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continued on the back inside cover

Cover: A bag worm with its beautiful heap of junk. Acrylics on 300 GSM paper by Dupati Poojitha based on a picture by Sanjay Molur.

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SHORT COMMUNICATION

Blackwellomyces pseudomilitaris (Hywel-Jones & Sivichai) Spatafora & Luangsa-ard, 2017 (Sordariomycetes: Hypocreales: Cordycipitaceae): first report from Western Ghats of India

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Abstract: A rare entomopathogenic fungus *Blackwellomyces pseudomilitaris* (Hywel-Jones & Sivichai) Spatafora & Luangsa-ard on lepidopteran larva is described for the first time from India. This fungus was formerly known as *Cordyceps pseudomilitaris* Hywel-Jones & Sivichai. Morphological, microscopic, and cultural characteristics with molecular identification has been discussed.

Keywords: Cordyceps, cultural studies, first record, fungal pigment, ITS sequencing, medicinal mushroom, phylogeny, taxonomy,

Cordyceps, a medicinal mushroom, is highly valuable in the market. There are two *Cordyceps* species in the market: *Ophiocordyceps sinensis* (Berk.) G.H.Sung, Hywel-Jones & Spatafora and *Cordyceps militaris* (L.) Link. Others include *Metacordyceps liangshanensis* (M.Zang, D.Liu & R.Hu) G.H.Sung, J.M.Sung, Hywel-Jones & Spatafora and *Ophiocordyceps nutans* (Pat.) G.H.Sung, Hywel-Jones & Spatafora. These formulations are available in the Chinese market and consumers are searching for more effective alternatives (Zha et al. 2018). *Cordyceps* species has gathered attention for its immunostimulatory properties. Numerous species exhibit biological actions such as anti-oxidant, anti-cancer, anti-hyperlipidemic, anti-diabetic, anti-fatigue, anti-aging, hypo-cholesterolemic, hypotensive, vasorelaxation, anti-depressant, aphrodisiac, and kidney protectant (Das et al. 2021). The first record of *C. pseudomilitaris* came from Thailand (Hywel-Jones 1994). Isaka et al. (1999) mentioned that *C. militaris* is known to produce several secondary metabolites including nucleosides antibiotic cordycepin, while from *C. pseudomilitaris* Cordyanhydrides A and B have been detected. Diversity of *Cordyceps* from Maharashtra is not well known, however Pande (2001) listed three species of *Cordyceps*, viz., *C. militaris, C. forquignoni*, and *C. unilateralis* from Maharashtra State. Fungi from India database (www.fungifromindia.com) shows no record of the genus *Blackwellomyces* from India till date.

Blackwellomyces was proposed as a new genus on the basis of ascospores which are irregularly septate, but do not separate into part-spores. In contrast, septation and disarticulation are frequent in other family members. Some species of *Cordyceps* due to irregular septation and no separation into part spores were transferred to the genus *Blackwellomyces*, viz., *B. pseudomilitaris* and *B. cardinalis* (Kepler et al. 2017). Genus *Blackwellomyces* is not recorded from India (Manoharachary et al. 2022).

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During the present study, mature fruiting bodies of *B. pseudomilitaris* were collected on lepidopteran larva from Pachal, Ratnagiri District, Maharashtra, India. Morphological and microscopic identification was carried out. Isolation of *B. pseudomilitaris* into pure cultures, ITS rDNA identification, and evaluation of the phylogenetic relationship have been completed.

Material and Methods

a) Collection and morphological analysis: The specimen was collected from Pachal, Ratnagiri District, Maharashtra, India on an unknown lepidopteran larva. Morphological studies and microscopic observations were conducted with a Lawrence and Mayo N-300M research microscope.

