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SHORT COMMUNICATION

Staphylotrichum boninense, a new hyphomycete (Chaetomiaceae) from soils in the Bonin Islands, Japan

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Abstract *Staphylotrichum boninense*, a new hyphomycete classified in the Chaetomiaceae (Ascomycota), was isolated from soils in the Bonin Islands, Japan. It is characterized morphologically by the production of yellow-orange colonies and subglobose holoblastic conidia. Morphologically the species is similar to *S. coccosporum*, but it is significantly different from *S. coccosporum* in phylogeny and also differs with respect to its secondary metabolite profile.

Keywords 28S rRNA gene D1/D2 region · Anamorph · Ascomycota · rRNA gene ITS region · Secondary metabolite

During an exploratory survey of soil-inhabiting fungi producing useful metabolites for the microbial industry, some interesting *Staphylotrichum* species, including *S. coccosporum* J.A. Mey. & Nicot (Nicot and Meyer 1956), were isolated from soil samples collected with roots of subtropical plants in the Bonin Islands and Izu Islands, Tokyo, Japan. Based on morphological, phylogenetic, and physiological studies, some isolates were found to be a new species of *Staphylotrichum*. Therefore, we report here the species with some notes on the taxonomy, phylogeny, and secondary metabolite profile.

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For isolating *Staphylotrichum* species at 25°C, potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) and Czapek yeast extract agar (CYA; Pitt 1979) plates were used with 50 mg/l rose bengal and 100 mg/l kanamycin. For observing morphological characteristics, isolates were incubated on PDA, oatmeal agar (OA; Difco), and potato carrot agar (PCA; Atlas 2010) at 25°C (also at 5° and 37°C on PDA) for 7 days in the dark. The *Methuen Handbook of Colour* (Kornerup and Wanscher 1978) was used to determine color names and hue numbers.

For microscopy, a Vanox-S AH-2 microscope equipped with a DP25 digital camera (Olympus, Tokyo, Japan) was used.

Genomic DNA of the strains was isolated using the PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Amplification of the 28S ribosomal RNA gene (rDNA) domain1/domain2 (D1/D2) region and rDNA internal transcribed spacer (ITS) region including 5.8S rDNA was performed using primers NL1 and NL4 (O'Donnell 1993) and primers ITS1 and ITS4 (White et al. 1990), respectively. Polymerase chain reaction (PCR) was performed according to the QIAGEN Fast Cycling PCR Kit protocol (Qiagen, Valencia, CA, USA).

Amplifications were performed in a PCR Verity 96-well thermal cycler (Applied Biosystems), programmed with denaturation at 95°C for 5 min, followed by 35 cycles consisting of denaturation at 96°C for 5 s, primer annealing at 50°C (for D1/D2) or 56°C (for ITS) for 5 s, extension at 68°C for 21 s, and a final elongation step at 72°C for 1 min. After amplification of the D1/D2 and the ITS region templates, excess primers and dNTPs were removed from the reaction mixture using a QIAquick PCR DNA Purification Kit (Qiagen). The PCR products were sequenced directly in both directions using primers NL1, NL4, ITS1,

and ITS4 using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The cycle sequencing reaction mixture had a total reaction volume of 10 μl and contained 2.5 μl of template DNA (10–15 ng/μl), 2 μl BigDye terminator premix, 4 μl ultrapure sterile water, and 0.5 μl primer (5 pmol/μl). Reactions were run in a PCR thermal cycler, programmed with denaturation at 96°C for 1 min, then by 25 cycles of denaturation at 96°C for 10 s, followed by primer annealing at 50°C for 5 s, and extension at 60°C for 4 min. Sequencing products were purified by ethanol/ethylenediaminetetraacetic acid (EDTA) precipitation, and samples were analyzed on an ABI PRISM

Table 1 *Staphylotrichum* isolates and the related taxa used for phylogenetic analyses

