube and carefully mixed. The tube was centrifuged at 13.000 rpm for

10 minutes at 4 ^{o}C. The upper layer was moved into a new tube, 2/3 volume of cold isopropanol (-20 ^{o}C) was added, gently mixed and incubated at -20 ^{o}C overnight. The DNA was precipitated by centrifugation at 13000 rpm for 20 minutes at 4 ^{o}C and washed in 1 ml of 70% ethanol 2 times. The DNA was dissolved in 50 µl of sterile deionized H_2O and kept at -20 ^{o}C (Doyle & Doyle, 1987).

PCR reactions

The ITS4 - ITS5 primer pairs were used to amplify the *ITS* complete sequence (Schoch et al., 2012). The PCR reactions (50 µL) contained 25 µl of PCR master mix, 1.5 µL of primer (10 pM) of each type, 19 µL of H_2O and 3 µl of DNA. The thermal cycles were 3 minutes of initial denaturation at 94 ^{o}C followed by 35 cycles of (1) denaturation at 94 ^{o}C for 40 seconds, (2) primers annealing at 48 ^{o}C in 40 seconds, (3) new strand extension at 72 ^{o}C in 80 seconds; and the final extension at 72 ^{o}C for 8 minutes. The PCR products were run in a 1% agarose gel containing 1.5 µL RedSafe at 80V for 60 minutes and visualized by UV lights.

DNA sequencing and phylogeny analysis

DNA sequencing was done by First Base company using the same PCR primers. The sequences were checked using Bioedit software (Hall, 2001). The relevant DNA sequences were retrieved from NCBI by using the Basic Local Alignment Search Tool (BLAST). The alignment of the sequences was done in ClustalX2 (Larkin et al., 2007). The phylogenetic trees were built in Mega11 software (Tamura et al., 2021). Sequences from GenBank used in the analysis are shown in the phylogenetic tree.

RESULTS

***Ophiocordyceps xuansonae* Nguyen DV & Duong ML sp. nov.** (Figs. 1 & 2)

Etymology: named after the Xuan Son (National Park) where the fungus was found.

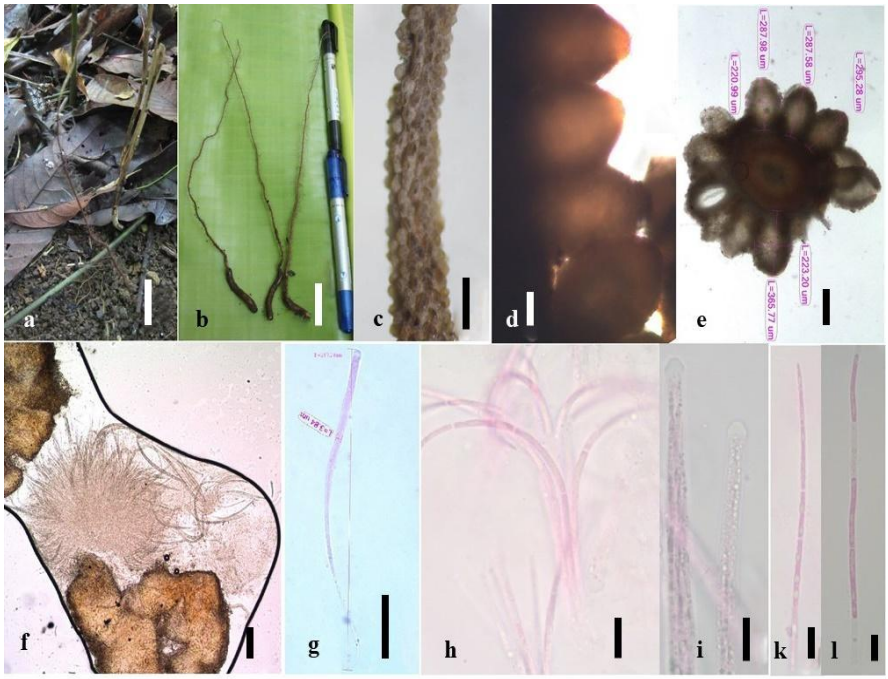

Figure 1. Morphological characteristics of *Ophiocordyceps xuansonae* Nguyen DV & Duong ML sp. nov**.** a, b- fungal strain Ophiocordyceps sp.3 on the host; b- stromata ; d, e- perithecia; f, g, i- asci ; h, k, l- ascospores. Size: a- 400 µm; c, d- 100 µm; e, f- 200 µm; g- 50 µm; h, i, k, l- 10 µm

