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SHORT COMMUNICATION

Penicillium viticola, a new species isolated from a grape in Japan

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Abstract A new monoverticillate *Penicillium* species (subgenus *Aspergilloides* Dierckx), *Penicillium viticola*, was isolated from a grape cultivated in Yamanashi Prefecture, Japan. Morphologically, *P. viticola* is characterized by the production of slightly roughened conidia, slightly roughened stipes, and rapid growth on 25% glycerol nitrate agar (G25N). This species is phylogenetically close to *Penicillium angulare* S.W. Peterson, E.M. Bayer & Wicklow, but differs with respect to colonial characteristics and conidia and penicilli morphology.

Keywords Calmodulin gene · *Penicillium viticola* sp. nov. · Taxonomy

We have isolated numerous fungal strains from soil and plant samples during the course of our screening program to discover antiinfective agents. A novel antimalarial agent was discovered from the secondary metabolites of a fungal strain designated FKI-4410. FKI-4410 was identified as belonging to the genus *Penicillium*, but it was not one of the known *Penicillium* species.

The fungus, which was isolated from a grape harvested in Yamanashi Prefecture, Japan, was incubated on potato dextrose agar (PDA) at 25°C.

For determination of the morphological characteristics, the methodology of Pitt (1979) was used.

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Digital photographs of colonies were taken with a PowerShot G10 digital camera (Canon, Tokyo, Japan) and adjusted using the PhotoStudio 4 software (Canon).

The *Color Harmony Manual*, 4th edition (Container Corporation of America, Chicago) was used to determine color names and hue numbers (Jacobson et al. 1958).

For the determination of micromorphological characteristics, microscope slides were examined with a Vanox-S AH-2 microscope (Olympus, Tokyo, Japan), and digital micrographs were taken with a DP25 digital camera (Olympus). For scanning electron microscopy (SEM) of the conidia and conidiophores, agar blocks (3 mm^2) were cut from a 7-day-old culture of the strain FKI-4410 growing on malt extract agar (MEA). The agar blocks were fixed with osmium tetroxide (TAAB, Berkshire, UK), air dried, and sputter coated with gold using a JFC-1200 Fine Coater (JEOL, Tokyo, Japan). The samples were observed under a JSM-5600 scanning electron microscope (JEOL).

Genomic DNA of the strain FKI-4410 was isolated using the PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Amplification of the partial calmodulin gene region, the partial beta-tubulin gene region, and internal transcribed spacer (ITS) and large subunit (LSU) rDNA (ID) regions were performed using primers CF1 and CF4 (Peterson et al. 2005), Bt2a and Bt2b (Glass and Donaldson 1995), and ITS1 and NL4 (White et al. 1990; O'Donnell 1993), respectively. Polymerase chain reactions (PCR) were performed with QIAGEN Fast Cycling PCR Kit protocol (Qiagen, Valencia, CA, USA).

Amplifications were performed in a PCR Verity 96-well thermal cycler (Applied Biosystems), programmed for 5 min with denaturation at 95°C, followed by 35 cycles consisting of denaturation at 96°C for 5 s, primer annealing for 5 s at 50°C, extension for 21 s at 68°C, and a final

1 min elongation step at 72°C. After amplification of each gene templates, excess primers and deoxynucleosides (dNTPs) were removed from the reaction mixture using a QIAquick PCR DNA Purification kit protocol (Qiagen). The PCR products were sequenced 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 ultra pure sterile water, and 0.5 µl primer (5 pmol/µl). Reactions were run in a PCR thermal cycler, programmed for 1 min at 96°C, then by 25 cycles of 10 s denaturation at 96°C, followed by primer annealing for 5 s at 50°C, and extension for 4 min at 60°C. Sequencing products were purified by ethanol/ethylenediaminetetraacetic acid (EDTA) precipitation, and samples were analyzed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Contigs were assembled using the forward and reverse sequences with the SeqMan and SeqBuilder programs from the Lasergene8 package (DNAStar, Madison, WI, USA). The sequences of the strain FKI-4410 were deposited at the DNA Data Bank of Japan (DDBJ) with accession number AB540173 (calmodulin), AB540174 (beta-tubulin), and AB606414 (ID).