Species	Strain number	Locality	Isolation source	Other strain number/ taxonomic information	GenBank accession no.	
					D1/D2	ITS
S. boninense	JCM 17908[a]	Chichi-jima, Bonin Islands, Tokyo, Japan	Soil under *Elaeagnus rotundata*	FKI-4751, ex-type	AB625568	AB625580
	JCM 17909[a]	Chichi-jima, Bonin Islands, Tokyo, Japan	Soil under *Clinostigma savoryanum*	FKI-4859	AB625569	AB625581
	JCM 17910[a]	Chichi-jima, Bonin Islands, Tokyo, Japan	Soil under *Ligustrum micranthum*	FKI-5336	AB625570	AB625582
S. coccosporum	JCM 17911[a]	Hachijo Island, Izu Islands, Tokyo, Japan	Soil under *Hibiscus rosa-sinensis*	FKI-5577	AB625571	AB625583
	CBS 364.58[b]	Yangambi, Zaire	Soil	Ex-type	AB625572	AB625584
	NBRC 31817[b]	–	Paddy field soil		AB625573	AB625585
	NBRC 33272[b]	Iriomote Island, Okinawa, Japan	Pineapple field soil		AB625574	AB625586
Botryotrichum piluliferum	NBRC 8277[b]	–	Horse dung		AB625575	AB625587
Chaetomium floriforume	IMI 368520	Thailand	Fallen leaves	MUCL 40181, ex-type	AF286402	–
C. globosum	ATCC 6205	Washington, DC, USA	Stored cotton		AF286403	EF524036
C. sphaerale	CBS 723.97[b]	–	Filter paper	MUCL 40089	AF286407	AB625588
Humicola fuscoatra	NBRC 9530[b]	–	–		AB625576	AB625589
H. grisea var. *grisea*	FO-2942[b]	Nagasaki, Japan	Soil		AB625577	AB625590
	CBS 119.14	Norway	Soil	DAOM 232586	–	AY706334
H. grisea var. *thermoidea*	NBRC 9854[b]	–	Lake sediment		AB625578	AB625591
H. insolens	MTCC 4617	–	–		EU257375	EF550968
	IMI 126330	–	–		–	AJ131857
H. nigrescens	CBS 208.55[b]	Norway	Soil in potato field	ATCC 22714, ex-type	AB625579	AB625592
Retroconis fusiformis	CBS 330.81	Multan, Pakistan	Seed of *Gossypium* sp.	IMI 170799	EU040239	EU040239
Zopfiella erostrata	CBS 255.71	Central African Republic	*Kobus defassa* dung	ATCC 22459	AY999110	AY999133
Z. karachiensis	NBRC 32902	–	Garden soil		–	AY999128

Dash (–), data not available

ATCC, American Type Culture Collection, Manassas, USA; CBS, Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, Utrecht, Netherlands; DAOM, Plant Research Institute, Department of Agriculture (Mycology), Ottawa, Canada; FKI & FO, Fungal Collection of Kitasato Institute for Life Sciences, Kitasato University, Tokyo, Japan; IMI, CABI Genetic Resource Collection, Egham, UK; JCM, Japan Collection of Microorganisms, Wako, Japan; MTCC, Microbial Type Culture Collection and Gene Bank, Chandigarh, India; MUCL, Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium; NBRC, NITE Biological Resource Center, Kisarazu, Japan

