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Molecular characterization, microscopic characteristics, and phylogenetic analysis of *Ophiocordyceps sinensis* from Sikkim, India

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ABSTRACT

The zombie fungi are a group of entomo-parasitic fungi comprising of 400 diverse species having tremendous pharmaceutical virtues. They have their use in traditional practice among Himalayan highlanders and inhabitants across the globe living in high altitude areas. *Ophiocordyceps sinensis* is one such representative of the entomo-parasitic group. The current study aimed to identify *Ophiocordyceps sinensis* from Sikkim, India, following classical, molecular taxonomic approaches, and culture. The classical approach involved a microscopic study of asci, stroma, and mycelia and the macroscopic characters of the stroma and larva. The molecular approach involved the amplification of internal transcribed spacer (*ITS*) region from the stroma, cytochrome oxidase subunit-I (*COI*), and cytochrome *b* (*Cytb*) from host larva for phylogenetic studies. The pure culture was established on potato dextrose agar (PDA). The sequences were edited with Bioedit version 7.2.5 and subjected to multiple alignments using fast fourier transform (MAFFT) database. Model testing was performed using MegaX version 10.2.5, and the best model was utilized to construct the maximum likelihood tree. To confirm the results of the maximum likelihood tree, a Bayesian tree was also constructed using MrBayes 3.2.7. Subsequently, the study confirmed that the collected specimen is *O. sinensis.* The main bioactive compounds of *O. sinensis* are cordycepin and adenosine which has been explored for different therapeutic applications including treatment of cancer, diabetes, anemia, inflammation etc. Thus, such study provides the platform for their exploration for extensive pharmaceutical and nutraceutical future studies.

1. Introduction

Ophiordyceps sinensis (Berk.) is an entomo-pathogenic fungus belonging to the genus *Ascomycota* [Zhou et al. (2014)]. It consists of >400 different species worldwide, which are parasitic, mainly on insects and larvae. Typically, it exists in two stages: an asexual stage (mitosporic fungi) and a sexual stage. The mitosporic fungi are parasitic on dead caterpillars of the moth *Hepialus* spp. The spores of *O. sinensis* germinate inside the caterpillars, colonizing with hyphae and producing a stalked ascomata (sexual stage) [Zeng, W., Yi, D.H. and Huang, T.F. (1998), Pu, Z.L. and Li, Z.Z. (1996)]. *Cordyceps* are mainly found in China, Nepal, and India at 3500 m above sea level. The fungus is a coveted medicinal utility in traditional Tibetan and Chinese medicine, which is commonly known in the West as "Himalayan Viagra." In India, the collection and trade of *Cordyceps* by the Bhotiya community have been reported from Garhwal, Uttarakhand [Caplins, L. B. (2016)]. It has also been reported

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from the hilly region of district Pithoragarh (Uttarakhand) at an altitude of 3200 m from the snow meadows of Brahamkot, Ultapara, Ghawardhappa, Chhipalakot, Najari in Dharchula, Chetri Bugyal, and ChiplaKedar (4000 m), as well as from Nagin Dhura, Ralam Bugyal at the base of Panchachuli Hills, Laspa, Tolatop, Darti, Mapa top, Burfu top, and Milam top in Johar Hills of Kumaun [Arora, R. K., (2014)]. Sikkim is the organic state of India, comprising a vast diversity of flora and fauna. The distribution of *O. sinensis* has been reported from Lachen (ca.2750 masl; 27.7167°N;88.5577°E), Lachung (ca.2700 masl; 276891°N;88.7430°E) in North Sikkim, and Gnathang valley (27.2986° N, 88.8173° E) in East Sikkim [Risley, H. H. (1894)]. *Lachenpas* and *Lachungpas* are primary dwellers of North Sikkim with a population of 3200 (Lachen) and 2495 (Lachung), according to the panchayat register of 2017 [Pradhan, B. K. et al. (2020)]. The Himalayan rural population relies on herbal medicinal plants and fungi for 3–58 percent of total yearly household income and 78 percent of cash revenue [Pradhan, B. K. et al. (2020)]. *Cordyceps* spp. has long been used to promote longevity, relieve exhaustion, and treat numerous diseases in Chinese traditional medicines [Russell, R.; Paterson, M. (2008)]. Recent studies have demonstrated that various species in this genus possess multiple pharmacological properties, including anti-tumor, anti-microbial, anti-inflammatory, and immunomodulatory effects [Holliday, J.; Cleaver, M. (2008), Agrawal, D. G., & Sandhu, S. S. (2020)].

