MORPHOLOGICAL AND MOLECULAR CHARACTERISTICS OF *Cordyceps* COLLECTED FROM XUAN SON NATIONAL PARK AND COPIA NATURAL RESERVE

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ABSTRACT

The *Cordyceps* is an important genus that includes over 183 recorded species and globally distributed. They have been applied a lot in medicine and agriculture. There has been 13 *Cordyceps* species recorded in Vietnam. However, the detailed descriptions of species found in Vietnam is limited. In this study, the fungal samples were collected from the Xuan Son National Park in Phu Tho province and the Copia Nature Reserve, Son La province were morphologically described and identified to species using morphological characteristics together with molecular analysis of *LSU, ITS, RPB1* sequences. The studied taxa were *Cordyceps cicadae, C. takaomontana, C. militaris* and two unidentified samples, *Cordyceps* sp. CPA64 and *Cordyceps* sp. XS142. The study gave useful insight of diversity and distribution of *Cordyceps* species in the two studied sites, Copia and Xuanson and also introducing new records of *C. cicadae* in Vietnam. The two unidentified samples might be new species to science and that needs to have further studies to clarify taxonomy of the two samples.

ĐẶC ĐIỂM HÌNH THÁI VÀ SINH HỌC PHÂN TỬ CỦA CÁC CHỦNG NẤM *Cordyceps* TẠI VƯỜN QUỐC GIA XUÂN SƠN VÀ KHU BẢO TỒN THIÊN NHIÊN COPIA

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TỪ KHÓA

Họ nấm Cordycipitaceae
Đông trùng hạ thảo
Copia - Sơn La
Xuân Sơn – Phú Thọ
Định loại phân tử

TÓM TẮT

C*ordyceps* là chi nấm ký sinh côn trùng quan trọng chiếm số lượng loài lớn, có vùng phân bố rộng trên toàn cầu, với 183 loài được công nhận và có nhiều ứng dụng trong y dược, nông nghiệp. Đã có 13 loài của chi C*ordyceps* được ghi nhận ở Việt Nam. Tuy nhiên, những miêu tả chi tiết các loài đã ghi nhận ở Việt Nam còn rất nghèo và thiếu thông tin. Trong nghiên cứu này, các mẫu nấm được thu thập từ Vườn quốc gia Xuân Sơn - Phú Thọ và Khu bảo tồn thiên nhiên Copia, được miêu tả chi tiết hình thái và định danh kết hợp giữa đặc điểm hình thái và sinh học phân tử thông qua phân tích trình tự các đoạn gen *LSU, ITS, RPB1*. Các loài được định danh là *Cordyceps cicadae, C. takaomontana, C. militaris* và hai mẫu chưa xác định được tên loài là *Cordyceps* sp. CPA64 và *Cordyceps* sp. XS142. Nghiên cứu đã cung cấp thông tin về đa dạng, phân bố của nấm *Cordyceps* tại Copia và Xuân Sơn và ghi nhận mới *C. cicadae*. Các mẫu chưa được định loại tới loài có thể là loài mới cho khoa học và cần có các nghiên cứu tiếp theo để xác định chính xác tên loài của hai mẫu này.

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1. Introduction

There have been more than 500 species described as *Cordyceps* in the Clavicipitaceae but 183 have been accepted by most mycologists [1]. Some species of the genus are traditionally used as medicine such as *C. militaris, C. cicadae*... Sung et al. (2007), based onmolecular and morphological features separated these species into three different clades representing for three families Clavicipitaceae, Cordycipitaceae and Ophiocordycipitaceae. The accepted *Cordyceps* species were moved to Cordycipitaceae, Hypocreales, Sordariomycetes, Ascomycota. The *Cordyceps* has soft stroma with pheomelanin. Perithecia are embedded or semi-embedded perpendicularly at the surface of the fertile portion of a stroma. Cylindrical asci have thickened apical cap, 8 string ascospores. Ascospores are cylindrical or polygon, disarticulated into part-spores [2]. Nevertheless, insect fungi have many morphological features in similar with high variability according to environment, geographical regions, their teleomorph or anamorph stages. Additionally, there have been a large number of species composition recorded. All of those make difficult to identify an insect fungus to species.

