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SHORT COMMUNICATION

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Hirsutella proturicola sp. nov. isolated from a proturan, *Baculentulus densus* (Protura, Hexapoda)

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Abstract A new species of *Hirsutella*, *H. proturicola*, isolated from a subterranean proturan (*Baculentulus densus*; Protura, Hexapoda), is described and illustrated. *Hirsutella proturicola* is characterized by producing monoblastic phialides of 24–51.5 × 2.5–5 μm with a slightly roughened neck, fusiform and curved conidia of 9–18 × 2.5–4 μm that have a truncate base and a papillate projection often capped with sheath-like mucilage, and pluricellular, globose to subglobose chlamydospores of 21–48 × 21–41.5 μm. This species is morphologically and phylogenetically close to *H. rostrata*, an acaropathogenic species, but can be distinguished from the size of the phialides and the size and shape of the conidia.

Key words Entomopathogenic fungi · Gamasida · *Hirsutella rostrata* · New species · Soil arthropods

Hirsutella Pat. is one of the anamorphic genera of *Ophiocordyceps* (Petch) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora (Ophiocordycipitaceae, Hypocreales) (Sung et al. 2007), and since Patouillard (1892) established the genus, nearly 80 species have been added to the genus (Index Fungorum; http://www.indexfungorum.org/; as of Sept. 12, 2007). Most species of *Hirsutella* are parasites of insects, mites, or nematodes (Seifert and Boulay 2004), and the species parasitizing small host arthropods such as mites generally do not produce synnemata on the host arthropods (Aoki 2003), whereas many of the hirsutellan species produce conspicuous synnemata on host arthropods (Minter and Brady 1980; Aoki 2003). Non-synnema-forming (mononematous) species are found relatively infrequently (Minter and Brady 1980), but include *H. thompsonii* F.E. Fisher, a famous pathogen of eriophyoid mites such as citrus rust mites (*Phyllocoptruta oleivora* (Ashmead)) (van der Geest et al. 2000). Minter and Brady (1980) proposed section *Mononematosa* Minter & B.L. Brady in the genus to accommodate the mononematous species; however, as pointed out by Fernández-García et al. (1990), the production of synnemata is changeable between in vitro and in vivo and would not be reliable as a character for dividing the sections of the genus.

The order Protura is the most ancestral group of hexapods dwelling in soil (Machida 2006), and 63 species of proturans have been found in Japan (Nakamura 1999). One of the species, *Baculentulus densus* Imadaté (Acerentomidae), is infected by various entomopathogenic fungi (Kurihara et al. 2006), but species belonging to *Hirsutella* have never been recorded from proturans, including *B. densus*. We found a mononematous species of *Hirsutella* from a laboratory-reared *B. densus*, and because the species was different from any other known species of *Hirsutella*, we describe and illustrate this species as a new species.

The host proturan, *B. densus*, was extracted from soil collected at Shinko-ji temple in Sanada, Ueda City, Nagano Prefecture, Japan in 2004 by a Tullgren funnel, and reared at the laboratory by Ms. M. Fukui and Dr. R. Machida. Rearing of proturans followed Machida and Takahashi (2004); namely, they were kept in a container at 0°–15°C, 100% relative humidity, under dark conditions. The fungus was isolated by transferring a conidium produced on a proturan cadaver that died while rearing to a cornmeal agar medium plate (Nissui Pharmaceutical, Tokyo, Japan).

Identification of the fungus was based on light microscopy and the sequences of internal transcribed spacer region (ITS) 1, 5.8S ribosomal DNA (rDNA), and ITS 2. The isolate was incubated on LCA medium (Miura and Kudo 1970) and malt agar medium plates (Nissui

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Pharmaceutical) at 26°C under dark/light conditions (L:D = 9:15) for 20 days for light microscopy and DNA extraction. Preparation of slides and measurements of each morphological feature followed Kurihara et al. (2000).