[a] New isolates sequenced in this study

[b] Sequenced in this study

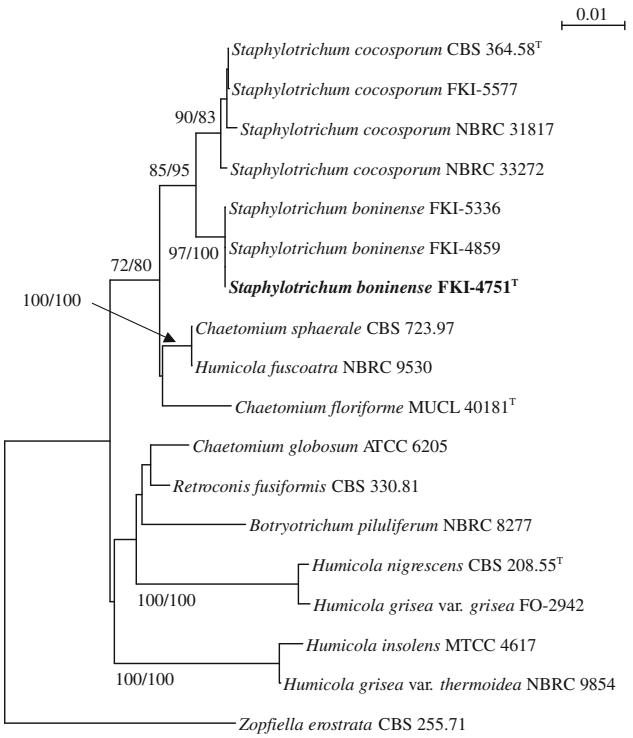

Fig. 1 Phylogenetic tree for *Staphylotrichum boninense* and the related species of the Chaetomiaceae, drawn from maximum-parsimony (MP) and neighbor-joining (NJ) analyses of the 28S rDNA D1/D2 region sequences. The outgroup is *Zopfiella erostrata* (Sordariales). The *numbers* shown in the branches represent bootstrap values above 70% (MP, *left*; NJ, *right*) based on 1,000 replicates. Strain numbers with a letter *T* are ex-type strains

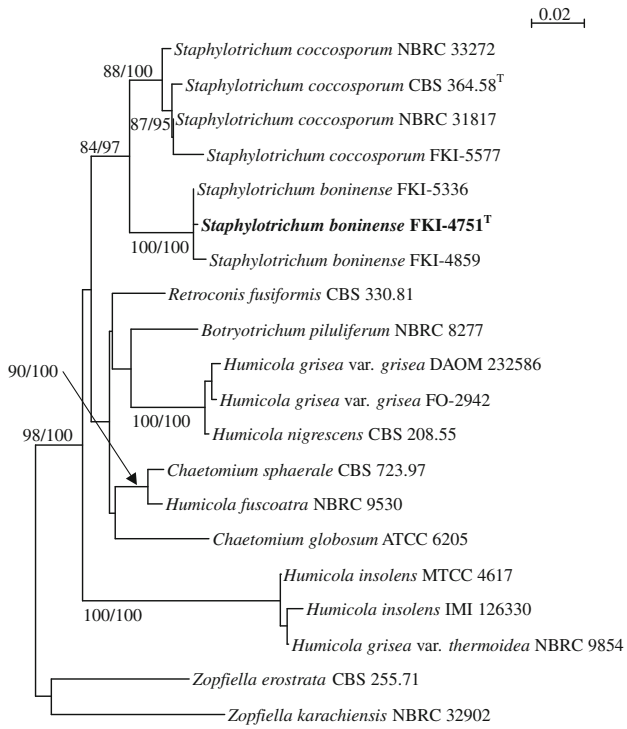

Fig. 2 Phylogenetic tree for *Staphylotrichum boninense* and the related species of Chaetomiaceae, drawn from MP and NJ analyses of the rDNA internal transcribed spacer (ITS) region sequences. The outgroups are *Zopfiella erostrata* and *Z. karachiensis* (Sordariales). The *numbers* shown in the branches represent bootstrap values above 80% (MP, *left*; NJ, *right*) based on 1,000 replicates. Strain numbers with a letter *T* are ex-type strains

3130 Genetic Analyzer (Applied Biosystems). Contigs were assembled using the forward and reverse sequences with the SeqMan and SeqBuilder programs from the Lasergene 8 package (DNAStar, Madison, WI, USA). The sequences determined in this study were deposited at the GenBank (Table 1).

Based on our preliminary phylogenetic analyses, a new *Staphylotrichum* species and *S. coccosporum* were found to be related to the Chaetomiaceae (Ascomycota). The D1/D2 and ITS sequences of the related species in the Chaetomiaceae were obtained from GenBank (Table 1) and aligned using MUSCLE 3.8 (Edgar 2004). Alignment was refined using SEAVIEW 4.2 (Gouy et al. 2010), and the alignment was deposited in TreeBASE (http://www.treebase.org/) with accession number S11921. Phylogenetic analyses were based on the neighbor-joining (NJ) method (Saitou and Nei 1987) using Clustal X 2.1 (Thompson et al. 1997) and the maximum-parsimony (MP) method using PAUP 4.0b10 software (Swofford 2002). Bootstrap analyses were performed on NJ and MP trees with 1,000 bootstrap replicates. The trees were rooted with *Zopfiella* species and viewed with NJplot (Perrière and Gouy 1996).