Status of the genus from India and the world: The genus *Blackwellomyces* has been recorded from different regions of the world – Brazil, Papua New Guinea, United States of America, Colombia, Thailand, Dominica, Cuba, Czechia, South Africa, Estonia, Ecuador, Puerto Rico, Japan, and Korea (GBIF.org 2024). There is no record of the genus *Blackwellomyces* from India until this study.

b) Isolation: Pure cultures were obtained on SDAY (Sabouraud Dextrose Agar with Yeast Extract), PDA, and PDA +chicken egg yolk. Before inoculation stromata were surface sterilized with 0.1% $HgCl_2$ (Table 1).

c) DNA extraction, PCR amplification, and sequencing: Pure cultures obtained were used for DNA extraction (Aamir et al. 2015); 30 mm colonies were crushed with liquid nitrogen and the powder was treated with 1 ml lysis buffer (100 mM Tris HCl [pH 8.0], 50 mM EDTA, 3% SDS). Shaking was done by inverting the tube and centrifuging at 10,000 rpm for 10 minutes. The supernatant was taken in a new Eppendorf tube and an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) (PCI) was added and centrifuged at 10,000 rpm for 10 minutes. The aqueous layer was separated in a new Eppendorf tube and an equal volume of Chloroform: Isoamyl alcohol (24:1) (CI) was added and centrifuged at 10,000 rpm for 10 minutes. The upper aqueous layer was separated in a new Eppendorf tube and an equal volume of 100% ethanol was added. It was kept at -20 °C for 20 minutes and centrifuged at 10,000 rpm for 10 minutes at 4 °C. The pellet was washed with 500 µL 70% ethanol and centrifuged at 10,000 rpm for 5 minutes at 4 °C. The pellet was dissolved in an elution buffer. 2 µl DNA was subjected to 0.6% agarose gel electrophoresis. It was observed under a gel documentation system and quantity was measured by nano-300 micro-spectrophotometer. For PCR and sequencing of DNA, the samples were sent to PEON laboratories, Kolhapur, India. Sequence was edited by BioEdit 7.2 software and Phylogram was obtained with MEGA 11 software.

Results and Discussion

Stromata 25–65 mm long and up to 1–5 mm wide, solitary, unbranched or branched arising directly from the head of the Lepidopteran larva. Stipe 10–35 mm long & 1–3 mm wide fleshy, brittle, flexible, solid, yellow to orange towards apex; whitish-cream towards the base. Stroma 12–25 mm long and 1–3 mm wide, cylindrical, often flattened, with blunt apex, yellow to orange, often bright orange towards apex. Perithecia superficial, immersed in the base, apex prominent, elongated-ellipsoid or elongate-ovoid, 289–574 x 122–241 µm with hyaline walls. Unitunicate ascus with ascus cap; asci 210–395 × 5–6 µm; eight ascospores not breaking into part-spores (Image 1a–f).

Collection examined: India, Maharashtra, District Ratnagiri, Tehsil Rajapur, Pachal (16.7038 ^{0}N, 73.7211 ^{0}E), on larvae buried in soil, 11 August 2022; Snehal Biranje & Yogesh Patil.

Remarks

Blackwellomyces pseudomilitaris was collected on Lepidopteran larva covered by hyphae around the dead diseased larvae that gathered into a loose network of rhizomorph like structures. *C. pseudomilitaris* was discovered from in the deciduous monsoon forest of Sam Lan National Park, Thailand. The species looked similar to *C. militaris* but on the basis of some distinguishing morphological characters, it was described as *C.*

Table 1. Isolation of *Blackwellomyces pseudomilitaris* on different media.

Name of medium	Granulated PDA	Dextrose	Peptone	Agar type I	Yeast extract	Magnesium sulphate	Egg yolk (Chicken)
SDAY	-	20 g/L	5 g/L	15 g/L	5 g/L	0.3 g/L	-
PDA	39 g/L	-	-	-	-	0.3 g/L	-
PDA + egg yolk	39 g/L	-	-	-	-	0.3 g/L	25 ml

SDAY—Sabouraud dextrose agar with yeast extract | PDA—Potato dextrose agar | PDA + egg yolk—Potato dextrose agar + egg yolk.