To determine the most closely related *Penicillium* species, the DNA sequences in the calmodulin gene region, the beta-tubulin gene region, and the ID region of FKI-4410 were compared to other sequences in the GenBank database by BLASTN 2.2.21 analysis. The calmodulin gene sequences of phylogenetically related *Penicillium* species were obtained from GenBank (see Fig. 1 for the GenBank accession numbers) and aligned using Clustal X 2.0.11 (Tompson et al. 1997) with the pairwise and multiple alignment parameters set at 15.0 for gap opening and 0.2 for gap extension. Alignment was refined using SeaView (Galtier et al. 1996), and the alignment was deposited in TreeBASE (http://www.treebase.org/) with accession number S11170. Phylogenetic analyses were based on the neighbor-joining (NJ) method (Saitou and Nei 1987) using Clustal X 2.0.11. Bootstrap analyses were performed on NJ trees with 1,000 bootstrap replicates. The trees were rooted with *Aspergillus* spp. and viewed with NJplot (Perrière and Gouy 1996).

Morphologically, FKI-4410 is classified (Pitt 1979) in the subgenus *Aspergilloides* sect. *Exilicaulis* Pitt based on monoverticillate penicilli that are nonvesiculate. However, FKI-4410 is different from all species of the section in having slightly roughened stipes and rapid growth on 25% glycerol nitrate agar (G25N) (>20 mm) (Pitt 1979; Peterson and Sigler 2002; Peterson et al. 2004; Peterson and Horn 2009). In contrast, the other newly described three species (*P. dravuni* Janso, *P. coffeae* S.W. Peterson, F.E. Vega, Posada & Nagai, and *P. macrosclerotiorum* L. Wang, X.M. Zhang & W.Y. Zhuang) belong to the section *Aspergilloides* (Janso et al. 2005; Peterson et al. 2005; Wang et al. 2007).

In a BLAST search using the blastn from the National Center for Biotechnology Information (NCBI) (Altschul et al. 1990), FKI-4410 had 91.5% similarity to the partial calmodulin gene sequences of *P. angulare* (EF198582). In the NJ analysis, FKI-4410 is most closely related to *P. angulare* (subgenus *Aspergilloides* sect. *Exilicaulis*), and the group was supported by a high bootstrap value (see Fig. 1). Nevertheless, FKI-4410 differs from *P. angulare* in colonial texture, growth rates, and length of stipes (Peterson et al. 2004). Conversely, in a BLAST search, FKI-4410 had 88.3% similarity to the partial beta-tubulin gene sequences of *P. multicolor* Grig.-Man. & Porad. (EU427265) and 98.4% similarity to the ID sequences of *P. sclerotiorum* J.F.H. Beyma (AF033404). However, the stipe of FKI-4410 (nonvesiculate) is different from those of *P. multicolor* and *P. sclerotiorum* (vesiculate). Therefore, FKI-4410 represents a novel species of the genus *Penicillium*, for which the name *Penicillium viticola* sp. nov. is proposed.

We discovered that the novel *P. viticola* produces the tropolone compounds puberulic acid and stipitatic acid and their novel analogues, viticolins A–C. In previous reports, puberulic acid has only been isolated from *Penicillium puberulum* Bainier belonging to the subgenus *Penicillium* (Birkinshaw and Raistrick 1932). We have observed that the compounds produced by *P. viticola*, notably puberulic acid, had antimalarial properties, and we reported these findings elsewhere (Iwatsuki et al. 2010).

Penicillium viticola Nonaka & Masuma, sp. nov.

Figs. 2–8

MycoBank no.: MB 516048

Coloniae in agaro CYA post 7 dies 25°C 34–35 mm diametro. Coloniae in agaro MEA post 7 dies 25°C 26–28 mm diametro. Coloniae in agaro G25N post 7 dies 25°C 20–21 mm diametro. Conidiophora plerumque ex hyphis basalibus. Stipes scabridis, 20–85 × 3.0–3.5 µm. Penicilli monoverticillati, phialides ampulliformes, 8.0–12.5 × (2.0–)3.0–4.2 µm. Conidia subglobosa ad ellipticus, leviter scabrida, 2.7–4.0(–4.3) × 2.2–3.7 µm.

Holotypus: TNS-F-38702 colonia exsiccata in cultura ex uva sativo (*Vitis* sp.), Yamanashi Pref., Japonia, 11.8.2006, a K. Nonaka isolata et in herbario fungorum TSN conservata; cultura viva ex holotypo in JCM ut 17636.