For the metabolite analyses, the strains FKI-4751 and CBS 364.58 (ex-type of *S. coccosporum*) were inoculated into each 10 ml of the seed medium in a test tube: 2.0% glucose, 0.5% Polypepton (Nihon Pharmaceutical, Tokyo, Japan), 0.2% yeast extract, 0.2% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% agar; adjusted to pH 6.0 before sterilization. The test tubes were incubated on a rotary shaker (300 rpm) at 27°C for 3 days. A 1-ml portion of each seed culture of FKI-4751 and CBS 364.58 was transferred to three 500-ml Erlenmeyer flasks each containing 100 ml production medium (3.0% soluble starch, 1.0% glycerol, 2.0% soybean meal, 0.3% dry yeast, 0.3% KCl, 0.2% $CaCO_3$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KH_2PO_4; adjusted to pH 6.5 before sterilization), and the fermentation on a rotary shaker (210 rpm) was carried out at 27°C for 6 days. Each culture broth (300 ml) was mixed with ethanol (300 ml), followed by centrifugation (3,000 rpm, 15 min). The mixtures were evaluated by analytical high performance liquid chromatography (HPLC) under the following conditions for the analysis of metabolites: column, Symmetry C18 (2.1 ø × 150 mm; Waters, Milford, MA, USA); mobile phase, acetonitrile–water with 0.05% phosphoric

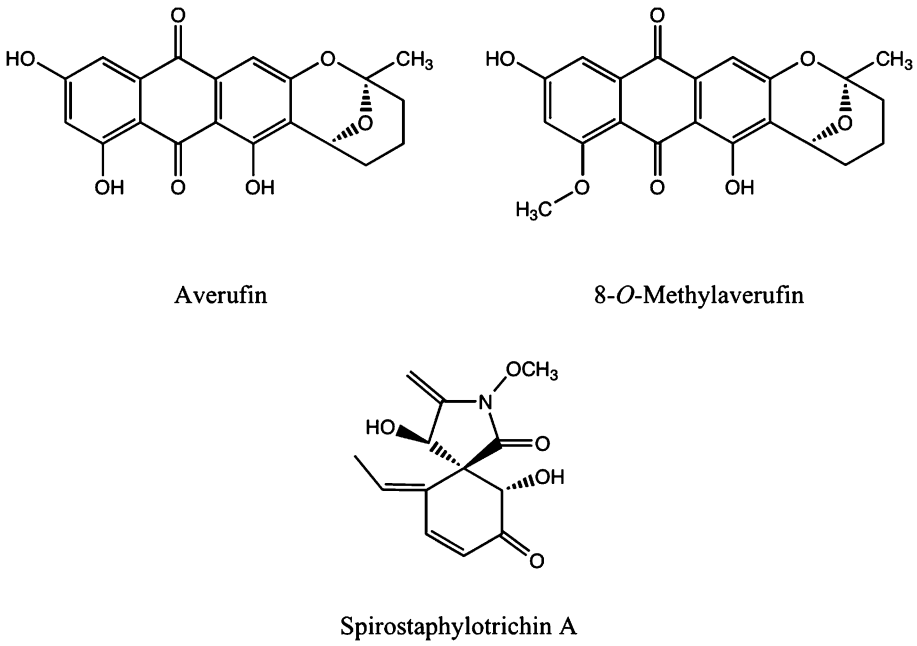

Fig. 3 Structures of averufin, 8-*O*-methylaverufin, and spirostaphylotrichin A (cf. Sandmeier and Tamm 1989)

acid, 5–100% (20 min); flow rate, 0.2 ml min^{-1}; detection, UV at 210 and 450 nm.