DNA extraction, polymerase chain reaction (PCR), sequencing reaction, and purification of PCR products were conducted as described by Kurihara et al. (2008). Namely, from total DNA extracted from a broken mycelium using a silica-gel membrane, the ITS region and the D1 and D2 domains of a large subunit of rDNA were amplified by PCR with KOD-Plus-DNA polymerase (Toyobo, Osaka, Japan) using a primer set ITS5 and NL4 (White et al. 1990; O'Donnell 1993). Sequencing reactions were conducted by the dye-terminator method using primers ITS5, ITS3, NL1, ITS2, ITS4, and NL4 (White et al. 1990; O'Donnell 1993) under the conditions described by Kurihara et al. (2008).

The sequences were assembled on "ATGC" (Windows Ver. 4.0.9; Genetyx, Tokyo, Japan), and the sequence of the ITS region was used for analysis. The assembled sequence was aligned with the 27 sequences of *Hirsutella*, *Ophiocordyceps*, and *Beauveria* downloaded from DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/index-e.html) using the Clustal X 1.83 package (Thompson et al. 1997) (Table 1). Phylogenetic trees were constructed by the neighbor-joining method (NJ method) and the maximum-parsimony method (MP method) using PAUP* 4.0b8 software (Swofford 2001). The NJ tree was constructed based on the HKY85 distance estimation model (Hasegawa et al. 1985), and the MP tree was constructed by heuristic search. The reliability of each branch was estimated by bootstrapping (Felsenstein 1985) in PAUP* 4.0b8 (Swofford 2001) with 1000 resamplings.

As a result, almost full sequences of the ITS region of the isolate were analyzed, and the result is available in the DDBJ/EMBL/GenBank databases under the accession number AB378557. A strict consensus of the 302 most parsimonious trees was constructed. Because the NJ tree and the strict consensus of MP trees were almost concordant with each other, only the MP tree is shown here (Fig. 1). In both NJ and MP trees, this fungus was in a sister-relationship with *H. rostrata* Bałazy & J. Wiśn. (accession number EF194150 in the DDBJ/EMBL/GenBank databases) with high bootstrap support (100%) (Fig. 1). They differed in 6 of 630 base pairs of the sequences of the ITS region. The sequences of the ITS region of some *Hirsutella/Ophiocordiceps* species are almost the same. For example, those of *H. gregis* Minter, B.L. Brady & R.A. Hall and *H. kirchneri* (O. Rostr.) Minter, B.L. Brady & R.A. Hall differ in only 2 of 521 base pairs. Therefore, we considered that the six-base substitution in the ITS region of this fungus can be a basis for discriminating a species. Also, because its microscopic features were distinct from all hitherto known species of the genus including *H. rostrata*, we describe this as a new species of *Hirsutella* as follows.

Table 1. Internal transcribed spacer (ITS) sequences of the species of *Hirsutella* and its related genera used for phylogenetic analysis

DDBJ/GenBank/ EMBL accession no.	Species	Strain no./specimen voucher no.
EF194156	*H. aphidis* Petch	SB3701
EF194155	*H. gregis* Minter, B.L. Brady & R.A. Hall	SB3661/3
DQ345595	*H. guyana* Minter & B.L. Brady	ARSEF 878
EF194154	*H. kirchneri* (O. Rostr.) Minter, B.L. Brady & R.A. Hall	SB3560/1a2
DQ345572	*H. minnesotensis* Sen Y. Chen, Xing Z. Liu & F.J. Chen	ARSEF 2799
EF194146	*H. nodulosa* Petch	SB3700
DQ345584	*H. rhossiliensis* Minter & B.L. Brady	CBS 113353
EF194150	*H. rostrata* Bałazy & J. Wiśn.	SB3697
AB378557*	*H. proturicola* Kurihara, Shirouzu, Tokum. & Harayama	NBRC 103239
DQ345579	*H. thompsonii* F.E. Fisher	CBS 420.82
AY365468	*H. uncinata* Seifert & H. Boulay	
DQ345581	*H. vermicola* M.C. Xiang & Xing Z. Liu	AS37879
DQ345594	*H. versicolor* Petch	ARSEF 1034
AY245626	*O. agriotidis* (A. Kawam.) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora	ARS 5692
AJ309363	*O. cantharelloides* (Samson & H.C. Evans) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora	K(M RS1175)
AB031196	*O. coccidiicola* (Kobayasi) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora	
AB027377	*O. cochlidiicola* (Kobayasi & Shimizu) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora	
AJ309347	*O. emeiensis* (A.Y. Liu & Z.Q. Liang) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora	G96031
AJ786560	*O. entomorrhiza* (Dicks.) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora	
AB113352	*O. heteropoda* (Kobayasi) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora	
AB031193	*O. konnoana* (Kobayasi & Shimizu) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora	
AJ309359	*O. multiaxialis* (M. Zang & Kinjo) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora	HKAS30258
AJ309358	*O. nepalensis* (M. Zang & Kinjo) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora	HKAS28095
AJ309335	*O. robertsii* (Hook.) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora	K(M)27083
AJ309361	*O. sinensis* (Berk.) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora	
AJ309326	*O. sobolifera* (Hill ex Watson) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora	
AB044643	*O. yakusimensis* (Kobayasi) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora	
AB027382	*B. bassiana* (Bals.-Criv.) Vuill.	NBRC 4848