The present study defines the taxonomic virtues of the collected sample, identified as *O. sinensis* by both classical and molecular taxonomy and characterized by mycelial culture. Also, the host larva of the *Cordyceps* spp. was characterized. Thus, this study could be significant for distinguishing *O. Sinensis* from-related *Cordyceps* spp. These features could be a measure for their systematic use as nutraceuticals for treatments and beneficial effects. The study can also be a tool to identify counterfeits to minimize or regulate the trade of *O. sinensis*. Therefore, the current study claims to be the first elaborate taxonomic study on *O. sinensis* from Sikkim.

2. Materials and Methods

Samples were collected from Yumesamdong (27°51'05.4"N 88°41'04.8"E), North Sikkim, in July 2021 and transported on an icebox to Bodoland University. Photographs were taken before and after cleaning the samples. DNeasy plant genomic, PCR purification, and gel extraction kits were purchased from Qiagen, Germany. Wizard SV Genomic purification system (Promega,USA) was obtained from the USA. Taq Polymerase was procured from ThermoFischer, USA, and 100-bp ladder was purchased from TaKaRa, Japan.

Isolation and Culture

The samples were cleaned with a sterile art brush and detached from the larva, followed by washing in sterile distilled water. 0.1% mercuric chloride was used for surface sterilization. Subsequently, the samples were cut vertically using a sterilized scalpel. The tissue from the center of the fruiting body was inoculated on potato dextrose agar (PDA) with 0.05% $MgCl_2$ [Barseghyan, G. S. et al. (2011)]. The culture plates were incubated at 18 °C in a Bio-oxygen Demand (BOD) incubator. Mycelial growth was observed after 4-5 days. In order to obtain pure culture, small chunks of mycelia were sub-cultured on PDA plates containing $MgCl_2$ and 500 mg/L ampicillin. The mycelia were then transferred to liquid media for DNA extraction to confirm the culture.

Microscopic Characteristics

The microscopic characteristics, such as the total diameter of the transverse section of stroma and the length and breadth of the asci and mycelia, were studied [Liu, H et al. (2011)].

DNA Barcoding Analysis

The genomic DNA of the sample was extracted from stroma (fruiting body), mycelia, and host (larva), separately. The DNA isolation from stroma and mycelia was carried out using the DNeasy plant genomic kit (Qiagen), and the isolation from the host was carried out with the Wizard SV Genomic purification kit (Promega) with some minor modifications. The DNA samples were quantified on Qubit4 fluorometer (Thermo Fisher Scientific). The genomic DNA was visualized on 0.8% agarose gel electrophoresis. The primers used for rDNA internal transcribed region (*ITS*) amplification were as follows: forward primer *ITS-5* (5'-GGAAGTAAAGTCGTAACAAGG-3') and reverse primer *ITS-4* (5'-TCCTCCGCTTATTGATATGC-3') [Wu, D. et al. (2016)]. The amplification of the cytochrome region from the genomic DNA of host larva was carried using forward primer *Cytb-1* (5'-TATGTACTACCATGAGGACAAATATC-3') and reverse primer *Cytb-2* (5'-ATTACACCTCCTAATTTATTAGGAAT-3') [Quan, X., & Zhou, S. L. (2011) and Wu, D. et al. (2016)]. The amplification of *COI gene* used forward primer *COI-F* (5'-GGTCAACAAATCATAAAGATATTG-3') and reverse primer *COI-R* (5'-TAAACTTCAGGGTGACCAAAAAAT3'). The polymerase chain reaction (PCR) for the *ITS* region of the genomic DNA from stroma (fruiting body) was as follows: an initial denaturation step of 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 51 °C for 2 min, and 72°C for 1 min, and a final extension step of 72 °C for 10 min. The PCR conditions for the amplification of *Cytb* gene of host larva consisted of an initial denaturation step of 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 50 °C for 2 min, and 72 °C