With respect to sequence distances determined by the MegAlign programs from the Lasergene 8 package, the isolate FKI-4751 of the new *Staphylotrichum* species had 99.0% similarity with the D1/D2 sequences of *S. coccosporum* CBS 364.58 (AB625572) and 95.0% similarity with the ITS sequences of *S. coccosporum* CBS 364.58 (AB625584). In the MP and NJ analyses based on the 28S rDNA D1/D2 region and rDNA ITS region sequences, the strain was most closely related to the examined strains of *S. coccosporum* with high bootstrap values (Figs. 1, 2). However, FKI-4751 showed substantial distance from *S. coccosporum*.

Analyses of HPLC chromatograms and UV spectra showed that averufin (Fig. 3; Pusey and Roberts 1963) and its derivatives [8-*O*-methylaverufin (Fig. 3; Maskey et al. 2003) and novel analogues (the structures not shown in this paper; cf., the right column in Fig. 4, retention time (r. t.) = 10.8, 11.4, 13.1, 15.8, and 16.7 min)] could be detected by UV at 450 nm in the culture broth of FKI-4751 (Fig. 4b), but not in the broth of *S. coccosporum* CBS 364.58 (Fig. 4d). The averufin and its derivatives isolated from FKI-4751 were identified by measurement of one-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) and mass spectrometry (MS) spectra. On the other hand, spirostaphylotricin A (Fig. 3) and its analogues could not be found in the culture broth of FKI-4751. The spirostaphylotricins have been isolated from *S. coccosporum* (Sandmeier and Tamm 1989). Details on the novel averufin analogues will be reported elsewhere. In this study, we clarified that the two different *Staphylotrichum* species produced structurally different compounds.

Staphylotrichum boninense Nonaka, Miyazaki & Masuma, sp. nov. Fig. 5

MycoBank no.: MB 561191

Coloniae in agaro PDA ad 25°C post 7 dies 54–59 mm diametro attingentes; coloniae in agaro OA ad 25°C post 7 dies 51–52 mm diametro attingentes; coloniae in agaro PCA ad 25°C post 7 dies 54 mm diametro attingentes. Conidiophora plerumque macronemata, mononemata, erecta, superne ramosa; stipites (380–)430–870 × 4.5–8.5 µm, inferne valde brunnei. Cellulae conidiogenae monoblasticae, cylindricae, 2.5–11.0(–16.5) × 1.5–3.0 µm. Conidia holoblastica, solitaria, unicellularia, hyalina vel dilute brunnea, globosa vel subglobosa, interdum ellipsoidea vel pyriformia, laevia, (8.3–)9.3–15.2 × (7.3–)8.2–13.7(–14.5) µm.

Holotypus: TNS-F-41734, colonia exsiccata in cultura (FKI-4751 = JCM 17908) ex solo sativo, Bonin Islands, Tokyo, Japonia, 15.6.2007, a K. Nonaka isolata.

Etymology: Latin, *boninense* = relating to Bonin Islands, referring to the type locality.

Colonies on PDA 54–59 mm in diameter after 7 days at 25°C, subelevated, floccose, golden yellow (5B7), covered with dark brown (6F4) conidia, with entire margin; exudate and soluble pigment not produced; reverse yellowish brown (5D6). Colonies on OA 51–52 mm in diameter after 7 days at 25°C, plane, floccose, greyish yellow (4B6), with entire margin; exudate sparse, pale brown; soluble pigment

