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SHORT COMMUNICATION

A new record of edible *Coprinopsis cinerea* (Family: Psathyrellaceae) naturally growing on arecanut husk in India

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ABSTRACT

A new record of edible *Coprinopsis cinerea,* naturally growing on arecanut husk, is being reported based on morphological and multi-gene-based molecular investigations. Morphological observations recorded a small, edible, culturable, short-lived mushroom on the arecanut husk. Multi-gene phylogenetic analysis of nuclear ribosomal internal transcribed spacer (nrITS) and nuclear ribosomal large subunit (nrLSU) DNA resulted in precise identification of the naturally growing mushroom as *Coprinopsis cinerea* for the first time on arecanut husk in India.

Keywords: Arecanut husk; Basidiomycota; Psathyrellaceae; Multi-gene phylogeny

INTRODUCTION

Arecanut (*Areca catechu* L., Arecaceae) holds significant agricultural importance in India as a key plantation crop, primarily cultivated for its kernel derived from the fruit. This crop is the primary source of betel nut, or '*supari*', mainly used as a masticatory. Beyond its agricultural role, arecanut carries economic, socio-cultural, religious, and medicinal significance in India and other South-East Asian nations. The palms, characterized by tall and upright growth, feature typically unbranched stems, reaching heights of 12-30 meters and diameters ranging from 30- 45 cm (Nagaraja et al., 2018).

The kernel possesses economic value and is enveloped by a lignocellulose husk, constituting a substantial proportion (65-80%) of the fruit's total fresh weight and volume. The lignocellulosic husk primarily comprises hemicellulose (35.0-64.8%), lignin (13.0- 26.0%), furfuraldehyde (18.8%), pectin (1.5-3.6%), and protopectin (1.5-2.1%) (Baruah et al., 1957; Govindarajan, 1968). Although less utilized, the husk holds potential for various applications, including as a fiber source. The fibers present in the inner layer form irregularly lignified cell groups known as 'hard fibers,' while soft fibers are situated in the middle layer below the outermost layer. Despite an annual availability of around 0.5 million tons of arecanut husks in India, their utilization is currently limited.

Macrofungi generate succulent fruiting bodies, commonly referred to as 'mushrooms,' which are abundant sources of proteins and provide outstanding nutritional benefits. The cultivation of mushrooms, a long-standing practice, frequently employs agricultural waste materials like wheat straw, rice bran, paddy straw, banana leaves, sawdust, and coffee waste as substrates to support their growth. Commercially, the arecanut leaf sheath has been utilized for cultivating oyster mushrooms (*Pleurotus sajor-caju*) (Chandramohanan and Moorthy, 1991; Moorthy, 1991).

Recently, we have encountered mushrooms that naturally grow on the arecanut husk in farmers' fields around the Sullia region of Dakshina Kannada District of Karnataka State, India (Fig. 1A-C). Locals have been consuming these mushrooms, which are extremely short-lived, for several

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Fig 1. (A) Farmer harvesting the mushroom growing on arecanut husk at the unopened stage in the evening hours for consumption purposes; (B) A cluster of mushroom growing in small groups on the arecanut husk; and (C) Basidiocarp with conical pileus, arising from the arecanut husk.

decades, immediately after harvest during evening hours. Despite this long-standing practice, there has been no systematic attempt to identify these naturally occurring mushrooms. The current study addresses this gap by systematically documenting macroscopic, microscopic, and molecular aspects through multi-gene analysis, specifically identifying these mushrooms.

MATERIALS AND METHODS

Sample collection

Fresh mushroom samples growing on arecanut husks from the farmer's field were collected in a sterile sample collection box and brought under laboratory conditions. The samples were subjected to morphological characterization using conventional tools as explained below.

Morphological studies

Conventional taxonomic tools were employed for the morphology-based identification of the specimen. Fresh basidiocarps were used to record the macroscopic features. The color of the different parts of the basidiocarps was recorded with the help of Kornerup and Wanscher (1978) (e.g., 4E1). The microscopic characteristics were recorded using thin sections obtained from different portions of the dried basidiocarps. The sections were stained with 1% aqueous solution of Congo Red and further mounted using 3% aqueous potassium hydroxide. The sections mounted in water were used to record the color and pigment topography of the microscopic structures. To assess the range of basidiospore size, at least 40 basidiospores obtained from the lamellae of each collection were measured for length, breadth, and width, respectively. Basidiospore measurements are represented as the length range × the breadth range × the width range. Q1 and Q2 values were determined as follows: Q1 = the length divided by the breadth, and Q2 = the length divided by the width (Ganga and Manimohan, 2018).