for 1. min, and a final extension step of 72 °C for 10 min. The PCR amplification of *COI* gene of host larva consisted of an initial denaturation step of 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 48 °C for 2 min, and 72°C for 1 min, and a final extension step of 72 °C for 10 min [Wu, D et al. (2016)]. The amplification products were purified using the Qiaquick PCR purification kit (Qiagen, Germany) and confirmed on 1.5% agarose gel electrophoresis with a 100-bp ladder. The fragments were excised and purified using QIAEX gel extraction kit (Qiagen), and the concentration was measured on a Qubit 4 Fluorometer (Invitrogen, USA). Subsequently, the DNA was used for sequencing in a AB13730XL, Applied Biosystem Sequencer following sanger sequencing method. The *ITS*, *Cytb*, and *COI* sequences were subjected to homology search by BLAST tool in the NCBI nucleotide blast portal (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE =BlastSearch). Finally, the sequences were submitted, and the accession number was obtained from the NCBI database

Phylogenetic analysis

Sequences were edited using BioEdit version 7.2.5. Phylogenetic analysis was performed using MegaXversion 10.2.5, and reference sequences were downloaded from NCBI. The evolutionary history was obtained using the maximum likelihood method and Kimura 2-parameter model [Kimura, M. (1980)]. The tree with the highest log likelihood (-3549.73) is shown in figure-2. The percentage of trees in which the associated taxa are clustered together is shown next to the branches. The initial tree(s) for the heuristic search were obtained by applying the neighbor-joining (NJ) method to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. A discrete gamma distribution was used to model the evolutionary rate differences among the sites (5 categories (+*G*, parameter = 1.1526)). This analysis involved 23 nucleotide sequences and a total of 880 positions in the final dataset. The evolutionary analysis was conducted in MEGA X10.2.5 [Kumar S et al, 2018].

3. Results and Discussion

The collected samples were submitted to Sikkim State Forest Herbarium (SSFH), Deorali, Sikkim vide (SSFH SK005007) and cultured in Petri plates containing PDA media supplemented with 0.5 g/L and 50 mg thiamine hydrochloride. The mycelia started to grow after 5–7 days, followed by a subculture to obtain a pure culture of the sample. Then, the collected samples were cleaned, and DNA was extracted (DNeasy Plant Genomic kit, Qiagen). The DNA from stroma, pure-cultured mycelia, and host larva was isolated independently and amplified on a Thermal Cycler (2720, Applied Biosystems). The PCR product of *ITS*, *COI*, and *Cyt b* amplification was 541 bp, 650 bp, and 419 bp, respectively. These were then sequenced (AB13730XL, Applied Biosystem Sequencer) following sanger sequencing method. The sequencing data of stroma, mycelia, and larva were blasted in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGETYPE=BlastSearch). The *ITS* amplicon of sample *Cordyceps* Bodoland University Sikkim-3 (CBUS3 fruiting body and culture) covered a query of 97% and 99%, which is identical with *O. sinensis*. The sequencing data of *COI* gene had query cover of 100% and 93% identity to *Thitarodes* spp, while *Cytb* gene had a 92% query coverage and 96% identity to *Thitarodes* spp. The presence of grouped, cylindrical, and embedded asci confirmed *O. sinensis*. The phylogenetic analysis of the *ITS* region of stroma and *COI gene* of the host was performed separately. A total of 22 sequences of *Cordyceps* spp. were retrieved from NCBI for the phylogenetic analysis of the *ITS* region of the stroma, and 19 sequences were retrieved for the phylogenetic analysis of the *COI* region of the host. The sequences were subject to multiple alignment using fast fourier transform (MAFFT) online tool. The alignment was optimized visually, and ambiguous regions were excluded from subsequent phylogenetic analyses. The best model was calculated by the model testing in Mega (version 10.2.5), and the K2+G model was chosen [Kimura, M. (1980)]. Pairwise distance matrices were generated using Kimura models of nucleotide substitutions [Kimura, M. (1980); Kumar S. et al. (2018); Swofford, D. L. (1998)], and the phylogenetic analysis was performed in the Mega version 10.2.5. An NJ tree [Jukes, T.H. and Cantor, C.R. (1969)] with bootstrapping was constructed with distance measured by the Jukes Cantor distance [Jukes, T.H. and Cantor, C.R. (1969)] model and Kimura's two-parameter distance. To assess the confidence of phylogenetic relationships, the bootstrap test [Felsenstein, J. (1985)] was conducted with 1000 resampling for NJ analysis. The phylogenetic relationships of *O. sinensis* were analyzed using the Bayesian method [Ronquist, F. et al. (2012)]. *Volvariella volvacea* is used as an outgroup for phylogenetic analysis of the *ITS* region and *Anthera assama* as an outgroup for *COI* region of host larva. The comparative phylogenetics results using *ITS* sequence indicated that the presence of the sample collected from Sikkim clubbed with the sequences of *O. Sinensis* sequences (China) retrieved from NCBI. The presence in the same clade diverging from the other related species confirmed that the sample belong to *O. Sinensis*, which is an entomo-parasitic fungus infecting larva of various genera (*Thitarodes* spp., *Endoclita* spp., *Napialus spp.*). The phylogenetic analysis was performed to confirm the larva. The sequence of *COI* of larva from the sample was compared to that of the common larva of *O. Sinensis.* The sequences were retrieved from NCBI, and phylogenetic analysis was carried out. Subsequently, the sample clubbed and grouped