pseudomilitaris. The ground-dwelling host lepidopteran larvae were often found 2–5 cm below the soil surface. The hyphae around the dead, diseased larvae gathered into a loose network of rhizomorph-like structures that encircled the caterpillar. These structures developed independently throughout the soil, periodically coming together and then splitting apart once more, until combining to create the stroma at the surface (Hywel-Jones 1994), stromata 12–25 mm long, rhizomorphs present, ascospores do not split into part-spores and asci 210–395 × 5–6 µm (Catania et al. 2018). The collections from the present specimen shows similarities to it with respect to morphological characteristics. *C. militaris* has been more frequently found on pupae of lepidopterans than the larvae. However, some researchers noted that *C. pseudomilitaris* was found only on the larvae (Mains 1958; Hywel-Jones 1994). *C. militaris* is usually found on pupae of many distinct families of moths. Contemporary molecular data also argues that *C. pseudomilitaris* distinct from *Cordyceps militaris* (Artjariyasripong et al. 2001). The microscopic characteristics of the non-disarticulating ascospore and host preference for lepidopteran larva of *C. pseudomilitaris* contrasts with the characteristics of *C. militaris* but resemble *C. cardinalis* (Sung & Spatafora 2004). The distinctive characteristics such as ascospores with irregularly spaced septa and non-disarticulating part-spores are used to identify *Blackwellomyces.* Two combinations are made in the genus *Blackwellomyces* i.e., *B. cardinalis* and *B. pseudomilitaris* (Kepler et al. 2017).

Cultural characteristics

The pure colonies isolated on PDA and SDAY are circular, white, umbonate with irregular margin and formed within two days of inoculation and achieving 20–30 mm diameter, release red pigmentation in the medium similar growth observed on PDA + egg but faster than PDA and SDAY (Image 1: g–l).

Previously some workers isolated *B. pseudomilitaris* into pure culture on PDA and MCM (Mushroom complete medium) which produced red pigments in two conditions, shaking and static (Sutthisa & Sanoamuang 2014). The production of reddish pigments diffusing in the agar medium and it can be used to identify species such as *B. aurantiacus*, *B. roseostromatus,* and *B. cardinalis*. *B. calendulinus*, *B. minutus*, and *B. pseudomilitaris* do not produce reddish pigments in agar medium (Mongkolsamrit et al. 2020). In the present study, there is secretion of red pigment by *B. pseudomilitaris* into the medium.

Phylogenetic analysis

Morphologically the present specimen shows similarities to *Cordyceps militaris* with some minor differences. The cultures released a red pigment in the medium. The 564 bp sequence obtained was deposited in the GenBank at the National Center for Biotechnology Information (NCBI) with Accession no. OR259389. GenBank BLAST search sequence showed 98.57% similarity with *Blackwellomyces pseudomilitaris* (MT000700) and 98.55 % similarity with *Cordyceps*

Figure 1. Collections of the genus *Blackwellomyces* from around the world (© GBIF | Global Biodiversity Information Facility).