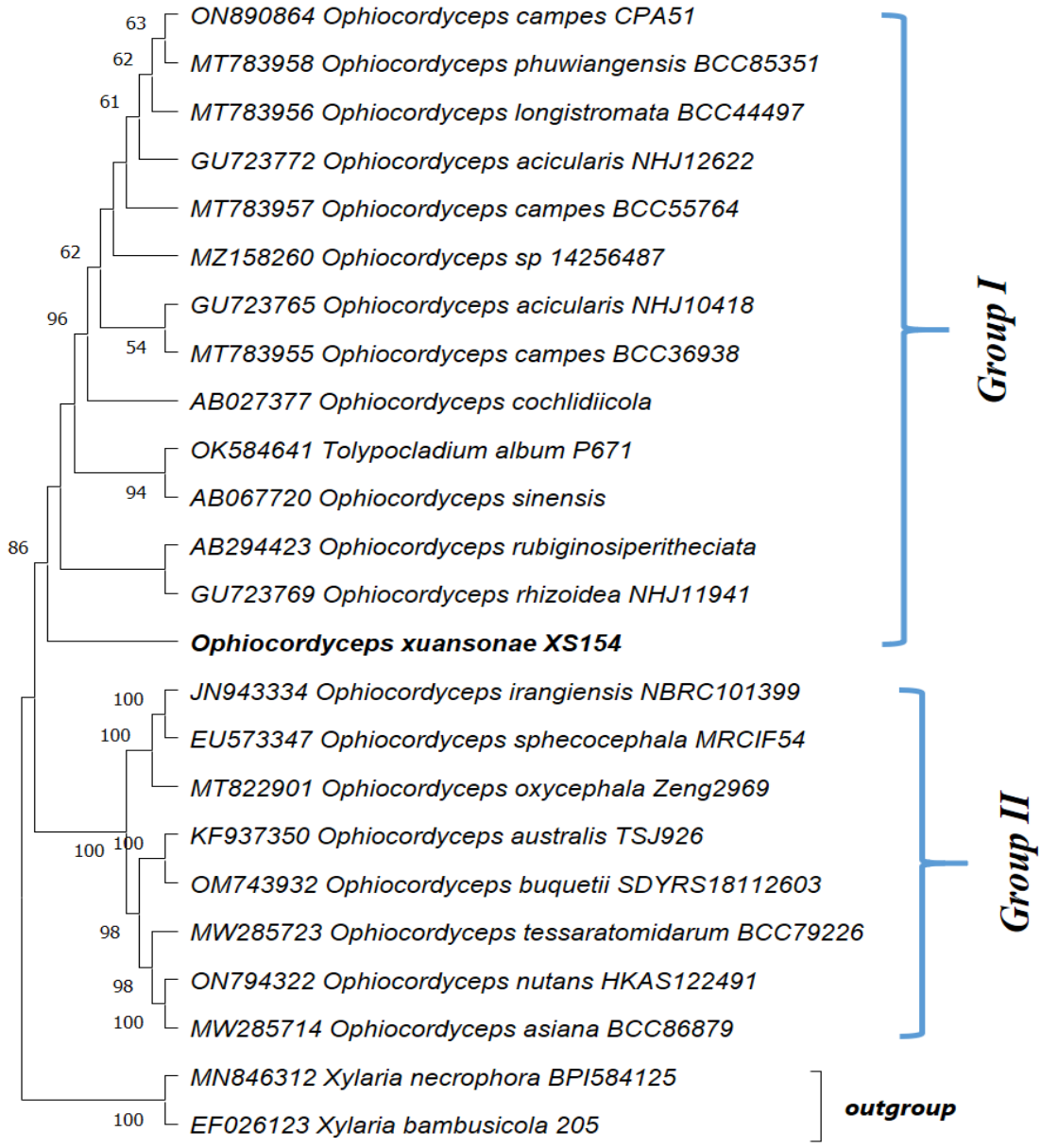

Figure 2. Evolutionary relationships of ITS sequences of the *Ophiocordyceps xuansonae* XS154 and closely related sequences in the GenBank

Habitat: on the larva of order Lepidoptera in a high-cover density forest.

Geographic distribution: Vietnam, only known from Xuan Son National Park, Phu Tho province

Specimen examined: **Vietnam**: Xuan Son National Park, Phu Tho province**,** at 21°13.835°N - 104°93.791°E; 1,200 m above sea level, on Lepidoptera larva, underground, 27/09/2018 Nguyen DV & Duong ML. **(**Holotype was deposited at The Biological Museum of Hanoi National University of Education, coded HNUE-Fu-0001).