with the sequences from *Ahamus* spp., *Hepialus* spp., and *Thitarodes* spp., which are synonyms, confirmed that the larva belongs to *Thitarodes* spp. The tree from Bayesian inference showed an identical tree topology. The recent studies have shown cordycepin from *cordyceps* spp. are capable of reducing pain, inflammation and joint pathology in rodent model [Ashraf, S, et al. (2019)] also cordycepin have been known to be involve in activation of AMPK and induction of apoptosis in prostate carcinoma cells [Hawley, S. A, et al. (2020); Lee, H. H. et al. (2013); Zhang, Y.et al. (2018); Li, S. Z. et al. (2019)]. Although *O. sinensis* has been reported, a detailed microscopic and phylogenetics analysis of *O. sinensis* from Sikkim is reported in this study. Thus, this study will be helpful in properly characterizing and differentiating different species of *cordyceps* which have diverse therapeutic potential.

4. Conclusions

The collection and trade of *O. sinensis* in Lachung and Lachen valley of North Sikkim is a common practice for livelihood management [Pradhan, B. K. et al. (2020)]. Traditional use of *O. sinensis* as aphrodisiac, treating fatigue, anti-inflammation, immune booster and treatment of respiratory ailments is a practice of choice by the traditional healers in Himalayan region [Panda, A. K., & Swain, K. C. (2011)]. A detailed scientific characterization and authentication of the collected sample of *O.sinensis* shall facilitate differentiation between other counterfeit species and establish the collected *Cordyceps spp.* from Sikkim to be *Ophiocordyceps sinensis*.

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6. Conflict of Interest

The authors declare no conflict of interest.

7. References

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Figure Legends

Fig 1 (A) *O. sinensis*. (B) *O. sinensis* cleaned. (C) *O. Sinensis*larva with 8 pairs of the legs on the abdomen and 4 pairs at the center. (D) Transverse section of the stroma. (E) Perithecia at 20x (F) Perithecia at 40x□ (G) Mycelial culture of *O. sinensis* grown on PDA media. (H) Mycelia of *O. sinensis*. (I) Central portion of the stroma.

Fig 2. Maximum likelihood tree for the *ITS* region of CBUS3.

Fig 3. Maximum likelihood tree for the *COI* region of CBUS3.

Table 1. Details of macro-morphological characters of *O. sinensis*

Species	Microscopic characteristics of stroma	Morphological characteristics of larva	Morphological and microscopic characteristics of mycelia

O. sinensis (CBUS3-Stroma), *Thitarodes sp.* (CBUS3-larva), *O. sinensis* (CBUS3-mycelia)	Stroma sparingly cylindrical, dark pinkish (fresh) and brown when dried, 38–40 mm in length, and about 2.5–3 mm in diameter. The total diameter of the transverse section is 298 µm, length and breadth of the asci were 84 µm and 33 µm, respectively. Perithecia oval to elliptical, elongated, and grouped at the fertile portion of stroma. Mycelia embedded with the asci. The outer layer of the stroma stained dark with Congo red.	Larva body resembling a silkworm, 29–32 mm in length and 4–5 mm in breadth. Yellowish in color with 8 pairs of the leg.	Light-creamish. Mycelia branched with 3-µm diameter.
Gene Bank Accession Number	MW990119	MZ956161	OK041477

