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NOTE

Coprinopsis cinerea from rice husks forming sclerotia in agar culture

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Abstract Sclerotia were formed in agar culture by a fungus with clamp connections isolated from rice husks at Tsukuba, Japan. The sclerotia were brown, globose to ellipsoidal, small, up to 200 μm in diameter, and composed of external rind tissue and internal medulla tissue. Such tiny sclerotia have not been commonly reported among basidiomycetous fungi in the literature. The fungus was identified as *Coprinopsis cinerea* on the basis of morphological characteristics together with molecular analyses. Three reference strains of *C. cinerea* formed sclerotia similarly under identical cultural conditions.

Keywords Basidiomycota · Coprinoid fungus · Molecular data · Morphology · Taxonomy

During studies on biodegradation of rice husks, brown globose to ellipsoidal tiny sclerotia (Fig. 1a, b) and hyphae with clamp connections were observed on 2% water agar (WA; Wako Pure Chemical Industries, Osaka, Japan) plated with rice husks. These sclerotia ranged from 65 to 180 μm in diameter. Basidiomes were also formed nearly 1 month later on Difco potato dextrose agar (PDA). The fungus was identified as one of the coprinoid fungi on the basis of deliquesced basidiomes with inky spore prints. However, most coprinoid fungi were reclassified into at least four genera and two families, i.e., *Coprinus* s. str. (Agaricaceae), *Coprinopsis* (Psathyrellaceae), *Coprinellus* (Psathyrellaceae), and *Parasola* (Psathyrellaceae) on the basis of molecular data (Hopple and Vilgalys 1999; Kirk et al. 2008; Redhead et al. 2001). Sclerotia formed by coprinoid fungi on agar cultures are not commonly reported, although there are some references on coprinoid fungi associated with rice plants in culture collection catalogues (ATCC 2010; NBRC 2010) including *Coprinus fimentarius* Fr. (ATCC36567) from rice straw, *Coprinopsis cinerea* (Schaeff.: Fr.) Redhead, Vilgalys & Moncalvo (ATCC42723 and ATCC42729) from rice compost and *C. cinerea* (NBRC 30628) from rice straw treated with urea, and *Coprinus patouillardii* Quél. (ATCC42762) from rice straw mat. Sclerotia of this fungus are comparatively studied with three reference strains from NBRC (2010), National Institute of Technology and Evaluation, Japan, in agar cultures.

Husks of rice (cv. Koshihikari) collected at Akatsuka, Tsukuba, Ibaraki, Japan, in September 2005 were used as samples. After washing samples under running tap water for more than 30 min and air-drying, they were plated onto 2% WA (5 husks/plate). The plates in plastic cases were placed on an open shelf and incubated under laboratory condition with constant temperature at 25°C and aeration, but light was not precisely controlled. Sclerotia (Fig. 1a, b) were noted in some plates within 30 days of incubation.

The three type strains (NBRC 30628, 31333, 100011) of *C. cinerea* from NBRC (2010) were comparatively tested for sclerotium formation.

Genomic DNA was extracted from mycelia in Difco potato dextrose broth (PDB) culture using the FastDNA SPIN Kit (Qbiogene, Carlsbad, CA, USA) following the manufacture's instruction. The internal transcribed spacer (ITS) regions and 5.8S rRNA gene were amplified with the primers NS1 (White et al. 1990; Kowalchuk et al. 1997) and NL4 (O'Donnell 1993). Polymerase chain reaction (PCR) cycling conditions were as follows: 2 min preheating at 95°C, followed by 40 cycles consisting of denaturation at