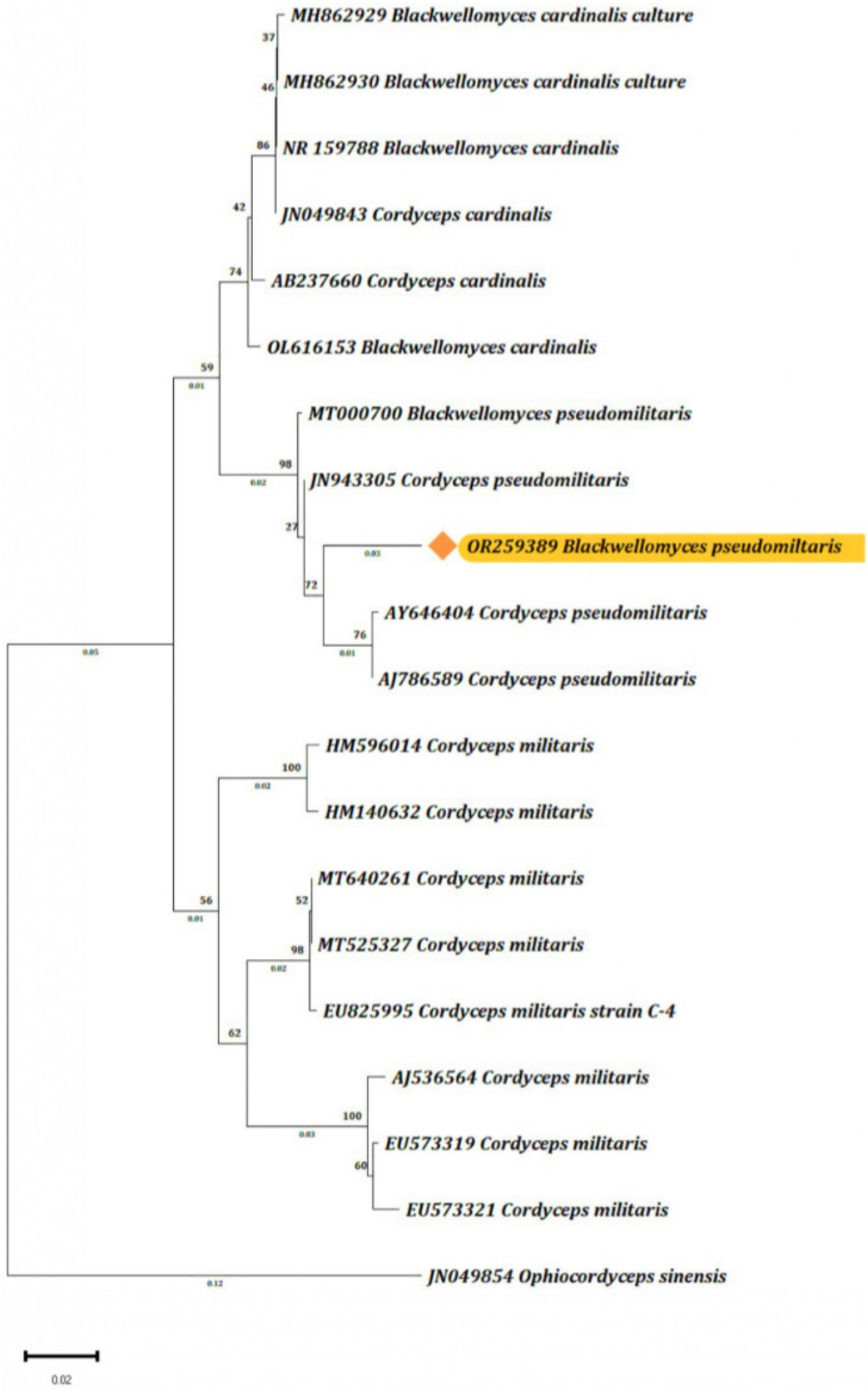

Figure 2. Phylogenetic tree of *Blackwellomyces pseudomilitaris* and other *Cordyceps* species based on rDNA internal transcribed spacer (ITS) sequences with neighbor-joining method with Kimura 2-parameter model with rapid bootstrapping algorithm of 100 replicate and *Ophiocordyceps sinensis* used as an outgroup.