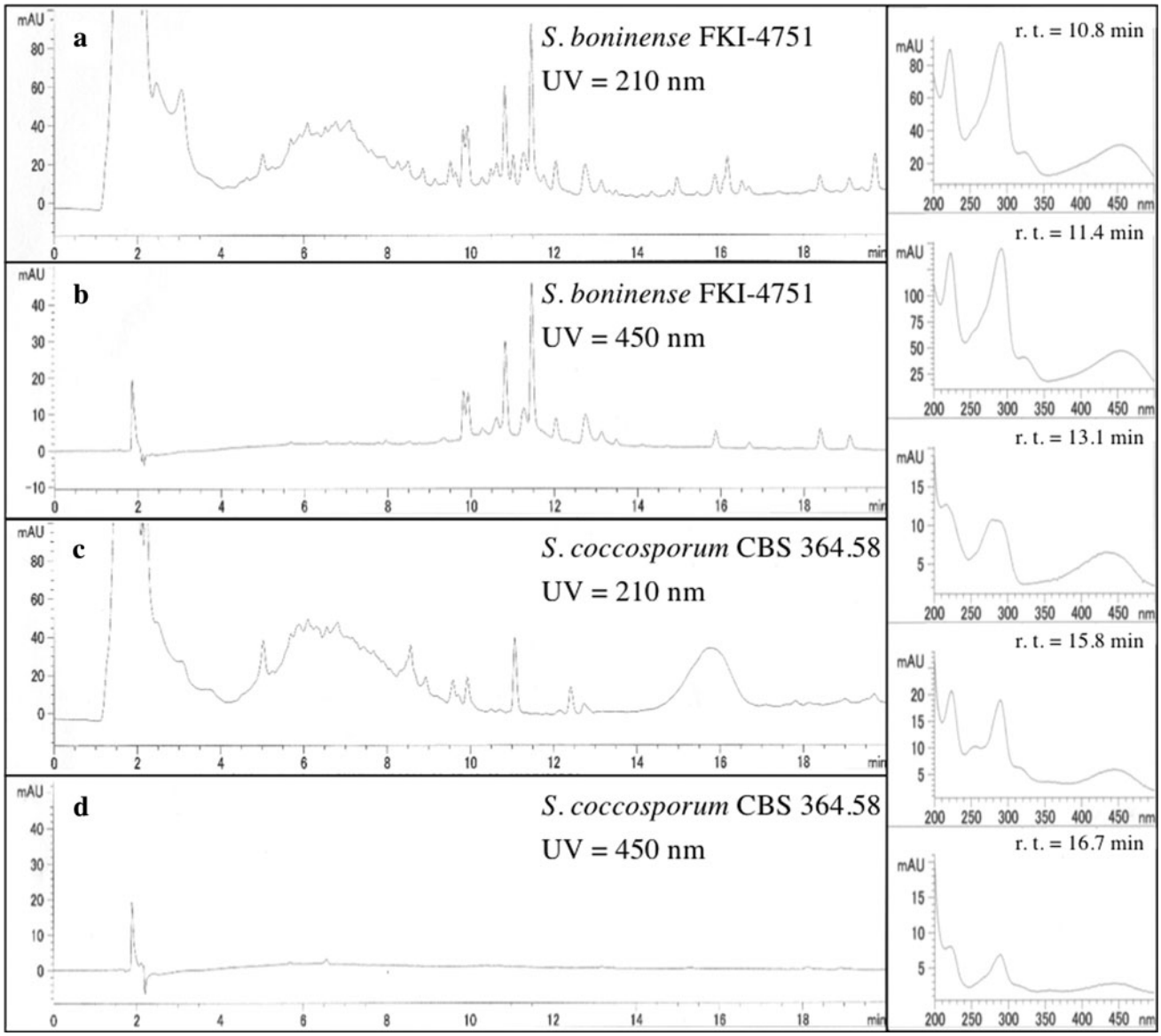

Fig. 4 HPLC chromatograms of extract derived from *Staphylotrichum boninense* FKI-4751 (ex-type; **a**, **b**) and *S. coccosporum* CBS 364.58 (ex-type; **c**, **d**) (**a**, **c** UV at 210 nm; **b**, **d** UV at 450 nm). UV spectrum data at the *right column* are for averufin analogues produced by FKI-4751

not produced; reverse orange yellow (4B8). Colonies on PCA about 54 mm in diameter after 7 days at 25°C, plane, floccose, white, covered with gray (5D1) conidia, with entire margin; exudate lacking; soluble pigment pink, produced after 10 days; reverse white.

No growth on PDA at 5°C and 37°C.