Etymology: Latin, *viticola*, referring to dweller on the vine (*Vitis* sp.)

Colonies on Czapek yeast extract agar (CYA) 34–35 mm in diameter after 7 days at 25°C (Fig. 2a), radially sulcate, velutinous, with white (a) mycelium at the margins, covered with olive gray (1 ig) conidia, exudate

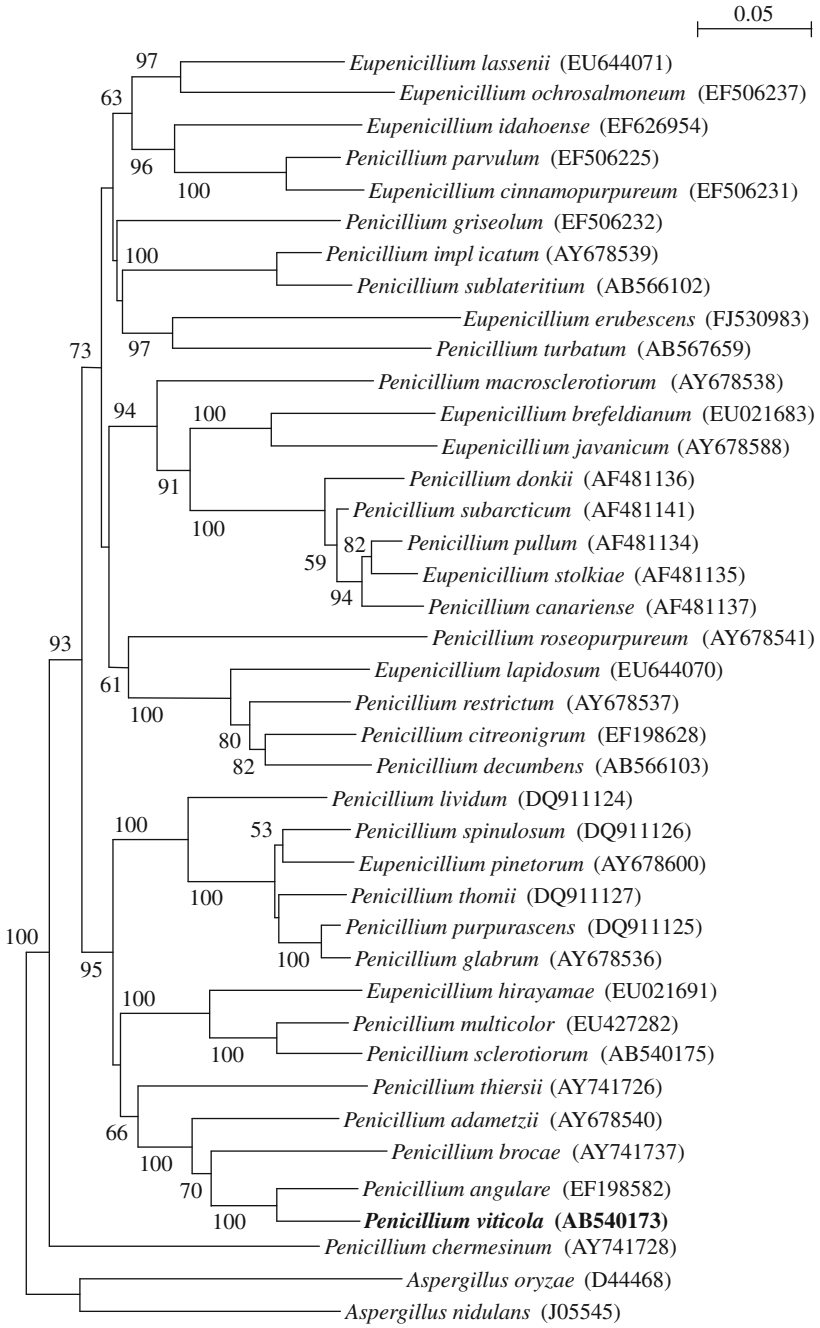


Fig. 1 Phylogenetic tree for *Penicillium viticola* and the related species of the genera *Penicillium* drawn from neighbor-joining (NJ) analysis of the partial calmodulin gene region sequences. The outgroups are *Aspergillus oryzae* and *Aspergillus nidulans*. The values shown in the *branches* represent bootstrap values based on 1,000 replicates above 50. The number of nucleotide changes between taxa is represented by the *branch length*

lacking, the reverse luggage tan (4 ne), the margin entire, soluble pigment not produced.