Vietnam has ideal conditions for growth and development of fungi with tropical monsoon climate, with high diversity of plants and animals. However, researches on C*ordyceps* have been limited with poor descriptions of some recorded species. The *Cordyceps gunnii* (Berk.), *C. takaomontana, C. crinalis, C. formicarum* were recorded in 2009-2011 [3], [4]. By 2014, there were 11 species of *Cordyceps* found in Vietnam including *C. cardinalis*, *C. formosana*, *C. gunnii*, *C. nelumboides*, *C. militaris* , *C. ninchukispora*, *C. neovolkiana*, *C. pseudomilitaris*, *C. takaomontana, C. elongatostromata, C. prolifica* [5]. Truong et al. collected 124 samples of entomopathogenic fungi and identified to species of *Cordyceps nevolkiana*, *C. pseudomilitaris*, *C. takaomontana, Isaria tenuipes* by using *ITS* complete sequences, of which, *C. neovolkiana* was introduced as a new species. The chemical composition of *C. neovolkiana* was also studied [6]. In 2020, the description on morphological features and molecular features of *Cordyceps* sp. V4 [7] was recorded at Copia. So, it can be seen that knowledge of *Cordyceps* in Vietnam is not going along with its potential diversity.

This paper introduces detail descriptions of the species found in the study and a new record of *Cordyceps cicadae* for Vietnam. The descriptions will be helpful for young mycologists in Vietnam to easily identify Vietnamese *Cordyceps* samples to species.

2. Methodologies

2.1. Materials

The samples were collected during 3/2016 to 12/2018 at Xuan Son National Park, Phu Tho province and Copia National Park, Son La province.

2.2. Methods

2.2.1. Fungal isolation and cultivation

The isolation was made by taking spores and diluted in 100 µl of sterile water and then poured onto both PDA (200 g Potato, 20 g Dextrose, 17 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. After 24-48 hours of incubation, germinated spores were inspected under the stereo microscope. The pure fungal colonies were then moved to tubes containing new PDA and SDAY media for further incubation of 4-5 days. The cultures then were kept at 4^{o}C and inoculated for further uses [8].

2.2.2. Morphological descriptions

Morphological characteristics are important in fungal identification to species or genus level. For insect fungi, some main characteristics that have to be recorded are hosts, stroma,

perithecium, asci, and ascospore, anamorphic stages [9]. The samples were directly observed under an Olympus SZ61 stereo microscope and microscope Olympus CX31 for morphological descriptions.

2.2.3. DNA isolation

300 mg of fungal biomass of each sample was crushed in an Eppendorf tube containing 500 µl 2X CTAB and incubated at 65ºC 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 min at 4ºC. The supernatant was moved into a new tube. The supernatant was collected and 2/3 volume of cold Isopropanol (-20ºC) was added, gently mixed and incubated at -20ºC overnight. The DNA precipitation was done by centrifuging at 13.000 rpm for 20 minutes at 4ºC and washed in 1 ml of 70% ethanol for 2 times. The DNA was dissolved in 50 µl of sterile deionized H_2O and kept at -20ºC for preservation [10].

2.2.4. PCR Reaction

ITS4 - ITS5, LROR- L7 and Crpb1A- RPB1Cr primer pairs were used to amplify the *ITS* complete sequences, *nrLSU* region and *Rpb1* region respectively [11]. 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°C followed by 35 cycles of (1) denaturation at 94°C for 40 seconds, (2) primers annealing at 48°C in 40 seconds, (3) new strand extension at 72°C in 80 seconds; and the final extension at 72°C for 8 minutes. The PCR products were run in an 1% agarose gel containing 1.5 µl red-safe at 80V for 60 minutes and visualized by UV lights.

2.2.5. DNA Sequencing and Phylogenetic analysis

DNA sequencing was done by First Base company using the PCR forward and reverse primers. The sequences were aligned using Bioedit software [12]. Relevant DNA sequences in analysis were taken from NCBI by using the Basic Local Alignment Search Tool (BLAST). The alignment of sequences was done in ClustalX 1.83 [13]. The phylogenetic trees were built in Mega X software [14].