B, *Beauveria*; H, *Hirsutella*; O, *Ophiocordyceps*
*Sequence analyzed in this study

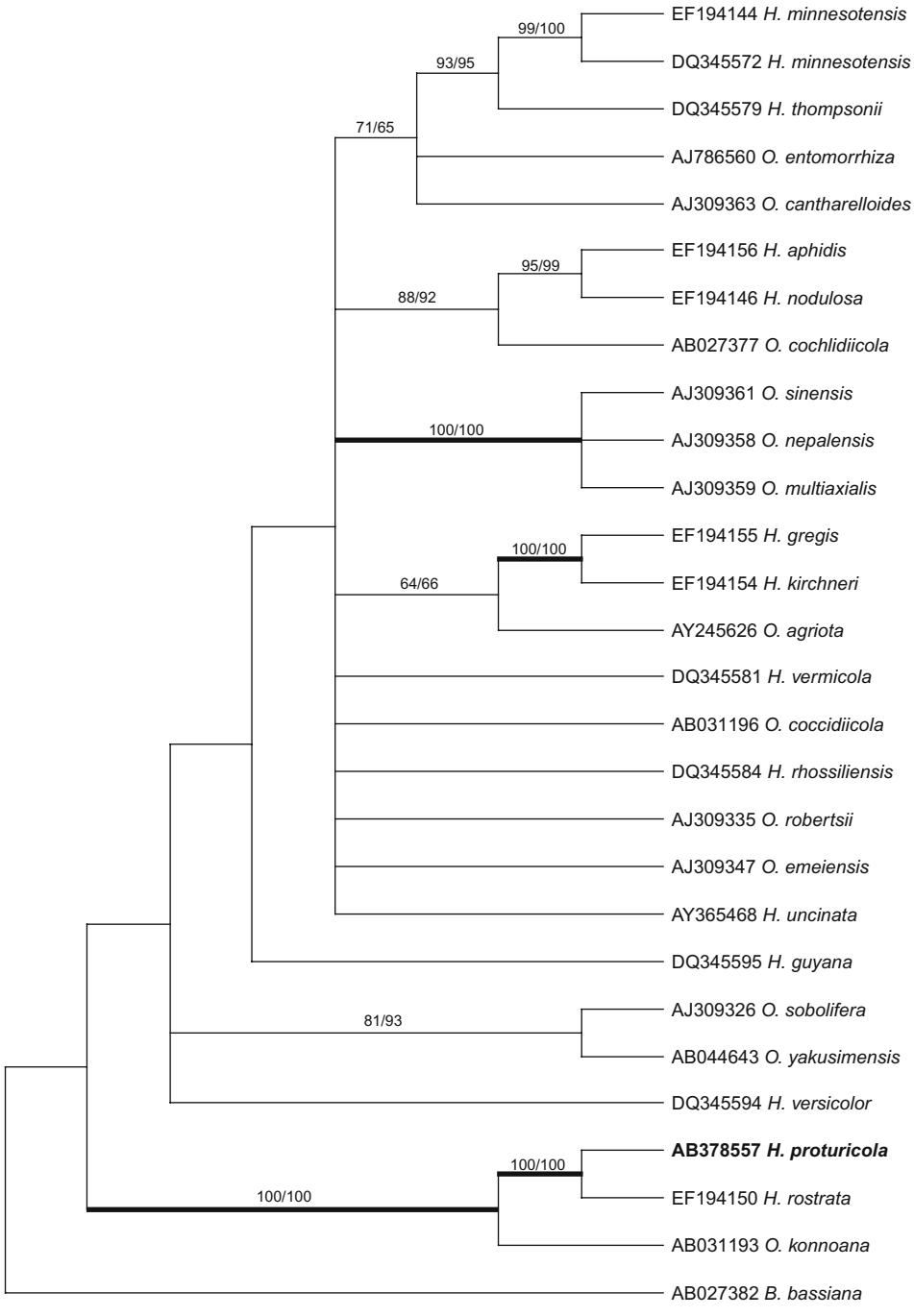

Fig. 1. Strict consensus of the 302 most parsimonious (MP) trees inferred from the sequences of the internal transcribed spacer region 1 (ITS 1), 5.8S ribosomal DNA, and ITS 2 of 28 taxa. Length = 287, CI = 0.606, RI = 0.660, RC = 0.400. The sequence determined in this study is shown in *bold*. Bootstrap values by MP/neighbor-joining (NJ) analyses are indicated *above nodes*. *Bold lines* indicate the parts of the tree with 100% consensus support. Frequencies below 50% are not indicated. *B*, *Beauveria*; *H*, *Hirsutella*; *O*, *Ophiocordyceps*

Taxonomy

Hirsutella protulicola Kurihara, Shirouzu, Tokum. & Harayama, sp. nov. Figs. 2, 3

Coloniae in LCA hyalinae, paginae coloniarum castaneae cum chlamydosporis. Hyphae hyalinae, septatae, 1.5–6 µm crassae. Phialides ex hyphis aeriis lateraliter vel terminaliter orientes, solitariae vel aliquando oppositae, sursum attenuatae in collum tenue vel infrequenter longe ampulliforme, 24–51.5×2.5–5 µm, monophialidicae, non vel aliquando septatae infra phialides, ad collum asperulae. Conidia continua, solitaria, 9–18 × 2.5–4 µm, hyalina, levia, fusiformia, curva, ad basim truncata, papillaria. Processus apicales conidiorum papillati, 1.5–3.5 × 1–1.5 µm, saepe cum vagina mucilagina obtecti. Chlamydosporae pluricellulares, intercalares, globosae vel subglobosae, laeves, pallide castaneae, 21–48 × 21–41.5 µm, cellulae chlamydosporarum laeves, subglobosae vel ellipsoideae, 1 (aliquando 2 vel 4) globulum contentae.

