Cryptogamie, Mycologie, 2005, 26 (2): 133-141

Geosmithia argillacea is the anamorph of *Talaromyces eburneus* as a heat resistant fungus

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Abstract – *Talaromyces eburneus*, previously unregarded as a heat resistant fungus, is re-described on new isolate from a spoilage outbreak that involved a pasteurized pineapple juice in Japan. Based on examination of the new isolate, type studies, and the D1/D2 region of 28S rDNA sequence analysis, we conclude that *Geosmithia argillacea* is assigned to the teleomorphic species *T. eburneus* as an anamorph. Heat resistant fungus identification such as this finding is important in the study of food spoilage.

Heat resistant fungi / *Talaromyces* / *Geosmithia* / *Penicillium* / systematics / 28S rDNA

INTRODUCTION

Heat resistant fungi are often reported as spoilage agents in fruit juices and other heat processed fruit based products (Samson *et al*., 1992; Tournas, 1994; Scholte *et al*., 2000; Udagawa, 2000). Frequently, the spoilage of fruit products by heat resistant fungi is mostly caused by ascospores because of their strong longevity more than mycelium and conidia. For ascospores of *Talaromyces flavus* (Klöcker) Stolk & Samson and *T. macrosporus* (Stolk & Samson) Frisvad *et al*., a D_{85} of 20-100 min and D_{90} of 2.5-11.1 min (Scholte *et al*., 2000), whereas conidia of the very common genera such as *Penicillium* and *Aspergillus*, *etc*. are killed after heating for 10 min at 60°C. Spoilage due to formation of heat resistant ascospores by some members of the genus *Byssochlamys*, *Eupenicillium*, *Hamigera*, *Neosartorya* and *Talaromyces* has occurred repeatedly. However, on the role of mitosporic fungi in spoilage of pasteurized products, information is often scattered. Heat resistant chlamydospores, thick-walled vegetative mycelium and sclerotia have been described for a few causal agents.

Problems caused by *Geosmithia* sp. were initially encountered in spoiled canned lemon tea drink in 1990, but the spoilage attributed to this fungus could not be recognized in the repeated test. The main reason was because there was no evidence of formation of heat resistant structures in the isolate culture. An out-

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break of fungal contamination of pasteurized pineapple juice in a beverage industry was recently occurred and an isolation of *Talaromyces eburneus* Yaguchi *et al.* with a *Geosmithia* anamorph (Yaguchi *et al.*, 1994) as its causal agent was the reason for our redescription of the fungus as a previously unregarded heat resistant fungus in this paper. Thus we presume that ascospores of *T. eburneus* are sufficiently resistant to survive on the thermal processes at pineapple juice products.

MATERIALS AND METHODS

Isolation and morphology: *Talaromyces eburneus* (with *Geosmithia* anamorph) SUM 3297 was isolated from a spoilage outbreak that involved a pasteurized pineapple juice at 70°C, 20 min, and identified based on morphological characteristics to species level, using Czapek Yeast Extract (CYA), Malt Extract (MEA), Oatmeal (OA) Agars according to the standard procedures (Pitt, 2000). A culture of the isolate was deposited at the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Inohana, Chuo-ku, Chiba 260-8673, Japan (IFM 53925). *Ex* type and authentic cultures of *T. eburneus* (CBM FA-940 ex type, IFM14455) and *Geosmithia argillacea* (Stolk *et al.*) Pitt (NBRC 31128 = CBS 101.69, *ex* holotype, NBRC 31148 = IMI 154253, and NBRC 32004) were examined.