3. Results and discussions

3.1. Morphological descriptions

***Cordyceps cicada,* specimen CPA60**

The fungus parasites in a Cicada at Copia Reserve, at 21,328541ºN – 103,584905ºE, 1000.5 m above sea level, where there was an agricultural habitat with no trees and many grasses. The sample were found underground in sexual reproduction stage. *Stromata* arose from the top of cicadas' head or neck, 3 - 6 cm long, orange, with two distinct parts, spore bearing (2-4 x 0.4-0.5 cm) and smooth stipe (1.5-2.0 x 0.2-0.3 cm) (Fig. 1: a, b, c). *Perithecia* tightly and vertically arranged with the stromatal surface, 202.29 µm - 222.20 µm x 505 µm - 507.22 µm, hard wall, mostly superficial, oblique, flask-shaped (Fig. 1. d, e, f). *Asci* were cylindrical 3.58 µm – 3.91 µm x 185.57 µm - 521.96 µm, with thin, transparent, smooth and fragile wall. Apical cap was hemi-spherical (1.85 µm x 2.85 µm), J^- in Melzer's reagent. Paraphyses were not found (Fig. 1: g, h, i). *Ascospores* elongates, 3.31 µm - 7.27 µm x 2.0 µm – 2.14 µm, easily decayed into part-spores at two ends (Fig. 1: k).

***Cordyceps* sp. CPA64, specimen CPA64**

The fungus parasites in a *Cicadas* at Copia Reserve, at 21,328541ºN – 103,585605ºE, 1.011,5 m above sea level, where there was an agricultural biotope. The specimen was found in soil, which was covered by grasses. The sample were found underground in sexual reproduction stage. *Stromata* arose from any part of the cicada body, 4 – 5 x 0.3 - 0.5 cm, white when young and

became pink when matured. Perithecium covered 1 cm on the cap of Stroma in orange color distinct from the white part of Stromata. (Figure 2. a, b). *Perithecia* tightly and vertically arranged with the stromatal surface, mostly immersed, 101,55 µm - 102.64 µm x 460.97 µm – 497.58 µm, hard wall, oblique, flask-shaped. (Fig. 2. b, c, d). *Asci* were cylindrical, elongated at two ends, 3.58 µm – 5.27 µm x 218.74 µm - 300 µm, with thin, transparent, smooth wall and round-shaped apical cap sizing 3.16 µm x 4.91 µm; J^- in Melzer's reagent. (Fig. 2. e, f, g). *Ascospores* elongate, 1.84 µm – 2.09 µm x 12.57 µm – 14.61 µm, easily decayed into part-spores at two ends. (Fig. 2. g, h).

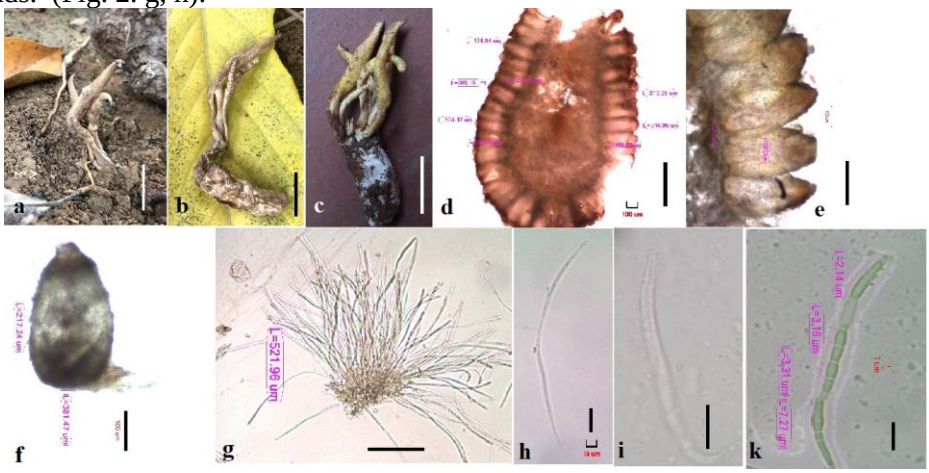

Fig. 1. *Cordyceps cicada CPA60; Scale bar: a, b, c = 2 cm; d = 500 µm; e =200 µm; f = 100 µm; g = 200 µm; h – 20 µm; i, k = 10 µm*