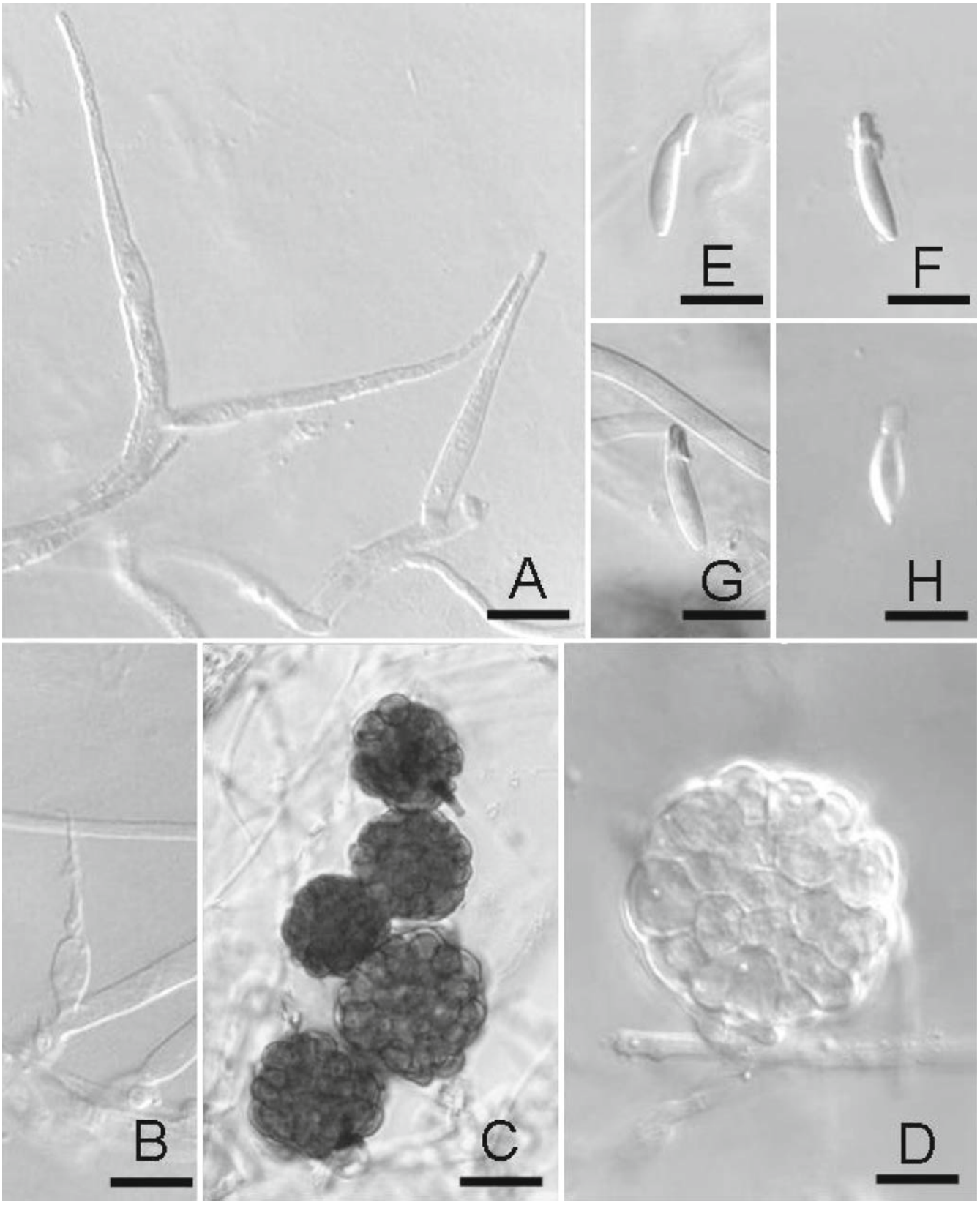

Fig. 2. *Hirsutella proturicola* (NBRC H-12641). **A** Three phialides. **B** Ampulliform phialide. **C** Five chestnut-brown chlamydospores. **D** Chlamydospore. **E–H** Conidia capped with sheath-like mucilage. **A–H** Cultured on LCA medium. *Bars* **A, B, D–H** 10 µm; **C** 25 µm