Molecular studies

Total genomic DNA was extracted from the pure culture of the mushroom using the Nucleospin Plant II DNA isolation kit (Macherey-Nagel, Germany) as per the protocol recommended by the manufacturer. To confirm the species identity, polymerase chain reaction (PCR) was performed with internal transcribed spacer (ITS) region of ribosomal DNA and nuclear ribosomal large subunit (nrLSU) DNA-specific primers, as given in Table 1.

PCR reactions were performed in 50 µl reaction volume containing 5.0 µl *Taq*A buffer (Genei, India), 2.0 µl each of forward and reverse primers (10 pmol µl-1), 2.0 µl of 10mM dNTP's, 2.0 µl DNA, 1.0 µl *Taq* DNA polymerase (Genei, India) and 36.0 µl sterile distilled water. PCR was performed in a T100 Thermocycler (Bio-Rad, USA) with initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR amplified products were electrophoresed on a 1.2% agarose gel (Sambrook and Russel, 2001) and analyzed through Sanger's sequencing (Biokart India Pvt. Ltd., India).

The DNA sequences were end trimmed using the BioEdit tool (Biological sequence alignment editor: http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and further, nBLAST analysis was performed in National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) to confirm the species identity. The DNA sequences of ITS and nrLSU for the representative isolate were deposited to GenBank. The reference sequences available in the NCBI database were used for the phylogenetic analysis. *Pleurotus ostreatus* was used as an outgroup taxon. A phylogenetic tree was constructed for the concatenated sequences using ITS and nrLSU sequences. The phylogenetic tree was constructed using the Maximum Likelihood (ML) method with the Tamura 3-parameter model (Tamura, 1992) for nucleotide substitutions with gamma distribution and bootstrap of 1000 replicates in MEGA11 (Tamura et al., 2021).

Artificial cultivation

To obtain the pure culture, gills on the pileus region (n=5) from five different basidiocarps collected from the field conditions were cut into small pieces (2 mm × 5 mm),

Table 1: List of genes amplified with their primer sequences

SN	Name of the gene	Primer	Primer sequence (5'-3')	Reference
1	Internal transcribed spacer (ITS) region of ribosomal DNA (rDNA)	ITS1_F	TCCGTAGGTGAACCTGCGG	White et al. (1990)
		ITS4_R	TCCTCCGCTTATTGATATGC	
2	Nuclear large subunit of ribosomal DNA (nrLSU)	LR0R_F	ACCCGCTAACTTAAGC	Vilgalys and Hester (1990)
		LR6_R	CGCCAGTTCTGCTTACC	

washed under running tap water, surface sterilized in 2% NaOCl for 30 s, rinsed in distilled water for three times, and allowed to air dry. A small portion of the mushroom gill tissues (~5mm) was placed on potato dextrose agar (PDA) medium and incubated at 28 ± 2°C for 5-7 days in a BOD incubator. The plates were constantly observed for the emergence of mycelium and, further, fruiting structures, *i.e.*, basidiocarp. The pure culture of the mushroom was maintained in PDA agar slants and glass jars for artificial cultivation of the natural mushroom under laboratory conditions.

RESULTS

Morphological studies

The stringent morphological observations of the mushroom conducted are described below:

Coprinopsis cinerea (Schaeff.) Redhead, Vilgalys and Moncalvo

Description

Basidiocarps small to medium-sized, fragile (Fig. 2 A-C). Pileus 5–15 × 2.5–7 mm when young, finally up to 40 mm diam., initially paraboloid to conical, expanding to almost applanate; surface white all over when young, becoming white at the center and on the velar remnants, grey (4E1) elsewhere at maturity, initially covered with a dense, matted-fibrillose veil all over, later the veil radially spitting into appressed to recurved hairy or fibrillose flocks all over, densely so at the center, smooth elsewhere at maturity, strongly deliquescent; margin initially straight, later collapses at maturity. Lamellae adnexed, crowded, initially white, becoming black with age; edge not observed due to deliquescence. Stipe 27–150 ×2–4 (6) mm, central, tapering towards the apex, hollow; surface white, smooth, slightly floccose towards the base; base subbulbous, often with a psuedorrhiza. Odor and taste not distinctive.