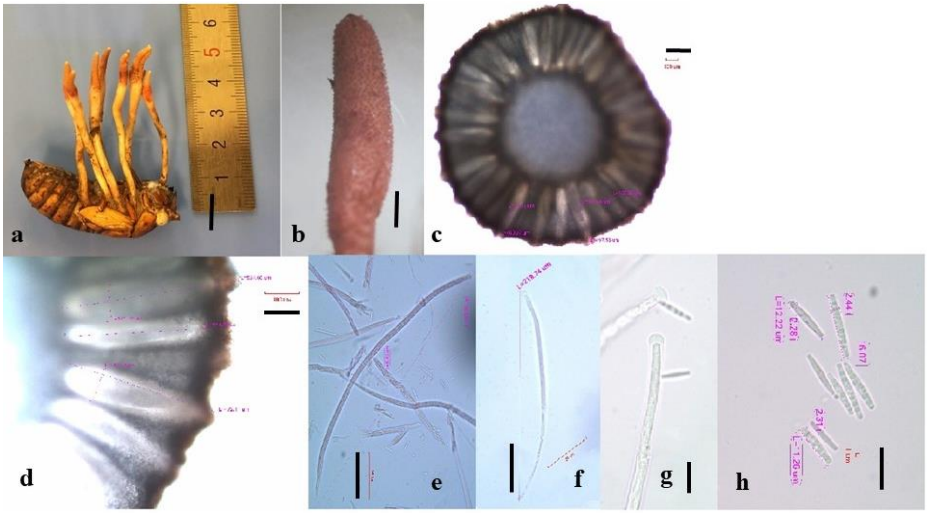

Fig. 2. *Cordyceps* sp. CPA64; *Scale bar: a, b = 1 cm; c = 200 µm; d = 100 µm; e, f = 50 µm; g, h = 10 µm*

***Cordyceps takaomontana* XS76, specimen XS76**

The fungus parasites in a Lepidoptera larva at the Xuan Son National Park, at 21º6170̓N – 104º56,292̓E, 500 m, which was high forest cover density. The sample were found underground in sexual reproduction stage. *Stromata* arose from the top of the host's head, 5 – 6 cm long, yellow, with two distinct parts, spore bearing (2-3 x 0.3-0.4 cm) and smooth stipe (2.0 - 3.0 x 0.2-0.3 cm). The stromata of this fungus was soft and porous. (Fig. 3. a, b). *Perithecia* loose and vertically arranged with stromatal surface, 142.85 µm – 214.28 µm x 285.71 µm – 357.14 µm, hard wall, pseudo-immersae, oblique, flask-shaped. (Fig. 3. b, c). *Asci* were cylindrical 2.86 µm –

3.03 µm x 318.74 µm - 500 µm, with thin, transparent, smooth and fragile wall. Apical cap was hemi-spherical (3.14 µm x4.0 µm), containing an 8-ascospore-string, J^- in Melzer's reagent. (Fig. 3. d, e, f, g). *Ascospores* elongated, easily decayed into rhomboid part-spores, average size goes around 0.6 µm – 0.8 µm x 4.05 µm – 7.61 µm (Fig. 3. h).

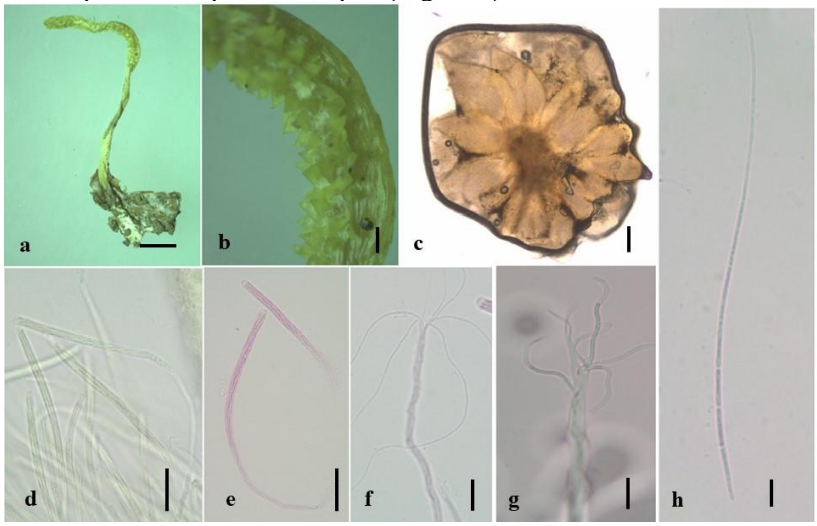

Fig. 3. *Cordyceps takaomontana* XS76; *Scale bar: a = 1 cm; b = 500 µm; c = 100 µm; d, e = 50 µm; f = 10 µm; g, h = 5 µm*