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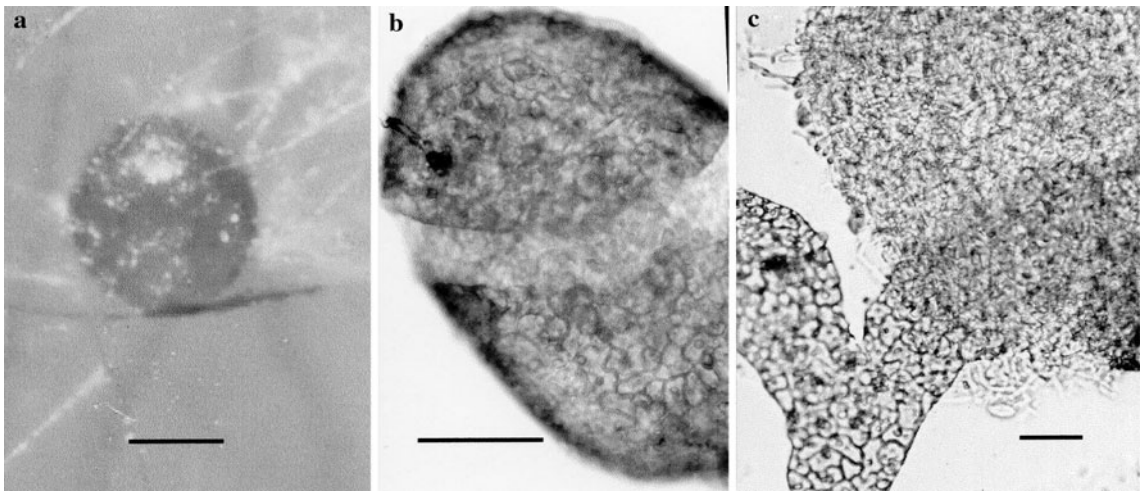

Fig. 1 Sclerotium morphology of *Coprinopsis cinerea* TW 06-150. **a** Globose sclerotium formed on water agar. **b** Ellipsoidal sclerotium crushed, composed of rind and medulla tissue. **c** Sclerotium composed of pseudoparenchymatous rind (*lower part*) and prosenchymatous medulla tissue (*upper part*). *Bars* **a** 50 μm; **b** 30 μm; **c** 10 μm

95°C for 10 s, annealing at 55°C for 15 s, extension at 72°C for 150 s, and a final 2 min extension at 72°C. The PCR product was sequenced with the DTCS-Quick Start kit (Beckman Coulter, Fullerton, CA, USA) and the CEQ 2000XL genetic analysis system (Beckman Coulter), using the primers 5.8S-F (5′-CATCGATGAAGAACGCAGC-3′, a modification of ITS3 (White et al. 1990), and 5.8S-R (5′-KRTGCGTTCAAAGATTCGAT-3′), designed in this study. A phylogenetic tree was constructed by the neighbor-joining method using CLUSTAL W on the basis of ITS and 5.8S rRNA gene sequences that were determined in this study, and sequences of other species collected from the public databases. Bootstrap resampling analysis for 1,000 replicates was performed, and the nucleotide sequence data in this study were deposited in the DDBJ/EMBL/GenBank databases (AB499044).

The culture (TW 06-150) (=AIST06150) derived from sclerotia was deposited at National Institute of Advanced Industrial Science and Technology, Ministry of Economy, Trade and Industry, Tsukuba, Ibaraki, Japan, and living subcultures were also deposited at NIAS Genebank, National Institute of Agrobiological Sciences, Ministry of Agriculture, Forestry and Fisheries (=MAFF 240343), Tsukuba, Japan.

Morphology of the fungus is as follows. Pileus columnar to bell-shaped, floccose, white to grayish, 3.5–8 mm long, 3–4 mm wide, then becoming flattened up to 18 mm wide, finally uplifted marginally or turning over and splitting with deliquescence after expansion. Lamellae 40–50 in number, straight or branched, pale brown, mostly 40–100 μm broad. Universal veils composed of chained hyaline cylindrical or ellipsoidal cells, 125–175 × 17.5–30 μm. Pleurocystidia hyaline, clavate, cylindrical or ellipsoidal, rarely horned, (60–)70–96(–106) × 22–30 μm. Cheilocystidia clavate to ellipsoidal, 96–130 × 77 μm, globose 16–26 μm in diameter. Basidia hyaline, globose, 20–25 × 8–8.5 μm in diameter, 4-spored, sterigmata 2–2.4 μm long. Spores dark brown, ellipsoidal to ovoid, apiculate and inflated apically, truncate basally, 8–11 × 5–6 μm, with central germ pores, nearly 2 μm wide, rarely cylindrical in side view. Stipes floccose, white to pale brown, up to 80 mm long, tapering from base towards apex, 1.5–3 mm wide basally, 0.8–1 mm wide apically, composed of cylindrical, often clamped, cells, 18–21(–24) μm broad. Sclerotia (Fig. 1a, b) often covered by hyphae, brown, smooth, spherical to ellipsoidal, 70–180(–200) μm in diameter, composed of external yellowish to dark brown pseudochymatous rind tissue (textual angularis, mostly 10 μm in diameter) and internal pale brown prosenchymatous medulla tissue (Fig. 1c). Hyphae clamped, 2–5.6 μm broad.