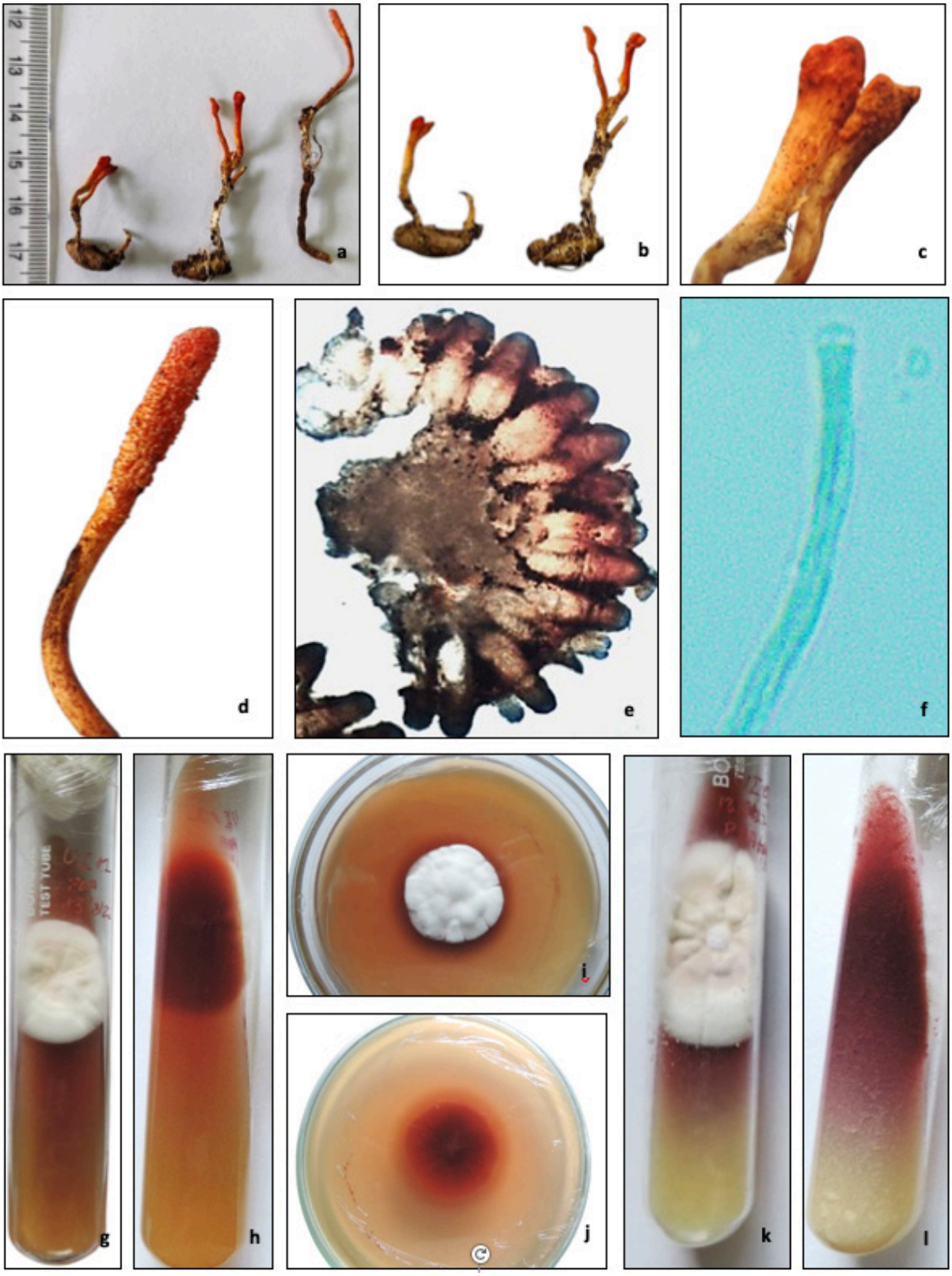

Image 1. ***Blackwellomyces pseudomilitaris*** **(Hywel-Jones & Sivichai) Spatafora & Luangsa-ard: a–b—Habit | c–d—Stromata with semi superficial perithecia | e—Perithecia 10X | f—Ascus 100X | g–h—Pure cultures growing on PDA | i–j—Pure cultures growing on SDAY | k–l—Pure cultures growing on PDA+ egg. © Snehal Biranje.**

pseudomilitaris (JN943305). For phylogenetic analysis, all available ITS rDNA sequences of reference such as *B. pseudomilitaris, B. cardinalis*, and *Cordyceps militaris* were retrieved from GenBank. *B. pseudomilitaris* (MT000700) and *C. pseudomilitaris* (JN943305) show maximum match which indicates that the isolated strain is *Blackwellomyces pseudomilitaris.* All retrieved sequences were aligned using the MEGA11 program. Phylogenetic tree was constructed using the neighbor-joining method with Kimura 2-parameter model in MEGA11 software. Bootstrap analysis was performed with 100 replications to determine and support the match (Figure 2).

Microscopic, cultural, and molecular data clearly indicate that *C. militaris* and *B. pseudomilitaris* are phylogenetically separate species. The present collection shows affinity towards *B. pseudomilitaris*. Thus, this makes a new record to the fungi of India. As it shares a close relation to *Cordyceps militaris* which is one of the important medicinal fungus, further studies will result in exploring the medicinal potential of the present specimen.

Conclusion and future prospective

B. pseudomilitaris is recorded for the first time from India. This species has been only reported from Thailand. It clearly indicates that, it is an extremely rare species. Morphology, microscopy, cultural studies, and ITS rDNA sequencing confirms the identity of the species.

Even though *C. militaris* and *C. cardinalis* show morphological similarity the molecular sequence shows the highest similarity with *B. pseudomilitaris*. Further biochemical characterization of cultures will lead to knowledge about its biological potential.

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