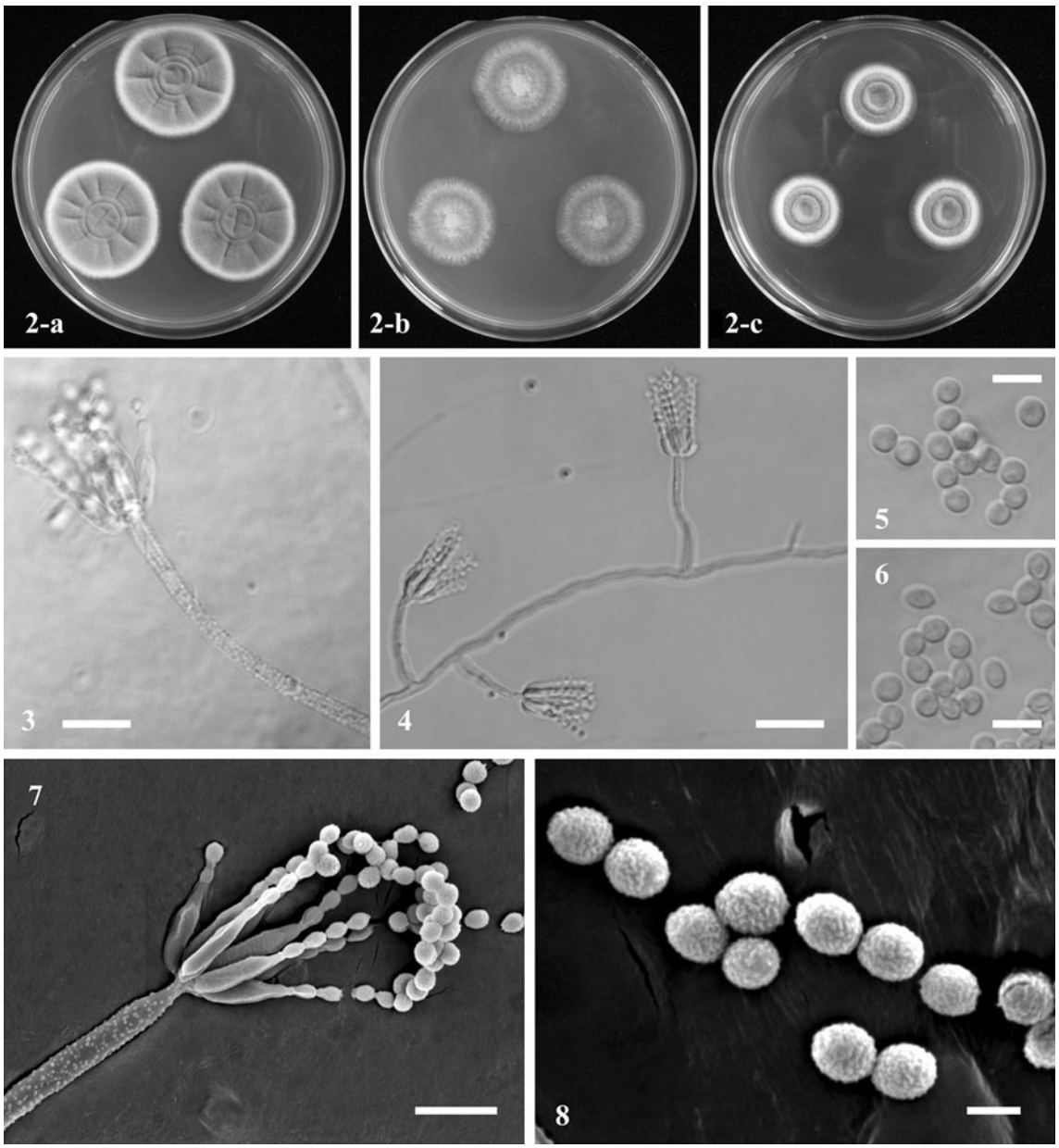
Colonies on malt extract agar (MEA) 26–28 mm in diameter after 7 days at 25°C (Fig. 2b), plane, with bamboo (2 gc) floccose aerial mycelium, covered with moss green (24 pi) conidia, exudate sparse clear drops, the reverse pale olive (1 ie), the margin entire, soluble pigment not produced.