Colonies on PDA after incubation for 7 days at 25°C were 44–54 mm in diameter, slightly aerial, white, brown and slightly raised partially and locally because of sclerotium formation; reverse pale brown.

In the molecular analysis, 688 bp of the sequences of the fungus were determined. It showed high sequence similarity (99.9%) to three *C. cinerea* strains (NBRC 31333, AB097562, AB097563) (Fig. 2). Thus, the fungus was identified as *C. cinerea* (Schaeff.: Fr.) Redhead, Vilgalys & Moncalvo (Redhead et al. 2001) on the basis of the BLAST analysis of the ITS sequences together with morphological characteristics (Buller 1924; Kües 2000; Uljé 2009). The phylogenetic analyses (Fig. 2) also support this result.

Sclerotia of *Coprinopsis cinerea* or *Coprinus lagopus* (Fr.) Fr. (syn.: *Coprinopsis cinerea*) were described by Moor and Jirjis (1976), and Waters et al. (1972, 1975a,b) (Table 1).

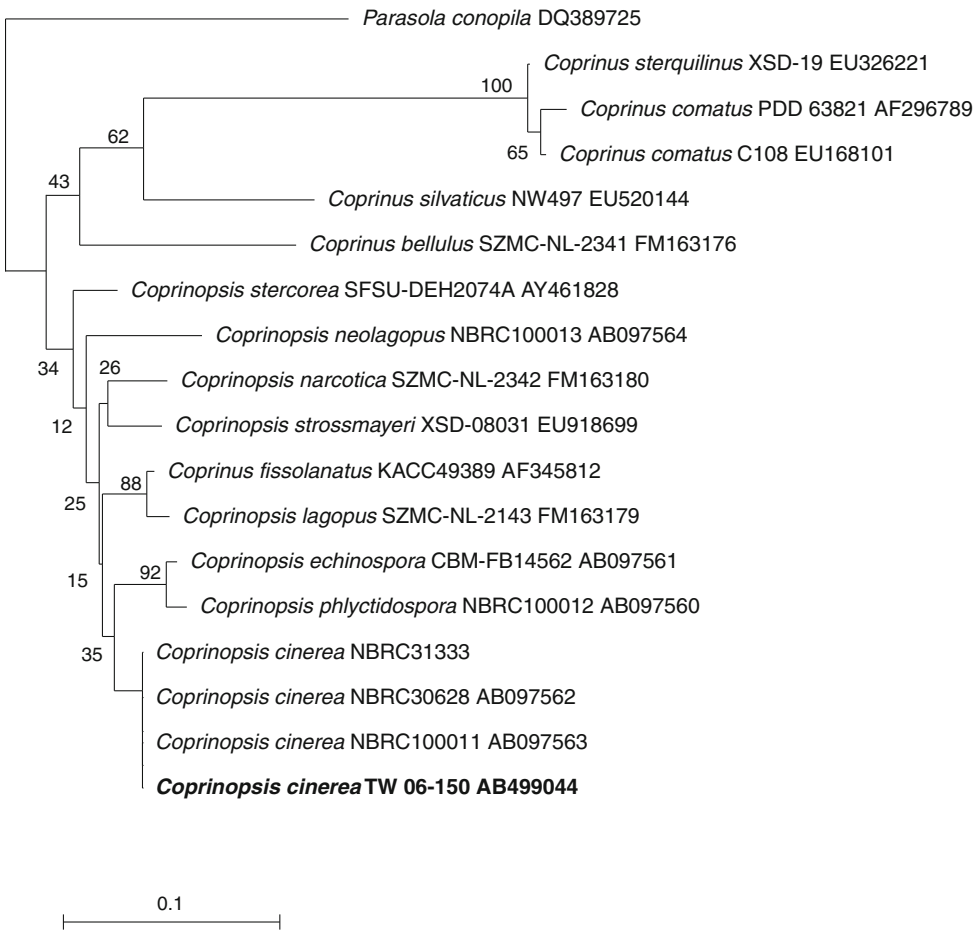

Fig. 2 A phylogenetic tree showing relationships of four strains of *Coprinopsis cinerea*, i.e., TW 06-150 (=MAFF 240343), NBRC 31333, NBRC 30628, and NBRC 100011, and related species of *Copinopsis* and *Coprinus* on the basis of internal transcribed spacer (ITS) regions and 5.8 S rRNA gene sequences, constructed using the neighbor-joining method. *Scale bar* indicates 0.1 substitutions per nucleotide position. Bootstrap values are shown at the *branch points*. *Parasola conopila* (DQ389725) was used as an outgroup

Table 1 Sclerotia of *Coprinopsis cinerea* or *Coprinopsis lagopus* (syn: *C. cinerea*) in culture

Taxon[a]	Color	Shape	Size	Remarks
Coprinopsis cinerea[b]	Black	Globose	~250 μm in diameter	Rind and medulla separated
Coprinopsis cinerea[c]	Black	Globose or ellipsoidal	<180 μm in diameter	Rind and medulla separated
Coprinopsis lagopus[d]	Pale brown to black	Globose	<1 mm in diameter	Rind and medulla separated
Coprinopsis lagopus[e]	Pale brown to black	Globose	100–250 μm in diameter	Aerial sclerotia composed of three basic tissue types: hyphal cells, rind, and medulla

[a] Names of taxon based on Redhead et al. 2001; [b] Moor and Jirjis 1976; [c] this study; [d] Waters et al. 1972, 1975a; [e] Waters et al. 1975b