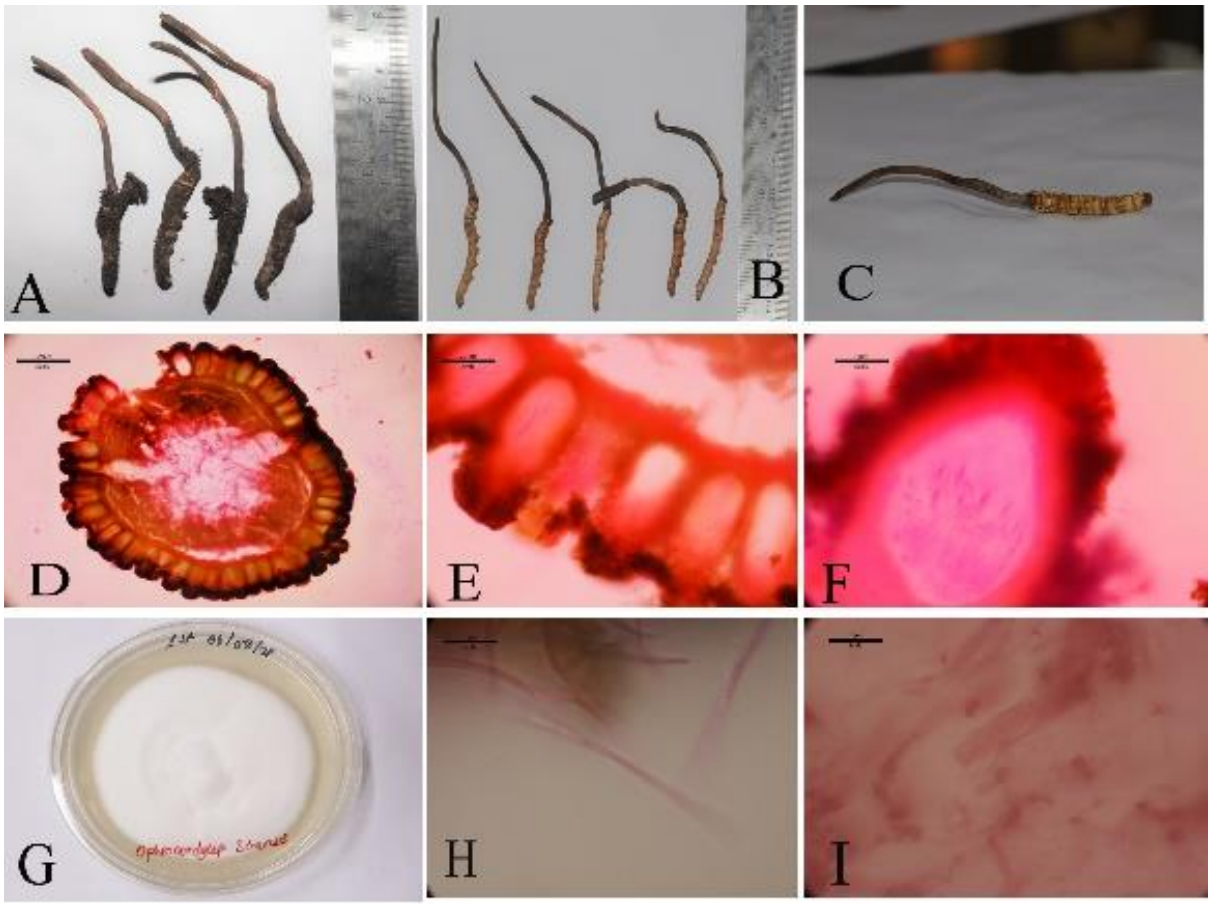


Fig 1. (A) *O. sinensis*. (B) *O. sinensis* cleaned. (C) *O. Sinensis*larva with 8 pairs of the legs on the abdomen and 4 pairs at the center. (D) Transverse section of the stroma. (E) Perithecia at 20x (F) Perithecia at 40x□ (G) Mycelial culture of *O. sinensis* grown on PDA media. (H) Mycelia of *O. sinensis*. (I) Central portion of the stroma.