The new name with a full description will be submitted to the Mycobank as soon as the paper is published. The fungus was found in

the sexual reproduction stage in the ground soil of a high forest cover density area. *Stromata* arose from the top of the host's, 20–23 cm long, brown-grey, branched. The stromata look like twigs with fruiting bodies covering the surface. (Figs. 1a, 1b, 1c); *Perithecia* vertically arranged on the stromata, mostly superficial, 233.20–295.28 µm × 287.58–365.77 µm, hard wall, oblique, ovoid, and brown color, (Figs. 1c, 1d, 1e); *Asci* cylindrical, tapered at both ends, 229–300 µm × 6.02–7.5 µm x, with a thin, clear, smooth wall. The apical cap was sporangium - round 5.0–5.5µm, inside contains 8 filiform spores parallelly arranged to each other; J^- in Melzer's reagent (Figs. 1f, 1g, 1h, 1i); *Ascospores* filiform, truncate at the ends, 112.0–150.0 µm × 1.77–2.0 µm (Figs. 1k, 1l & Fig. 2).

Molecular analysis

The ITS sequence of the fungus was used to search for similar available sequences in the GenBank and 21 sequences of different species were selected for the analyses. Two sequences of Xylaria necrophora and Xylaria bambusicola were chosen to take part as the outgroup. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA11 (Fig. 2).

DISCUSSION

The morphological identification led to the most similar species *Ophiocordyceps spataforae* as both with superficial, ovoid, dark brown perithecia. However, the studied sample is different from *O. spataforae* in stromata (with very few and scattered perithecia at the bottom of the stroma) and hosts (insect versus leaf litter) (Samson et al., 1988; Luangsa-ard et al., 2018). The morphological identification could not lead to any described species of the genus and it suggested the studied sample could be a new species named *O. xuansonae* Nguyen DV, Duong ML sp. nov. as it was found in Xuan Son primary forests.

The ITS sequence of the *O. xuansonae* XS154 (ON890872.1) with 532 nucleotides was used to search for the closely related sequences in the GenBank (NCBI). The most similar sequences at 100% of coverage were of the *Typocladium album* with only 82.84% of similarity (OK584641.1). The search result showed that the study sequence was new and much different from the available ITS sequences in GenBank. However, the 21 most similar sequences (in the nBlast) of 17 species (*Ophiocordyceps acicularis, O. asiana, O. australis, O. buquetii, O. campes, O. cochlidiicola, O. irangiensis, O. longistromata, O. nutans, Ophiocordyceps. oxycephala, O. phuwiangensis, O. rhizoidea, O. rubiginosiperitheciata, Ophiocordyceps sinensis, O. sphecocephala, O. tessaratomidarum* and *Tolypocladium album*) were selected for the phylogenetic analysis.

Phylogenetic trees of the *O. xuansonae* XS154 showed that the sequences were clustered into two groups with strong support bootstrap values (100% for group II and 86% for group I). The members of each group were with some similarities in morphologies.

Group I included *O. acicularis, O. campes, O. cochlidiicola, O. longistromata, O. phuwiangensis, O. rhizoidea, O. rubiginosiperitheciata, O. sinensis, Tolypocladium album* and *O. xuansonae*. All the species of the group I possess some common characteristics such as dark color stromata, hard-wall, semi-

immersed or superficial ascomata. Their ascospores are filiform, hyaline, and multiseptate but not breaking into part-spores (Luangsa-ar et al., 2018; Tasanathai et al., 2019, 2020).

Group II included *O. asiana*, *O. australis*, *O. buquetii*, *O. irangiensis*, *O. nutans*, *O. oxycephala*, *O. sphecocephala* and *O. tessaratomidarum.* All species in this group have soft and bright stromata, with pheomelanin. Their perithecia are immersed or semi-immersed perpendicularly arranged with stromata. The fertile parts are normally short on a long sterile stype. Cylindrical asci with thickened apical cap, 8 string ascospores. Ascospores are cylindrical or polygon and disarticulated into part-spores (Sung et al., 2007).

CONCLUSION

In this study, morphological observations were important and significant to confirm the identification of *O. xuansonae* XS154 as a novel species. The molecular markers, ITS sequences were used and the analysis supported the morphological identification.

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