Sclerotia were formed by the three reference strains (NBRC 30628, NBRC 31333, and NBRC 100011) of *C. cinerea* from NBRC (2010) aerially or embedded in aerial hyphae in 8 days on both WA and PDA media, although NBRC 31333 formed sclerotia 36–46 days after start of culture (Table 2). These sclerotia were morphologically identical to one another, and ranged 75–200 μm in diameter of globose sclerotia or largest length of ellipsoidal sclerotia. The respective 50 sclerotia randomly selected were 114.4–126.0 μm on average on WA and 115.7–135.4 μm on PDA.

Basidiome formation on agar cultures was often too erratic in each strain, but all the fungi tested formed basidiomes within 30–37 days on PDA, or sometimes on WA, except the NBRC 100011 strain. Mature basidiospores of these fungi are almost similar in shape and size.

Fungi are traditionally described on the basis of field-collected materials. Therefore, sclerotia, rhizoids, or other morphology formed in vitro have often been neglected or overlooked. However, this work started from sclerotia, and the fungus was almost impossible to identify before the basidiomes were observed, but it was identified

Table 2 Sclerotia of four strains of *Coprinopsis cinerea* formed aerially or embedded in aerial hyphae on water agar (WA) and potato dextrose agar (PDA) cultures

Strain	Medium	Formed (in days)	Range (μm)	Average ± SD (μm)[a]	Remarks
TW 06-150	WA	8	80–190	115.7 ± 17.2	Aerial sclerotia
	PDA	8	75–200	126.8 ± 24.6	Aerial sclerotia
NBRC 30628	WA	8	100–190	126.0 ± 19.7	Sclerotia embedded in aerial hyphae
	PDA	8	85–185	135.4 ± 23.9	Sclerotia embedded in aerial hyphae
NBRC 31333	WA	36	65–160	117.1 ± 26.2	Sclerotia embedded in aerial hyphae
	PDA	46	85–170	130.8 ± 18.3	Sclerotia embedded in aerial hyphae
NBRC 100011	WA	8	70–175	114.4 ± 24.2	Aerial sclerotia
	PDA	8	75–175	132.8 ± 28.6	Aerial sclerotia

Sizes (μm) (diameter or largest length) of globose or ellipsoidal sclerotia

[a] Average values (μm) of 50 sclerotia ± standard deviation (SD) for the respective cultures

morphologically, and also confirmed by the molecular data.

In the classical work on *Coprinus lagopus* (syn. *Coprinopsis cinerea*) (Buller 1924), the basidiomes formed in both light and complete dark conditions were observed, and they were various in size and shape, but no sclerotia described.