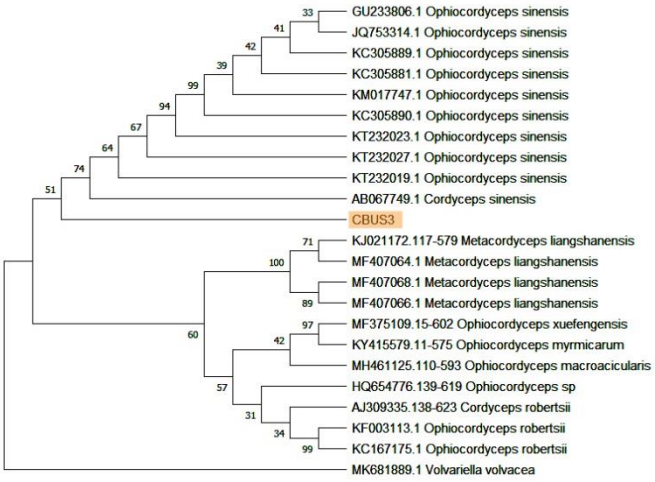


Fig 2. Maximum likelihood tree for the *ITS* region of CBUS3.

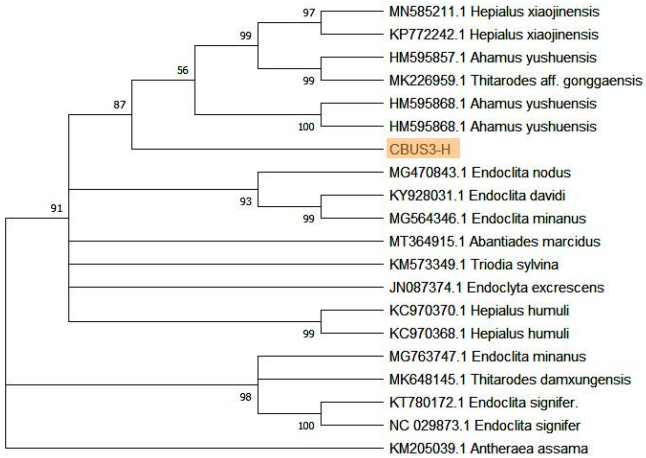
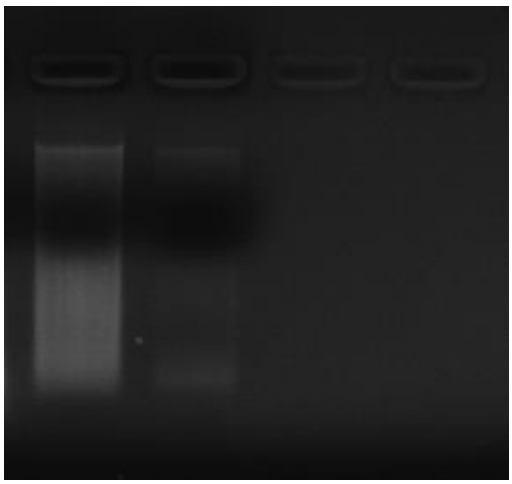
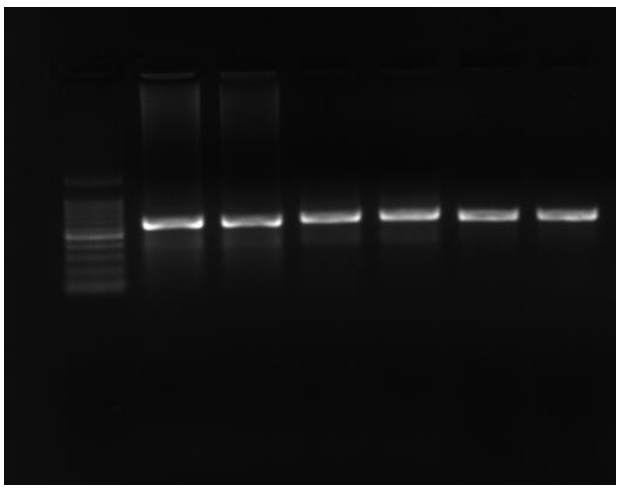


Fig 3. Maximum likelihood tree for the *COI* region of CBUS3.