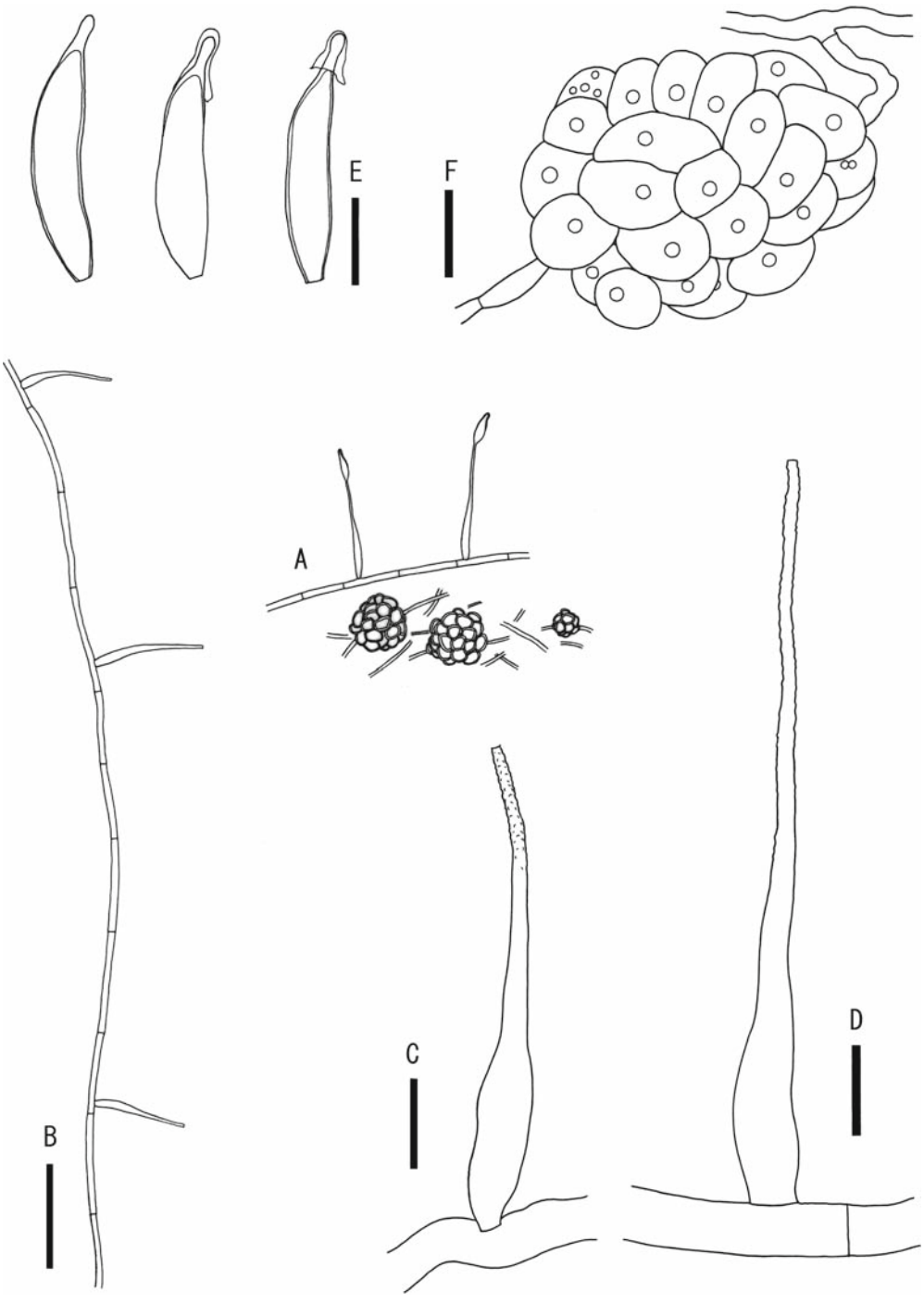

Fig. 3. *Hirsutella proturicola* (NBRC H-12641).
A Diagrammatic sketch of the habit of two sporulating phialides and three chlamydospores (not to scale). **B** Three phialides produced along an aerial hypha. **C, D** Phialides after segregation of conidia. Note slightly rough surface of the necks. **E** Three conidia; the papillate tips of the *right* two conidia capped with sheath-like mucilage. **F** Chlamydospore. **A–F** Cultured on LCA medium. *Bars* **B** 40 µm; **C–E** 5 µm; **F** 10 µm

Teleomorphus ignotus.

Holotypus. NBRC H-12641, colonia exsiccata in cultura ex proture, Shinko-ji, Sanada, Ueda, Nagano Pref., Japonia, Nov. 2004, M. Fukui et R. Machida leg., T. Shirouzu isol., in Herbario NBRC conservata. Culture viva: NBRC 103239, = KYK00196.