Sequence analysis: *Talaromyces eburneus* is known to produce a *Geosmithia* anamorph (Yaguchi *et al.*, 1994). Type examination shows our heat resistant isolate SUM 3297 to be identical to *T. eburneus*, and its anamorph is regarded as *G. argillacea* morphologically (Stolk *et al.*, 1969; Pitt, 1979). To infer the taxonomic clarification of the heat resistant isolate and its anamorphic affinities of *G. argillacea*, DNA was extracted from potato-dextrose agar cultures of all the examined strains with a DNA extraction kit (Dr, GenTLE^{TM} , Takara Bio Inc., Shiga, Japan). Two µl of DNA extract, a piece of Ready-to-Go beads (Amersham Pharmacia Tokyo, Japan), 2 µl of 10 pM of the primers NL-1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL-4 (5'-GGT CCG TGT TTC AAG ACG G-3') (Kurtzman & Robnett, 1997) in 19 µl of distilled water were mixed. The reaction mixture was subjected to 1 cycle of denaturation at 95°C for 4 min, 30 cycles of amplification at 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and a final extension cycle at 72°C for 10 min with a PCR Thermal Cycler MP (TaKaRa). The PCR-amplified samples were purified by PCR purification kit (QIAquick®, Qiagen Co. Ltd., Tokyo, Japan), labeled by with BigDye® terminator Ver. 1.1 (Applied Biosystems, Foster City, CA., USA) following to the manufacture's protocol and using primers NL-1 and NL-4 by a following amplification method: 96°C for 1 min, thereafter, 25 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes. The labeled samples were directly sequenced by ABI PRISM® 3100 (Applied Biosystems, Foster City, CA., USA) sequencer.

Molecular phylogenetic analysis: The sequences were aligned by using Clustal X software (Thompson *et al.*, 1997). For the neighbor-joining analysis (Saitou & Nei, 1987), the distances between sequences were calculated using Kimura's two-parameter model (Kimura, 1980). A bootstrap analysis was conducted with 1000 replications (Felsenstein, 1985).

Table 1. List of taxa sequenced in this study and additional taxa included in the analysis.

Taxon	*Strain number*	*DDBJ* accession number*
Talaromyces eburneus Yaguchi *et al.* (*ex* type)	IFM 14455 (= CBM FA-940)	AB196357
Talaromyces eburneus	IFM 53925 (= SUM 3297)	AB196358
Talaromyces emersonii Stolk (*ex* type)	CBS 393.64	AB196359
Talaromyces flavus (Klöcker) Stolk & Samson (*ex* type)	CBS 310.38	AB196360
Geosmithia argillacea (Stolk *et al.*) Pitt (*ex* type)	NBRC 31128 (= CBS 101.69)	AB047236**
Geosmithia argillacea	NBRC 3148 (= IMI 154253)	AB047237**
Geosmithia argillacea	NBRC 32004	AB047238**

*: DNA Data Bank of Japan.
**: Data of Ogawa *et al.*

RESULTS

DNA sequences of the D1/D2 region of 28S rDNA of the strains listed in Table 1 were determined. New sequences were deposited in the DNA Data Bank of Japan (DDBJ), and the accession numbers were listed in Table 1. In this analysis (Fig. 1), the heat resistant isolate and *T. eburneus* were strongly supported as conspecific (the sequence homology = 99.7%). Moreover, the three strains of *G. argillacea* (including the *ex* type culture NBRC 31128 (= CBS 101.69)) showed identical sequence in this region, and could be the same that was identified with the anamorph of *T. eburneus*.

TAXONOMY

***Talaromyces eburneus* Yaguchi, Someya & Udagawa, *Mycoscience*, 35: 249. 1994.**
Figs. 2-8

Anamorph: *Penicillium argillaceum* Stolk, Evans & Nilsson, *Trans. Br. Mycol. Soc.*, 53: 307. 1969.
Geosmithia argillacea (Stolk *et al.*) Pitt, *Can. J. Bot.*, 57: 2026. 1979 (Basionym).
Geosmithia eburnea Yaguchi *et al.*, *Mycoscience*, 35: 249. 1994.

Colonies on MEA growing rapidly, attaining a diameter of 50-52 mm in 7 days at 30°C, floccose, plane, consisting of a thin basal felt, Greyish Yellow (M. 4B4, after Kornerup & Wanscher, 1978) to Brownish Orange (M. 5C4), becoming Pale Yellow (M. 2A3) in 30 days from the later development of abundant ascomata which are embedded in the mycelial felt; margins thin, broad, entire; conidiogenesis moderate; exudate and soluble pigment absent; reverse uncolored to Greyish Yellow (M. 4B4).