***Cordyceps* sp. XS142, specimen XS142**

The fungus parasites in a Lepidoptera larva at the Xuan Son National Park, at 21°6170'N – 104°56,292'E, 1200 m above sea level, which was a pine forest environment with high cover density. The sample were found underground in sexual reproduction stage. *Stromata* arose from the top of the host's head, 2.8 cm long, yellow. (Fig. 4. a, b). *Perithecia* tightly and vertically arranged with the stromatal surface, 120.35 µm – 260.44 µm x 372.51 µm – 574.00 µm, hard wall, pseudo-immersae, oblique, flask-shaped. (Fig. 4. b, c). *Asci* were cylindrical, 3.70 µm – 4.86 µm x 400 µm – 638 µm, with thin, transparent, smooth and fragile wall. Apical cap was hemi-spherical (2.91 µm x 4.28 µm), J^- in Melzer's reagent. (Fig. 4. d, e, g). *Ascospores* are rectangular, 1.0 µm – 1.8 µm x 2.91 µm – 4.5 µm, easily decayed into part-spores at two ends. (Fig. 4. f).

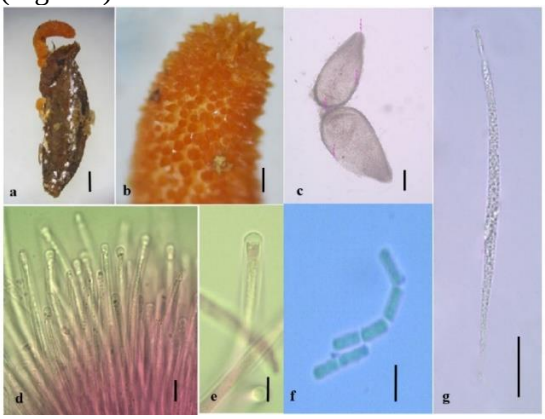

Fig. 4. *Cordyceps* sp. XS142; *Scale bar: a = 1 cm; b = 500 µm; c = 100 µm; d = 10µm; e, f = 5 µm; g = 100 µm*

Fig. 5. *Cordyceps militaris* CPA70; *Scale bar: a = 2 cm; b, c = 100 µm; d =5 µm; e = 10 µm; f =1 cm*

***Cordyceps militaris* CPA70, specimen CPA70**

The fungus parasited in a Lepidoptera larva at the Copia Nature Reserve, at 21.328541°N – 103.585605°E, 1500.5 m above sea level, was found in sexual reproduction stage in the ground soil of a high forest cover density. *Stromata* arose from the top of the host's abdomen, 6 – 8 cm long, oranger yellow, with two distinct parts, spore bearing spore bearing (3-4 x 0.4-0.5 cm) and smooth stipe (2.0-4.0 x 0.4-0.4 cm). (Fig. 5. a). *Perithecia* was tightly and vertically arranged with the stromatal surface, 300 µm – 315.14 µm x 500 µm – 539.50 µm, hard wall, pseudo-immersae, oblique, flask-shaped. (Fig. 5. b). *Asci* were cylindrical, 5.0 µm – 5.99 µm x 323.03 µm – 450 µm, with thin, transparent, smooth and fragile wall. Apical cap was hemi-spherical (3.0 µm x 3.27 µm), J^- in Melzer's reagent. Paraphyses were not found. (Fig. 5. c, d). *Ascospores* were rectangular, 2.47 µm – 2.94 µm x 5.76 µm – 8.53 µm, easily decayed into part-spores at two ends. (Fig. 5. e). *The culture* grew well on PDA and gradually changed from white to yellow at the 14th day of incubation at 25 °C (Fig. 5. f).