Hyphae on PCA hyaline to pale brown, straight or sinuous, branched, often anastomosed, septate, smooth-walled. Conidiophores usually macronematous, mononematous, erect, with branches and branchlets at the upper portion to form as conidial head, arising from a thick T-shaped foot cell (Fig. 5c); stipes straight or slightly flexuous, (380–)430–870 × 4.5–8.5 µm, thick-walled, dark brown in the lower part, becoming paler to hyaline upward; branches and branchlets formed at right angles to the main axis (Fig. 5a). Conidiogenous cells monoblastic, integrated, borne on the ends of the upper branches of the conidiophore or directly on short branches of the aerial hyphae, cylindrical, 2.5–11.0 (–16.5) × 1.5–3.0 µm, hyaline, smooth-walled, septate near the base (Fig. 5b). Conidia holoblastic, solitary, 1-celled, hyaline to pale brown, globose to subglobose, sometimes ellipsoidal or pyriform, smooth-walled, (8.3–)9.3–15.2 × (7.3–)8.2–13.7(–14.5) µm (Fig. 5d, e).

Materials examined: TNS-F-41734 (holotype), a dried culture (FKI-4751 = JCM 17908), from soil under

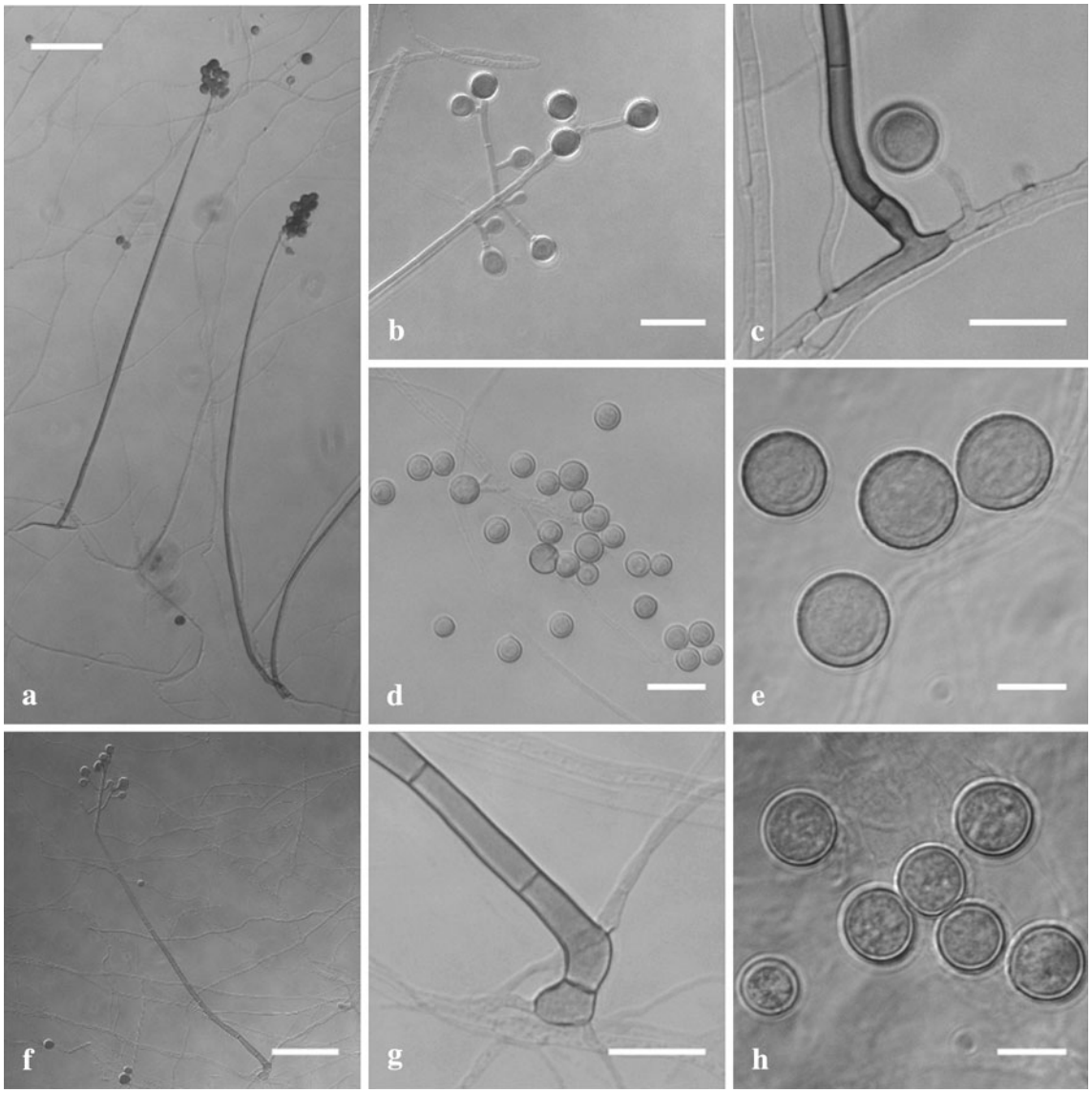

Fig. 5 *Staphylotrichum boninense* FKI-4751 (ex-type) (**a**–**e**) and *S. coccosporum* CBS 364.58 (ex-type) (**f**–**h**) on potato carrot agar (PCA). **a**, **f** Conidiophores. **b** Upper part of a conidiophore. **c**, **g** Foot cells of conidiophores. **d**, **e**, **h** Conidia. *Bars* **a** 100 μm; **b**, **f** 50 μm; **c**, **d**, **g** 20 μm; **e**, **h** 10 μm

Table 2 Morphological and physiological comparison among the species of *Staphylotrichum*

	S. boninense	*S. coccosporum*	*S. subramanianii*[a]
Stipes (μm)	(380–)430–870 × 4.5–8.5	(360–)450–700 × (5.0–)5.5–9.3(–10.0)	75–200 × 3–4
Conidiogenous cells (μm)	2.5–11.0(–16.5) × 1.5–3.0	(3.0–)3.5–11.0(–16.0) × 1.5–2.7(–3.5)	6–14 × 1–2.5
Conidia (μm)	(8.3–)9.3–15.2 × (7.3–)8.2–13.7(–14.5)	(7–)10.5–12(–14) diameter	6–8 × 5–7
	Smooth	Smooth	Spiral bands