Etymology. *Protura* + *-cola* (Latin), referring to isolation source of the fungus.

Colonies on LCA hyaline, surface chestnut-brown with chlamydospores. Hyphae hyaline, septate, 1.5–6 µm wide. Phialides arising from aerial hyphae laterally or terminally, solitary or sometimes opposite, tapering to a slender neck, or rarely long ampulliform, 24–51.5 × 2.5–5 µm, monophialidic, nonseptate or sometimes septate below, slightly roughened at the neck. Conidia one-celled, produced solitary, 9–18 × 2.5–4 µm, hyaline, smooth, fusiform, curved, truncate at the base, with a papillate projection. Papillate apical projection of conidia, 1.5–3.5 × 1–1.5 µm, often capped with sheath-like mucilage. Chlamydospores pluricellular, produced on aerial hyphae or agar surface, intercalary, globose to subglobose, smooth, pale brown at maturity, 21–48 × 21–41.5 µm, chlamydospore component

Table 2. Microscopic morphological differences between *Hirsutella proturicola* and *H. rostrata*

	H. proturicola	*H. rostrata*[a,b]
Host arthropods	Protura	Gamasida (Acari), Coleoptera[a,b]
Size of phialides	24–51.5 × 2.5–4 μm	18.5–28.5 × 2.3–3.3 μm[a]
Surface of phialide necks	Slightly rough	Smooth?[a]
Size of conidia	9–18 × 2.5–4 μm	(7.7–)9.4–10.4(–13.3) × (2.3–)2.4–2.5(–3.1) μm; average, 9.9 × 2.5 μm on host[a]; 8.5–12 × 2.2–3 μm[b]
Shape of conidia	Fusiform, curved, truncate at the base, with a papillate apical projection	Fusiform, curved, not truncate at base, with a beak-like apical projection[a]
Mucilage of conidia	Sheath-like, covering the apical projection	Sheath-like, covering the apical projection[b]
Chlamydospores	21–48 × 21–41.5 μm (subglobose to globose)	Diameter (25–)35–75 μm (globose) or 50–120 × 40–65 μm (ovoid)[b]

Differences in ITS sequences of *H. proturicola* and *H. rostrata* are 6/631 bp
[a] Bałazy and Wiśniewski (1986)
[b] Bałazy et al. (2008)

cells smooth, subglobose to ellipsoid, containing 1 nucleus (sometimes 2 or 4 nuclei).

Teleomorph not found.

Holotype. NBRC H-12641, dried colony of a culture (NBRC 103239, = KYK00196) of the fungus on LCA isolated from a proturan *Baculentulus densus* (Protura), Shinko-ji Temple, Sanada, Ueda, Nagano Pref., Japan, Nov. 2004, coll. M. Fukui and R. Machida, isol. T. Shirouzu, deposited in the herbarium of NBRC.

Note. *Hirsutella proturicola* resembles *H. rostrata* in producing curved conidia with apical elongation on monophialidic conidiophores and pluricellular chlamydospores on agar media. However, *H. proturicola* can be distinguished from *H. rostrata* by the size and shape of the conidia and longer phialides, the neck surface of which is slightly rough (Table 2). Conidia of *H. proturicola* are 9–18 × 2.5–4 μm and have a truncate base and papillate projection, whereas those of *H. rostrata* are shorter (9.9 × 2.5 μm on host mite) than those of *H. proturicola*, not truncate at base, and the apical part of conidia is beak-like (Bałazy and Wiśniewski 1986) (Table 2). Phialides of *H. proturicola* (24–51.5 × 2.5–5 μm) are longer than those of *H. rostrata* (18.5–28.5 × 2.3–3.3 μm), and the slightly rough surface of the phialide neck observed for *H. proturicola* has not been seen in *H. rostrata* (Bałazy and Wiśniewski 1986) (Table 2).

The original description of *H. rostrata* is based on the fungal material growing on a host gamasid mite fixed in 70% alcohol, and the mucilage around the conidia was lost artifactually in the material (Bałazy and Wiśniewski 1986). Recently, Bałazy et al. (2008) found sheath-like mucilage capped on the apical tip of conidia in fresh materials of *H. rostrata*, and they also found carmine-red chlamydospores abundantly produced on agar media that were not produced on host mites.