Basidiospores 9–12 × 6–7 × 5–6 µm, on an average 10.9 × 6.1× 5.45 µm, Q1 = 1.5–2.0, Q1avg = 1.8, Q2 = 1.5–2.2, Q2avg = 1.94, lenticular, oblong-ellipsoid to ovo- ellipsoid in face view, oblong in side view with an apical cap or ridge, dark brown, thick-walled, with a central germ-pore up to 2 µm wide (Fig. 2D). Basidia 14–22 × 8– 11 µm, clavate to pedicellate-clavate, hyaline, slightly thick-walled, surrounded by 5–6 pseudoparaphyses, 4-spored; sterigmata up to 3 µm long (Fig. 2E & 2F). Pleurocystidia 50–80 × 30–36 µm, abundant, clavate to subcylindrical, hyaline, thin-walled (Fig. 2H). Lamella-edge sterile. Cheilocystidia 20–45 × 13–22 µm, abundant, often pedicellate-clavate, sometimes subglobose to clavate, hyaline, thin-walled (Fig. 2G). Pileipellis a cutis overlaid with chains of velar elements; hyphae 6–11 µm wide, hyaline, thin-walled; velar elements 20–180 × 7–30 µm, cylindrical to sausage-shaped, often constricted at the septa, hyaline, thin-walled, occasionally coated with hyaline resinous substances (Fig. 2I). Stipitipellis a cutis with scattered patches of velar hyphae; hyphae 6–10 µm wide, hyaline, thin-walled; velar hyphae 2– 11 µm wide, hyaline, thin-walled (Fig. 2J). Clamp connections were observed only on velar elements on the pileipellis and at the base of cheilocystidia.

Habit and Habitat: In small groups, on decaying husks of arecanut.

Molecular studies

On amplification with the ITS and nrLSU primer sets, amplicons of 620 and 1100 bp lengths, respectively, were observed in gel electrophoresis. The sequences were BLAST searched in the NCBI database, and the ITS sequence had 100% similarity with *Coprinopsis cinerea* (MW081311), whereas the LSU sequence matched closely with *Coprinopsis calospora* (NG_058776 - 99.45%) and *C. cinerea* (JX118797 - 100%). The sequences were submitted to the NCBI database with the accession numbers OR648089 (ITS) and OR857074 (nrLSU). Based on the phylogenetic tree developed from the maximum likelihood (ML) method using the ITS - nrLSU dataset, the sequence under study grouped with the *C. cinerea* (MH858630) with 99% bootstrap support (Fig. 3).

Artificial cultivation

A fungus consistently isolated from the inoculated tissues of the naturally collected mushroom on PDA plates exhibited fluffy, whitish aerial mycelium on both the upper and lower sides (Fig. 4 A and B). The basidiocarp was emerging from the PDA in Petri plates under laboratory conditions. Mushrooms were arising in groups on PDA medium in test tubes and glass jars, which are morphologically similar to the naturally growing mushrooms on the arecanut husk (Fig. 4 C and D). A reference specimen labeled as