Sclerotia formed by *C. cinerea* or *C. lagopus* as reported in the literature (see Table 1) are 100–200 μm or more in diameter, but the rice husks fungus and three NBRC reference strains formed slightly smaller sclerotia 65–190(–200) μm in diameter in culture (see Table 2). Formation of two anatomically distinct types of sclerotum, a loosely organized form that arises in the submerged mycelium and a compact highly organized structure which was developed in the aerial mycelium by *C. lagopus* (syn. *C. cinereus*), were described by Waters et al. (1975a,b), and the aerial sclerotia, 100–250 μm in diameter, consist of three basic tissue types arranged concentrically: the outermost layer of thin-walled dead hyphal cells, the second layer of the rind, and the central medullary region. However, sclerotia in our study (Fig. 1c) were composed of two layers, i.e., external rind and internal medulla tissues.

Some coprinoid species are known to be associated with plants, causing damage to the plants (Traquair and Smith 1982), or parasitizing them without any injurious effects (Hanna 1939). *Coprinopsis cinerea* from rice husks was not pathogenic to rice plants in the preliminary inoculation test (data not shown), although the nature of the association has not been studied. The ecological implications of the fungus are interesting to note for future work.

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References

ATCC (2010) ATCC Advanced Catalog Search http://www.atcc.org/ATCCAdvancedCatalogSearch/tabit/454/Def

Buller AHR (1924) Researches on fungi III. The production and liberation of spores in hymenomycetes and Uredineae. Hafner, New York

Hanna WF (1939) *Coprinus urticicola* on stems of Marquis wheat. Mycologia 31:250–257

Hopple JS, Vilgalys R (1999) Phylogenetic relationships in the mushroom genus *Coprinus* and dark-spored allies based on sequence data from the nuclear gene coding for the large ribosomal subunit RNA: divergent domains, outgroups, and monophyly. Mol Phylogenet Evol 13:1–19

Kirk PM, Cannon PF, Minter DW, Stalpers JA (2008) Dictionary of Fungi, 10th edn. CAB International, Wallingford

Kowalchuk GA, Gerards S, Woldendorp JW (1997) Detection and characterization of fungal infections of *Ammophila arenaria* (marram grass) roots by denaturing gradient gel electrophoresis of specifically amplified 18 s rDNA. Appl Environ Microbiol 63:3858–3865

Kües U (2000) Life history and developmental processes in the basidiomycete *Coprinus cinereus*. Microbiol Mol Biol Rev 64:316–353

Moor D, Jirjis RI (1976) Regulation of sclerotium production by primary metabolites in *Coprinus cinereus* (=*C. lagopus* sensu Lewis). Trans Br Mycol Soc 66:377–382

NBRC (2010) Catalogue of biological resources, 2nd edn. National Institute of Technology and Evaluation (NITE), Japan. http://www.nbrc.nite.go.jp/doc/catalogue/NBRC2010–223–584fungi.pdf

O'Donnell K (1993) *Fusarium* and its near relatives. In: Reynolds DR, Taylor JW (eds) The fungal holomorph: mitotic meiotic and pleomorphic speciation in fungal systematics. CAB International, Wallingford, pp 225–233

Redhead SA, Vilgalys R, Moncalvo JM, Johnson J, Hopple JS Jr (2001) *Coprinus* Pers. and the disposition of *Coprinus* species sensu lato. Taxon 50:203–241

Traquair JA, Smith JD (1982) Sclerotial strains of *Coprinus psychromorbidus*, a snow mold basidiomycete. Can J Plant Pathol 4:27–36

Uljé CB (2009) Kees Uljé *Coprinus* site. http://www.grzyby.pl/coprinus-site-Kees-Uljee/species/cinereus.htm

Waters H, Butler RD, Moore D (1972) Thick-walled sclerotial medullary cells in *Coprinus lagopus*. Trans Br Mycol Soc 59:167–169

Waters H, Butler RD, Moore D (1975a) Structure of aerial and submerged sclerotia of *Coprinus lagopus*. New Phytol 74:199–205

Waters H, Moore D, Butler RD (1975b) Morphogenesis of aerial sclerotia of *Coprinus lagopus*. New Phytol 74:207–213

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, pp 315–322