Many hirsutellan species exhibit host specificity to arthropod orders (Aoki 2003). *Hirsutella rostrata*, which phylogenetically and morphologically resembles *H. proturicola*, is isolated from various gamasid mites living under bark and in anthills and a predacious coleopteran larva (Ciidae?), but it did not infect larvae of *Scolytus ratzeburgi* Jans. (Coleoptera) and *Cydia pomonella* L. (Lepidoptera) and *Globodera rostochiensis* Wollenweber (Nematoda) by an artificial infection treatment (Bałazy et al. 2008). Infection tests of *H. proturicola* to other arthropods including Gamasida are required to clarify the host ranges of the species.

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References

Aoki J (2003) Kaitei konchu byogenkin no kensaku (in Japanese). Zenkoku Noson Kyoiku Kyokai, Tokyo

Bałazy S, Wiśniewski J (1986) Two new species of *Hirsutella* infecting mites in Poland. Trans Br Mycol Soc 86:629–635

Bałazy S, Wrzosek M, Sosnowska D, Tkaczuk C, Muszewska A (2008) Laboratory trials to infect insects and nematodes by some acaropathogenic *Hirsutella* strains (Mycota: Clavicipitaceous anamorphs). J Invertebr Pathol 97:103–113

Fernández-García E, Evans HC, Samson RA (1990) *Hirsutella cryptosclerotium* sp. nov., an entomopathogen of the mealybug pest *Rastrococcus invadens*, in West Africa. Mycol Res 94:1111–1117

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Hasegawa M, Kishino H, Yano T (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J Mol Evol 22:160–174

Kurihara Y, Tokumasu S, Chien CY (2000) *Coemansia furcata* sp. nov. and its distribution in Japan and Taiwan. Mycoscience 41:579–583

Kurihara Y, Machida R, Fukui M, Okuda T, Harayama S (2006) Entomopathogenic fungi isolated from laboratory-reared *Baculentulus densus* (Acerentomidae, Protura). Edaphologia 80:25–28

Kurihara Y, Sukarno N, Ilyas M, Yuniarti E, Mangunwardoyo W, Saraswati R, Park JY, Inaba S, Widyastuti Y, Ando K (2008) Entomopathogenic fungi isolated from suspended-soil inhabiting arthropods in East Kalimantan, Indonesia. Mycoscience 49:241–249

Machida R (2006) Evidence from embryology for reconstructing the relationships of hexapod basal clades. Arthropod Syst Phylog 64: 95–104

Machida R, Takahashi I (2004) Rearing technique for proturans (Hexapoda: Protura). Pedobiologia 48:227–229

Minter DW, Brady BL (1980) Mononematous species of *Hirsutella*. Trans Br Mycol Soc 74:271–282

Miura K, Kudo MY (1970) An agar medium for aquatic hyphomycetes (in Japanese). Trans Mycol Soc Jpn 11:116–118

Nakamura O (1999) Protura. In: Aoki J (ed) Pictorial keys to soil animals of Japan (in Japanese). Tokai University Press, Tokyo, pp 713–723

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

Patouillard N (1892) Une Clavariée eutinigène. Revue Mycol (Toulouse) 14:67–70

Seifert KA, Boulay H (2004) *Hirsutella uncinata*, a new hyphomycete from Australia. Mycologia 96:929–934

Sung GH, Hywel-Jones NL, Sung JM, Luangsaard JJ, Shrestha B, Spatafora JW (2007) Phylogenetic classification of *Cordyceps* and the clavicipitaceous fungi. Stud Mycol 57:1–63

Swofford DL (2001) PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4.0b8. Sinauer Associates, Sunderland, MA

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

van der Geest LPS, Elliot SL, Breeuwer JAJ, Beerling EAM (2000) Diseases of mites. Exp Appl Acarol 24:497–560

White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols. Academic Press, San Diego, pp 315–322