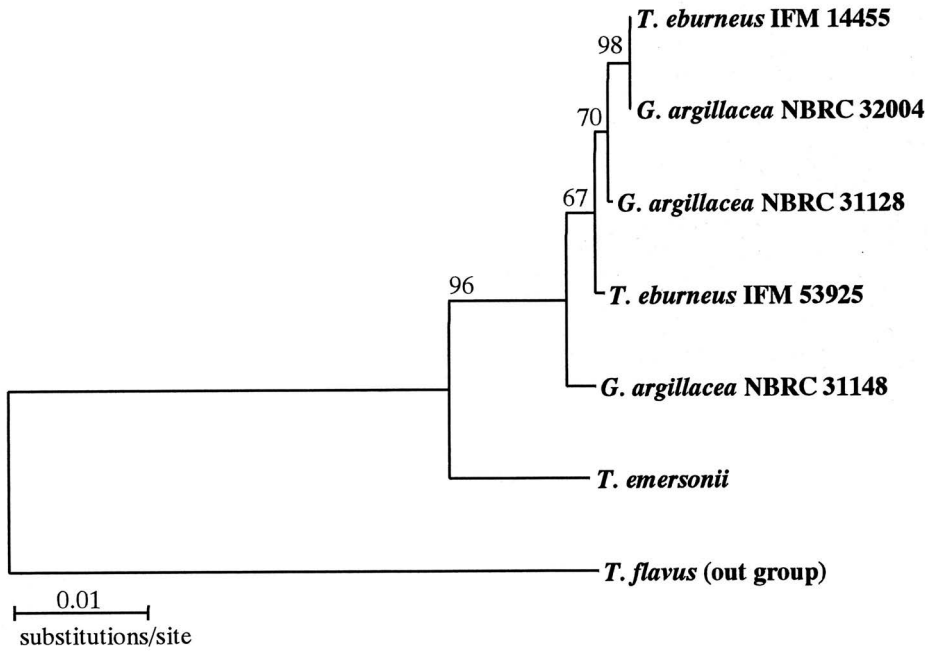


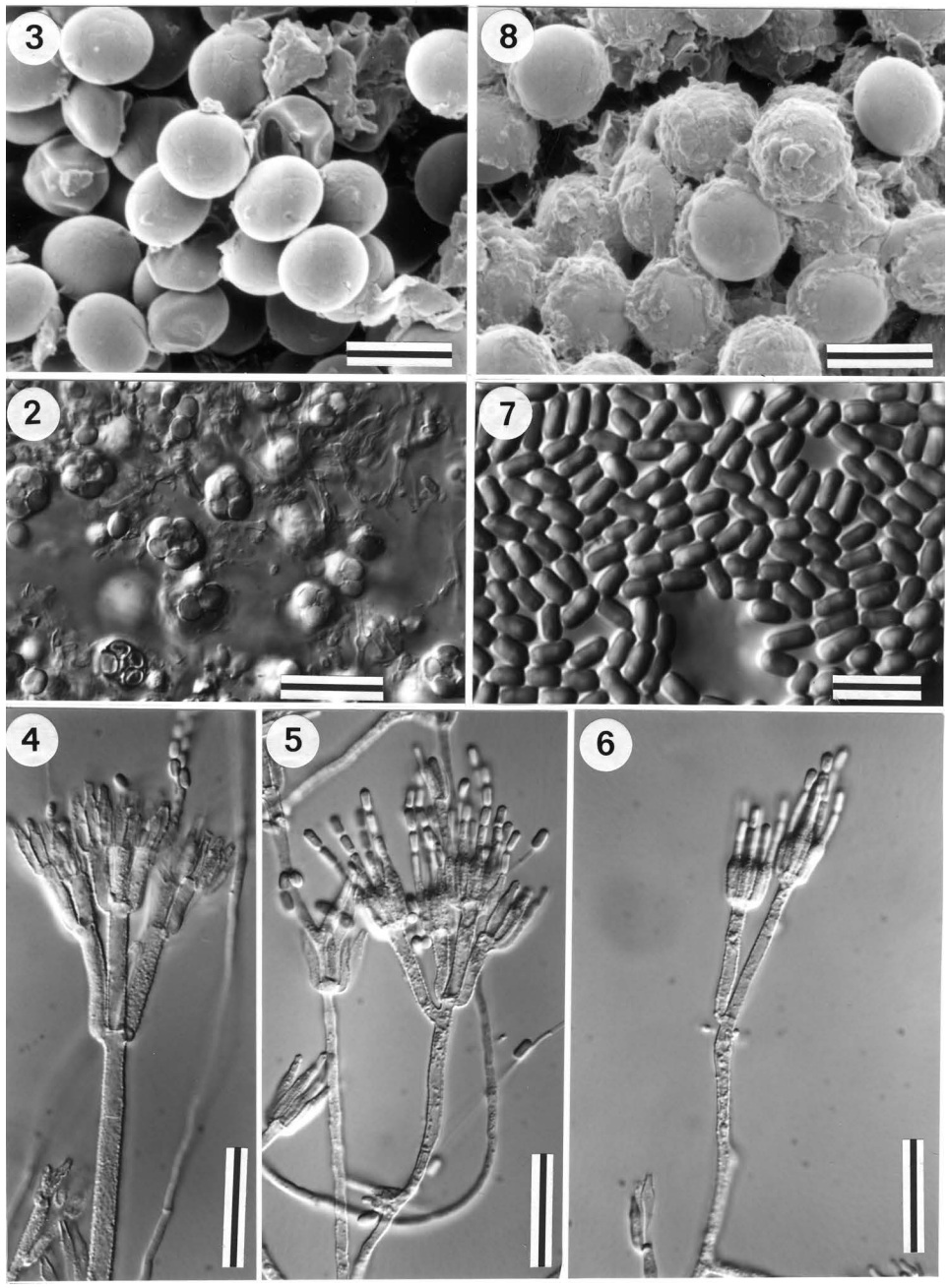
Fig. 1. Neighbor-joining tree from sequences of D1/D2 region of 28S rDNA. Each number indicates the percentage of bootstrap samplings, derived from 1000 samples, supporting the internal branches of 50% or higher.

Colonies on OA growing rapidly, 45-46 mm in 7days at 30°C, velvety, plane, thin, vegetative mycelium submerged; ascomata later scattered on the mycelial felt, off-white in color; conidiogenesis abundant, Yellowish Brown (M. 5D4); exudate small, clear; reverse uncolored.

Colonies on CYA growing fairly rapidly, 33-35 mm in 7days at 30°C, velvety to floccose, plane, thin, Brownish Orange (M. 6C3); ascomata lacking; conidiogenesis abundant, more or less powdery; reverse Greyish Orange (M. 6B3).

Ascomata scattered or irregularly confluent, non-ostiolate, pale yellow, maturing slowly within 28 to 35 days, globose to subglobose, 70-125 μm in diameter, soft, covered by hyaline to pale yellow, encrusted, septate hyphae. Ascomatal initials consisting of a swollen branching hyphae but often indistinct. Asci 8-spored, borne singly, subglobose to ovoid, or pyriform, 10.5-13(-15) × 8-9.5(-11) μm, evanescent. Ascospores pale yellow, subglobose to somewhat ovoid, 4-5 × 4-4.5 μm, thick-walled, smooth but occasionally with foveolations (Fig. 8) with an equatorial thickening (under SEM).