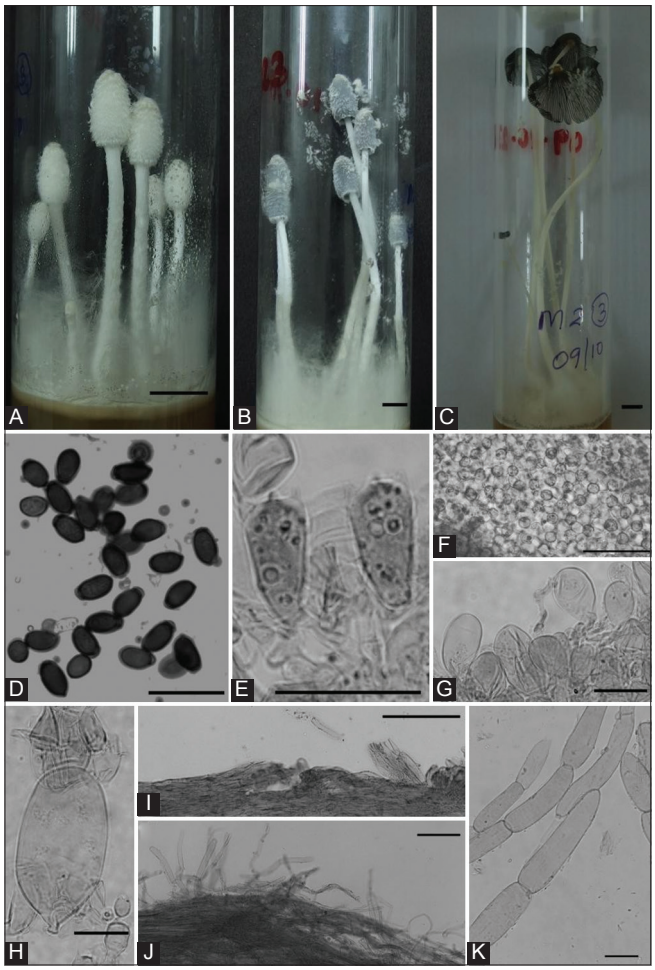

Fig 2. *Coprinopsis cinerea*. A-C. Basidiocarps. D. Basidiospores. E. Basidia. F. Pseudoparaphyses. G. Cheilocystidia. H. Pleurocystidium. I. Pileipellis. J. Stipitipellis. K. Velar elements on the pileipellis. Scale Bars: A-C = 10 mm; D, E, G, H&K = 20 µm; F& J = 50 µm; I = 100 µm.

'*Coprinopsis cinerea* CCBVH1' is being maintained at the Plant Pathology Repository, ICAR-CPCRI, RS, Vittal, Karnataka, India.

DISCUSSION

Coprinopsis cinerea (Schaeff.) Redhead, Vilgalys & Moncalvo (Basidiomycetes), known as the ink cap mushroom, has been widely used as a model organism in genetic studies to investigate multi-cellular development, mating types and fruiting body development in fungi (Lau et al., 2018). It has been reported earlier from diverse geographic regions in India (Manjula, 1983; Ulje, 2005). The mushroom reported in this study, found to be naturally growing on the arecanut husk, matches well with almost all morphological characters of *C. cinerea*. Small to medium-sized, white to greyish basidiocarps, oblong-ellipsoid to ovo-ellipsoid, dark brown basidiospores, 5-6 pseudoparaphyses surrounding each basidium, a hymenium with abundant pleuro and

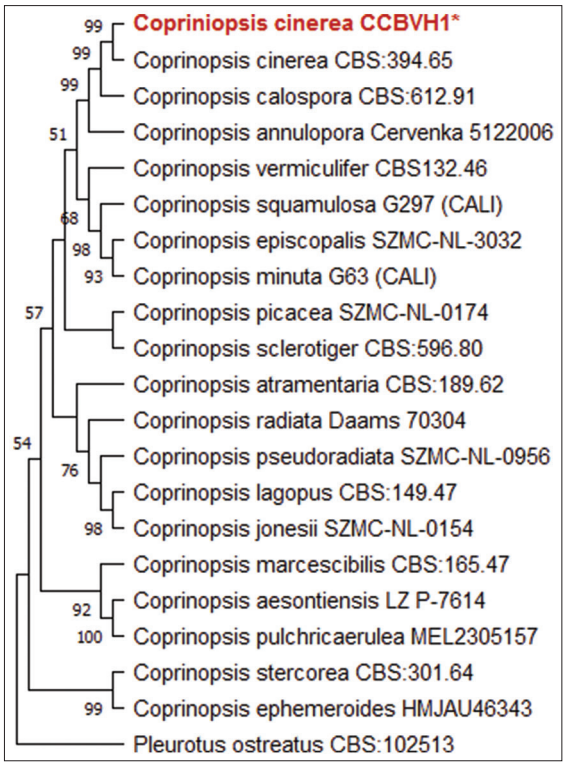

Fig 3. Phylogenetic tree generated based on the combined ITS + nrLSU dataset from Maximum Likelihood (ML) method with 1000 bootstrap replicates in MEGA 11.

cheilocystidia and a cutis-type pileipellis overlaid with chains of velar elements occasionally coated with hyaline resinous substances are the characteristic features of the present species. Our results match well with almost all morphological characters of *C. cinerea* reported earlier (Manjula, 1983; Ulje, 2005; Amandeep et al., 2014) except for the occasional presence of hyaline resinous substances on the velar elements of the pileipellis.

The ITS sequence of *C. cinerea* isolate in this study matched with *C. cinerea* through NCBI BLAST results (MW081311). The isolate in the current study was isolated from arecanut husk, whereas the isolate from NCBI (MW081311) was from a hot-dip galvanized sheet. The nrLSU dataset results closely matched *C. calospora* (NG_058776) and *C. cinerea* (JX118797). So, a combined dataset was used for phylogenetic analysis with the selected different *Coprinopsis* spp. (Table 2), which inferred that the isolate used in the current study grouped with *C. cinerea* only. In earlier studies, individual datasets of ITS alone (Ganga et al., 2022) or combined data sets with ITS and nrLSU (Nagy et al., 2013) were used to identify *Coprinopsis* species.