	Globose to subglobose, sometimes ellipsoidal or pyriform	Globose to subglobose	Globose to subglobose, sometimes ellipsoidal or pyriform
Secondary metabolites	Averufin, 8-*O*-methylaverufin	Spirostaphylotrichins[b]	Not reported

[a] Data from Udagawa (1997)

[b] Data from Sandmeier and Tamm (1989)

Elaeagnus rotundata, Chichi-jima, the Bonin Islands, Tokyo, Japan, 15 June 2007, isolated by K. Nonaka. The holotype and ex-type strain were deposited in the National Museum of Nature and Science (TNS), Tsukuba and Japan Collection of Microorganisms (JCM), Wako, respectively. FKI-4859 (=JCM 17909) and FKI-5336 (=JCM 17910), from soils under *Clinostigma savoryanum* and *Ligustrum micranthum*, respectively, Chichi-jima, the Bonin Islands.

The genus *Staphylotrichum* consists of only two species (Seifert et al. 2011): i.e., *S. coccosporum* (Nicot and Meyer 1956) and *S. subramanianii* Udagawa (Udagawa 1997). In this study we examined the ex-type and authentic strains of *S. coccosporum*. Morphologically, *S. boninense* resembles *S. coccosporum*. The ex-type strain of *S. boninense* FKI-4751, however, showed 5% sequence difference in the ITS region from *S. coccosporum* CBS 364.58 (ex-type; AB625584), and phylogenetic trees clearly separated the two from each other (Figs. 1, 2). Also, the clade of *S. boninense* was shown with high bootstrap value support. The ex-type strain (SUM 3034; Udagawa 1997) of *S. subramanianii* was unfortunately unavailable for this study. Therefore, we were not able to compare the sequences of *S. boninense* and *S. subramanianii.* However, the conidial surface of *S. boninense* (smooth-walled under a light microscope) is quite different from those of *S. subramanianii* (spiral bands), and the conidia of *S. boninense* (9.4–15.2 × 8.2–13.7 μm) are larger than those of *S. subramanianii* (6–8 × 5–7 μm) (Table 2).

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