Conidiophores arising primarily from the basal mycelium, but also as perpendicular branches from aerial and trailing hyphae or the main axis of conidiophores; stipes (20-)50-400 × (2-)3-4 μm, verrucose, occasionally smooth. Penicilli variable, mostly biverticillate but with terverticillate or sometimes monoverticillate. Rami 1-3 per stipe, 10-30 × 3-4 μm, verrucose. Ramuli 10-15 × 3-4 μm. Metulae mostly appressed verticils of 2-6, verrucose, 8-20 × 2-4 μm, often with enlarged apices, verrucose to smooth. Phialides cylindrical, appressed, 2-10 in the



Figs 2-8. *Talaromyces eburneus* (2-7 from IFM 53925 and 8 from IFM 14455). 2. Asci. 3. Ascospores. 4-6. Penicilli. 7. Conidia. 8. Ascospores. Scale bars: 2 = 20 µm; 3 = 5 µm; 4-6 = 20 µm; 7 = 10 µm; 8 = 5 µm.

verticil, (8-)10-16 × 2-3 μm, verruculose, sometimes smooth, tapering gradually to long collula. Conidia hyaline, at first cylindrical or ovoid, (2.5-) 3-5(-7) × 1-2 μm, later ellipsoidal or ovoid, 2.5-4 × 2-3 μm, smooth-walled, borne in disordered chains up to 250 μm or more long.

Growth temperatures: minimum ca. 15°C, optimum 35°C, maximum ca. 50°C (thermotolerant).

Source of strains: IFM 53925 (= SUM 3297), isolated by 45°C culture from a spoiled pineapple juice that was pasteurized at 70°C, 20 min, Tokyo, Japan, April 2004, by S. Udagawa; IFM 14455 (= CBM FA-0940), *ex* type culture of *T. eburneus*, isolated from soil, Taipei, Taiwan, 1968, by T. Yaguchi. For *G. argillacea*, NBRC 31128 (= CBS 101.69), *ex* holotype, isolated from mine tips with very high surface temperature, Stratfordshire, UK, by H.C. Evans; NBRC 31148 (= IMI 154253), isolated from bagasse, Trinidad, by J. Lacey; and NBRC 32004, in Sake brewery, Japan, by T. Ito, 1986, examined.

The distinctive characteristics of *T. eburneus* are its thermotolerant growth, off-white to yellowish brown colony, pale yellow and slow-developing ascomata, subglobose to ovoid, smooth to slightly ornamented ascospores, verrucose, long conidiophores up to 400 μm long, variously verticillate penicilli, and cylindrical to ovoid, 2-2.5 μm wide conidia.

There are two other species of *Talaromyces* known to produce a *Geosmithia* anamorph: *T. bacillisporus* (Swift) C.R. Benjamin (anam. *G. swiftii* Pitt) and *T. emersonii* Stolk (anam. *G. emersonii*) (Stolk & Samson, 1972; Pitt, 1979).

Talaromyces bacillisporus is weakly thermotolerant (the maximum growth temperature: about 45°C). In addition, *T. bacillisporus* differs by dark green colony reverse color, rather rapidly ripening (14 days) ascomata, globose and spinulose ascospores, and very narrow, cylindrical conidia.

Talaromyces emersonii is strongly thermophilic (minimum and maximum growth temperatures are near 30°C and 55-60°C, respectively). Its ascomata are reddish to orange brown, ripening within 7 days. Ascospores are subglobose to ovoid, but without a sign of ornamentation.

The morphology of the anamorph of *T. eburneus* was compared to the published descriptions (Stolk *et al*., 1969; Pitt, 1979), and to the NBRC strains of *G. argillacea*. No evidence of a teleomorph was detected on the culture of NBRC 31128 (the *ex* type strain), but based on morphological features of the conidiogenous cells and conidia as well as the molecular data derived by the almost identical sequences (99.5-99.8) in the D1/D2 region of 28S rDNA analysis, we concluded that the anamorph of *T. eburneus* is conspecific to *G. argillacea*.

DISCUSSION

Until recently, the eight species of *Geosmithia* were accepted and listed in "List of accepted species and their synonyms in the family Trichocomaceae" (Pitt *et al*., 2000). When Pitt (1979) erected the genus to accommodate species of the *Penicillium pallidum* series, he separated it from *Penicillium* by following characters: colonies with conidia in colors other than green, penicilli with all elements roughened, and with phialides and conidia cylindrical.