The fruiting bodies of *C. cinerea* are typical mushrooms which can be produced synchronously on defined media in the laboratory. We have successfully cultivated the *C. cinerea* mushroom on a PDA medium under controlled laboratory conditions.

Fig 4. A-B. Cultural characteristics of *Coprinopsis cinerea* CCVBH1 grown on potato dextrose agar medium (PDA); (C) *C. cinerea* CCVBH1 basidiocarp emerging out of potato dextrose agar medium in Petri plate; and (D) and a cluster of basidiocarps successfully cultivated on PDA in test tubes.

Table 2: List of species included in the phylogenetic analysis, with their geographical origin and GenBank accession numbers. The species used in this study is indicated in asterisk (*)

SN	Species name	Strain or Voucher No.	Country of origin	GenBank accession No.	
				ITS	nrLSU
1.	*Copriniopsis cinerea*	CCBVH1*	India	OR648089	OR857074
2.	*Coprinopsis cinerea*	CBS: 394.65	Netherlands	MH858630	MH870270
3.	*Coprinopsis calospora*	CBS: 612.91	Netherlands	GQ249275	GQ249284
4.	*Coprinopsis annulopora*	Cervenka 5122006	Hungary	JX118680	JX118811
5.	*Coprinopsis squamulosa*	G297 (CALI)	India	OP549278	OP549708
6.	*Coprinopsis minuta*	G63 (CALI)	India	OP549280	OP549279
7.	*Coprinopsis sclerotiger*	CBS: 596.80	Netherlands	GQ249277	GQ249286
8.	*Coprinopsis vermiculifer*	CBS: 132.46	Netherlands	GQ249279	MH867658
9.	*Coprinopsis atramentaria*	CBS: 189.62	Netherlands	MH858138	MH869723
10.	*Coprinopsis lagopus*	CBS: 149.47	Netherlands	MH856194	MH867720
11.	*Coprinopsis radiata*	Daams 70304	Hungary	JX118673	JX118745
12.	*Coprinopsis aesontiensis*	LZ P-7614	Italy	KY554753	KY554752
13.	*Coprinopsis marcescibilis*	CBS: 302.47	Netherlands	MH856199	MH867797
14.	*Coprinopsis stercorea*	CBS: 301.64	Netherlands	MH858437	MH870068
15.	*Coprinopsis ephemeroides*	HMJAU46343	China	MW832859	OL375252
16.	*Coprinopsis pseudoradiata*	SZMC-NL-0956	Hungary	JX118687	JX118755
17.	*Coprinopsis pulchricaerulea*	MEL2305157	Australia	MZ997384	MZ997375
18.	*Coprinopsis picacea*	SZMC-NL-0174	USA	JN943110	JQ045885
19.	*Coprinopsis jonesii*	SZMC-NL-0154	Hungary	JX118726	JX118788
20.	*Coprinopsis episcopalis*	SZMC-NL-3032	Hungary	FN396118	FN396200
21.	*Pleurotus ostreatus*	CBS 102513	Netherlands	MH862794	MH874388

The mushroom is edible but should be consumed swiftly after harvest. Despite being rich in nutrients, the rapid autolysis of the mushroom cap during maturation diminishes its potential nutritional value. *Coprinopsis cinerea* produces a repertoire of enzymes like laccases, chitinases and glucanases, which can be useful in industrial and biotechnological applications (You et al., 2014; Zhou et al., 2016). Furthermore, *C. cinerea* produces copsin, a protein

which possesses antibiotic properties and is effective against Gram-negative bacteria (Sabotic et al., 2016).

CONCLUSION

To conclude, this study adds to the existing knowledge by providing a new record of *C. cinerea* in terms of substrate, habit, habitat and geographical location. The isolate was characterized through morphological examinations and phylogenetic analyses. *Coprinopsis* is a genus of mushrooms primarily known for their ecological role in decomposing organic matter. There are some potential uses and ongoing research in utilizing *Coprinopsis* spp. in various industrial processes. They could be used in bioremediation processes to clean up environments contaminated with pollutants such as oil, pesticides, and industrial chemicals. Some species can produce ligninolytic enzymes, which are important in breaking down lignin, a complex organic polymer found in plant cell walls. These enzymes have applications in industries such as paper and pulp. Research is also ongoing to explore their potential in areas such as immuno-modulation, anti-inflammatory effects, and as a source of bioactive compounds for pharmaceutical purposes.

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