3.2. Phylogenetic analysis

The figure 6 showed that the sequences of 3 families Cordycipitaceae, Clavicipitaceae, Ophiocordycipitaceae were well separated. These families were revealed and rearranged by Sung, 2007 [2]. Morphologically, Cordycipitaceae and Ophiocordycipitaceae can be distinguished by the arrangement of perithecia, shape of asci, ascospores, and part-spores. The species in *Cordyceps* have soft texture and strong colored stromata, orderly arranged, partly or completely immersed. Asci are hyaline, which have long and breakable walls, containing cylindrical ascospores. The Ophiocordycipitaceae stromata are hard and strong or light/bright colored. Perithecia immerse inside or distributed sporadically outside of the stromata at oblique angle. The asci are with a thick cap. The ascospores can be disarticulated into part-spores upon maturity. The species in Clavicipitaceae normally have bright, spongy or hard stromata. The ascospores are cylindrical or polygonal shape and can be disarticulated into part-spores upon maturity [2].

The species in this study were distributed into 5 strongly supported groups (figure 6). Group I includes some closely related taxa to the *Cordyceps* sp. XS76 including *C. takaomontana* BCC 12688, *C. tenuipes* with bootstrapping value of 99% (figure 6). Therein, *C. tenuipes* was described as sexual state of *Isaria tenuipe*s. *Paecilomyces tenuipes* (Peck) Samson is a synonym of *C. tenuipes*. Based on the combination of host, morphological and molecular features, we concluded that XS76 is *Cordyceps takaomontana.* Group II includes the species that are closely related to the *Cordyceps* sp. CPA60, including *C. cicadae* IFO 33259, *C. cicadae* JGS150805, *C. cicadae* GACP 14061604, *C. cicadae* GACP 07071701 (fig. 6). *Cordyceps cicadae* has been reported from South Asia (including Vietnam), North America, and South America, China [15]. The morphological and sequences analysis proved that the *Cordyceps* sp. CPA60 is *Cordyceps cicadae.* This was the first time the species found and recorded for fungal flora in Vietnam. Group III includes the *Cordyceps* sp. CPA70 and *C. militaris* NBRC 9787, *C. militaris* NBRC 100741, *C. militaris* NBRC 30377 (figure 6). In Vietnam, *C. militaris* were found in Hoang Lien Son forests. The morphological and molecular data confirned that the Cordyceps sp. CPA70 is *Cordyceps militaris*. Group IV contained the *Cordyceps* sp. XS142 and *Isaria farinosa* MY01338, *I. farinosa* J5, *Cordyceps farinosa* CBS 156.65, *C. farinosa* CBS 127996, *Samsoniella inthanonensis* TBRC 7915, *S. inthanonensis* TBRC 7916, *S. aurantia* TBRC 7271. Morphologically, *Samsoniella aurantia* TBRC, *S. inthanonensis* TBRC 7915, *S. inthanonensis* TBRC 7916 has egg-shaped superficial perithecia, which was different from that of *Cordyceps* sp. XS142. It was not possible to identify the studied *Cordyceps* to species level at the moment. Group V included *Cordyceps* sp. CPA64 together with *Cordyceps kanzashiana, C. nipponica* NBRC 101406, *C nipponica* NBRC 101405. However, stromata of *C. nipponica* were single or branched into two, or three arising from the head of the Heteroptera larva (mostly) while it was single and arose from different part of the insect in *Cordyceps* sp. CPA64. The asci and

ascospores of *C. nipponica* were much bigger than that of the *Cordyceps* sp. CPA64. The combination of morphology and melocular data was not strong enough to support the identification of the sample to species.