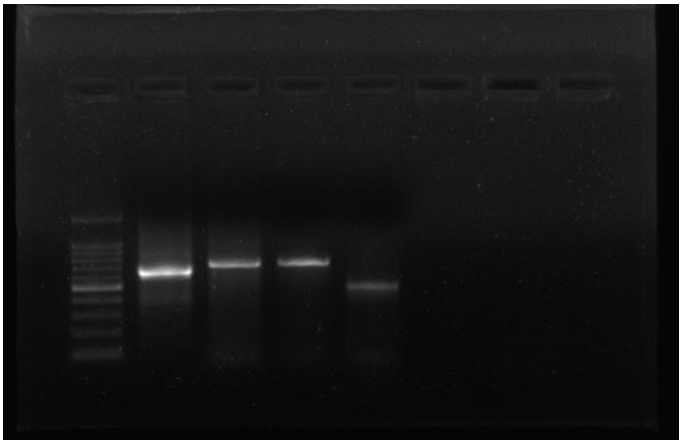
Supplementary material



Supplimentary Fig 1. Genomic DNA of CBUS3



Supplimentary Fig 2. PCR amplicon of *ITS* region of CBUS3 L1- 100 bp ladder, L2-L7- PCR product



Supplimentary Fig 3. PCR amplicon CBUS3 L1- 100 bp ladder, L2- *COI gene*, L3, L4- *ITS gene*, L5- *Cytb gene*

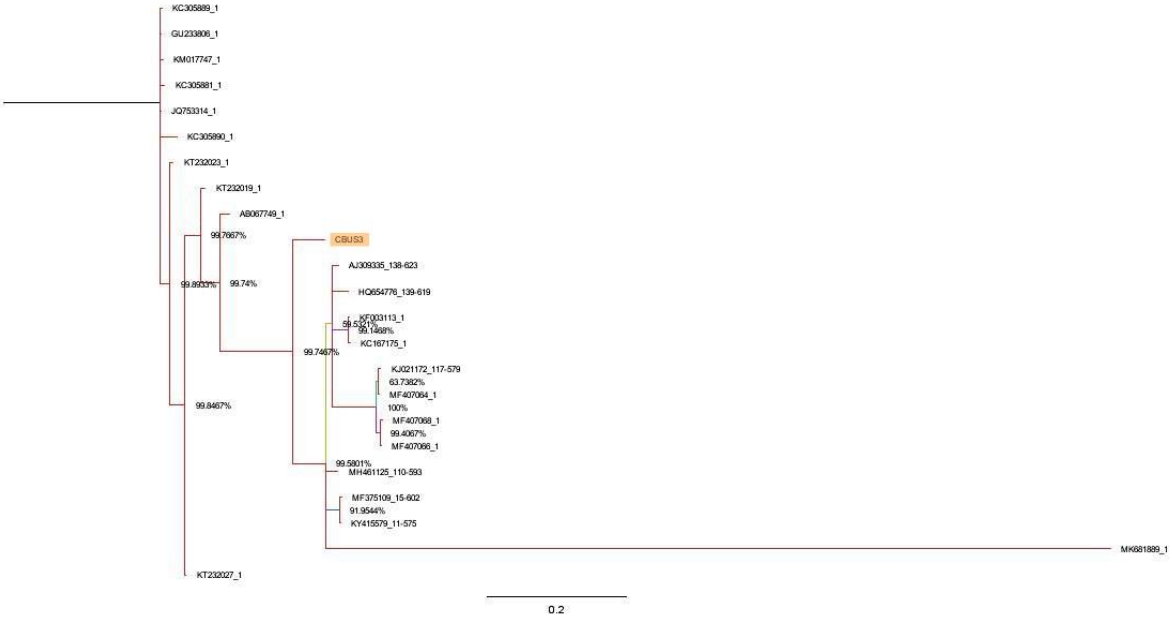


Fig 4. Bayesian tree of *ITS* CBUS3 constructed of Mr Bayes version 3.2.7a