However, based on their phylogenetic study using 18S, 5S and 28S rDNA sequence analysis, Ogawa *et al*. (1997) concluded that *Geosmithia* species are not monophyletic and *G. lavendula* (Raper & Fennell) Pitt, the type species of the genus, and *G. putterillii* (Thom) Pitt are placed within the pyrenomycete lineage comprising the hypocrean fungi such as *Gliocladium*-producing *Hypocrea lutea* (Tode) Petch. The hypocrean *Geosmithia* members are often associated with bark beetles and other subcorticolous insects, and not known to produce a teleomorph either on natural substrate or after prolonged incubation on agar plates (Kolařik *et al*., 2004). In the latest paper of Kolařik *et al*., a study of bark beetle associated *Geosmithia* isolates by RAPD and ITS sequence analysis revealed eight groups, including new and previously synonymized species. Thus, for a group of isolates formerly identified as *G. putterillii*, the new species *G. flava* (G. smith) Kolařik *et al*. was proposed based on a characteristic RAPD-type, a unique ITS sequences and a different phenotype.

The remaining species including *Geosmithia* anamorphs of three *Talaromyces* species and *T. macrosporus* (anam. *Penicillium macrospoum* Frisvad *et al.*) are placed in the monophyletic family Trichocomaceae of the plectomycete lineage with 100% bootstrap support (Ogawa *et al*., 1997; Ogawa & Sugiyama, 2000). Hence the name *Penicillium argillaceum* is now the correct name for the anamorph of *T. eburneus*.

Spoilage of heat processed food products by *Talaromyces* species has been recognized and documented in several countries (Beuchat, 1986; Scott & Bernard, 1987; King & Halbrook, 1987; King & Whitehand, 1990; Enigl *et al.*, 1993; Jesenská *et al*., 1991; Samson *et al.*, 1992; Tournas, 1994; Pitt & Hocking, 1997; Scholte *et al*., 2000; Udagawa, 2000). In the genus, *T. flavus, T. macrosporus* and *T. trachyspermus* (Shear) Stolk & Samson are important spoilage organisms which are capable of surviving pasteurization heat treatments given to fruit juices and fruit-based products. *Talaromyces bacillisporus*, *T. striatus* (Raper & Fennell) C.R. Benjamin and *Hamigera avellanea* (Thom & Turesson) Stolk & Samson (≡ *Talaromyces avellaneus*) have now become common in spoilage of heat-processed foods (Samson *et al.*, 1992; Pitt & Hocking, 1997; Udagawa, 2000). Most of these fungi are widely distributed in soil (Domsch *et al*., 1980; Fravel & Adams, 1986; Jesenská *et al*., 1993), and consequently may cause spoilage problems in food products containing fruits which are readily contaminated by soil, *e.g*. apples, berry fruits, mangoes, passion fruits, pineapples, or tomatoes.

Penicillium argillaceum is principally of soil origin (Minoura *et al*., 1973), but also occurs upon self-heating plant materials undergoing natural aerobic decomposition. It is rather common in soft wood chip piles where high temperatures 30-35°C up to 50°C (Stolk *et al*., 1969). There is little evidence of *P. argillaceum* being found on foods; Ramirez (1982) isolated it from peppers, Madrid, in Spain, and we recorded it as *Geosmithia* sp. from spoiled canned black tea drinks (Udagawa, 1991). The finding of teleomorphic form of *P. argillaceum* in this paper is significant, because the organism has little known as heat-resistant and has previously not been described to produce ascospores. The heat resistance for the isolate of *T. eburneus* from spoiled pineapple juice (after pasteurization at 70°C and 20 min) indicates that this organism may survive commercial processes if sufficient ascospores are present. However, it should be noted that this organism has been the cause of spoilage only infrequently. It is not known if this is related to the limited distribution in nature, to small amounts of ascospore formation or to other factor.

As an approach to establish a good practice of processing and handling of fruit juices, further information on thermoresistance and heat inactivation of *T. eburneus* ascospores is required.

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