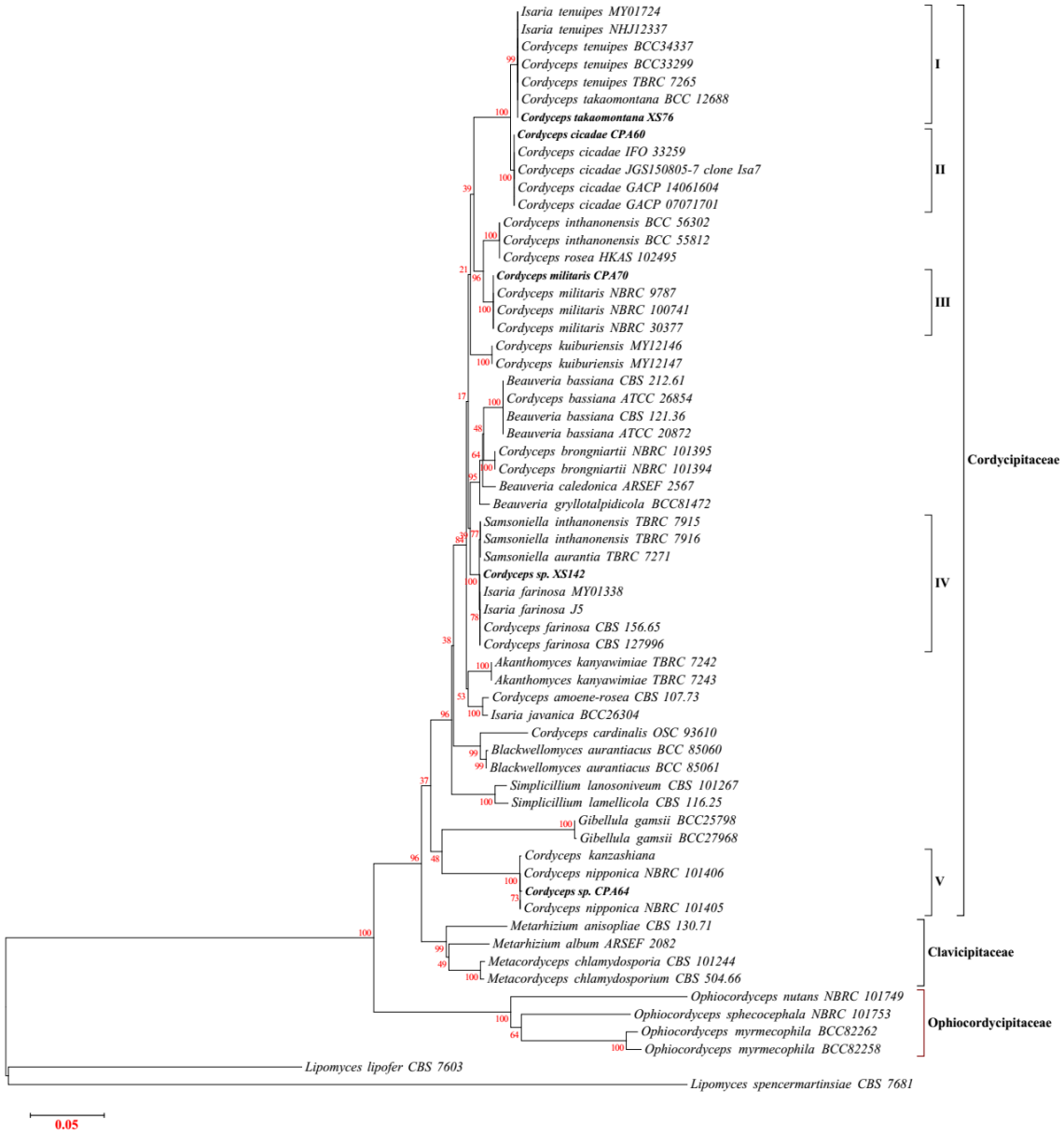

Fig. 6. *Neighbor Joining phylogenetic tree of studied taxa based on the combination of ITS, LSU and Rpb1 sequence analysis*

4. Conclusion

The detail descriptions of the five *Cordyceps* samples collected in Xuan Son, Phu Tho province and Copia, Son La province were presented. Based on morphological and molecular characteristics, three of the five were identified to species, *Cordyceps cicadae* CPA60, *C. takaomontana* XS76 and *C. militaris* CPA70. Of which *Cordyceps cicadae* was new record for fungal flora in Vietnam. The *Cordyceps* sp.XS142 and *Cordyceps* sp. CPA64 were unable to identified to species even the morphologies and DNA sequences of the three markers (ITS, LSU

and RBP1) were analysed. The two samples were possible new to sciences and further studies are needed.

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