Colonies on G25N 20–21 mm in diameter after 7 days at 25°C (Fig. 2c), zonate, velutinous, with white (a) mycelium at the margins, covered with olive gray (1 ig) conidia, exudate lacking, the reverse luggage tan (4 ne), the margin entire, soluble pigment not produced.

Colonies on CYA at 5°C, 37°C: no growth.

Conidiophores on MEA borne on a basal felted hyphae or directly from the agar, stipes were simple, slightly roughened, nonvesiculate, 20–85 × 3.0–3.5 µm, with a heavy wall. Penicilli typically monoverticillate (Figs. 3, 4, 7).

Phialides ampulliform, 8.0–12.5 × (2.0–)3.0–4.2 µm, collula 0.5–1.5 µm wide. Conidia borne in chains,



Figs. 2–8 *Penicillium viticola* FKI-4410. **a** Colonies on Czapek yeast extract agar (CYA) at 25°C for 7 days. **b** Colonies on malt extract agar (MEA) at 25°C for 7 days. **c** Colonies on 25% glycerol nitrate agar (G25N) at 25°C for 7 days. **3**, **4** Penicilli. **5**, **6** Conidia. **7** Penicillus, scanning electron microscopy (SEM). **8** Conidia, SEM. *Bars* **3**, **7** 10 μm; **4** 20 μm; **5**, **6** 5 μm; **8** 2 μm

subglobose to ellipsoidal, less commonly globose, slightly roughened, 2.7–4.0(–4.3) × 2.2–3.7 μm in size (Figs. 5, 6, 8). Sclerotia not produced.

Specimen, culture examined: TNS-F-38702 (holotype), a dried culture derived from the isolate (FKI-4410) from a grape, Yamanashi, Japan, 11 July 2006, isolated by K. Nonaka. The holotype deposited in the herbarium at The National Science Museum, Ibaraki, Japan (TNS). JCM 17636 (ex-type) deposited at the Japan Collection of Microorganisms (JCM), Saitama, Japan.

Note. *Penicillium viticola* is classified (Pitt 1979) in the subgenus *Aspergilloides* sect. *Exilicaulis* ser. *Citreonigra* Pitt based on typically monoverticillate penicilli, stipes nonvesiculate, and moderate growth on CYA and MEA for 7 days at 25°C. Morphologically, *P. viticola* resembles *P. decumbens* Thom and *P. adametzii* K.M. Zalessky: these all have a similar growth rate on CYA and MEA and short stipes (Pitt 1979). However, the colonial texture of *P. viticola* (velutinous) differs from that of *P. adametzii* (funiculose) and the conidial shape of *P. viticola*

Table 1 Comparison of *Penicillium viticola* FKI-4410 with the related taxa

	P. viticola	*P. decumbens*[a]	*P. adametzii*[a]	*P. angulare*[b]
CYA				
Diameter (mm)	34–35	20–30	25–35	13–18
Texture	Velutinous	Velutinous	Funiculose	Velutinous
Margin	Entire	Broad and deep	Entire	Irregular
Colonial color	Olive gray	Dull green	Dull green	Light celandine
Reverse color	Luggage tan	Pale, dull yellow brown	Pale to yellow brown	Baryta yellow
Soluble pigment	Not produced	Not produced	Not produced	Not produced
MEA diameter (mm)	26–28	25–40	30–35	9–15
G25N diameter (mm)	20–21	11–16	10–13	10–12
Stipe (μm)	20–85 × 3.0–3.5	20–60(–100) × 1.8–2.2(–2.5)	15–30(–50) × 1.8–2.2	100–150 × 3–4
	Nonvesiculate	Nonvesiculate	Nonvesiculate	Nonvesiculate
	Slightly roughened	Smooth	Smooth	Smooth
Phialides (μm)	8.0–12.5 × (2.0–)3.0–4.2	8–11(–14) × 2.2–2.5(–3.0)	6–8 × 2.0–2.2	8–10 × 2–3
Conidia (μm)	2.7–4.0(–4.3) × 2.2–3.7	2.5–3.0(–4.0) × 2.0–2.5(–3.0)	1.8–2.5 diameter	2.8–3.6 × 2.0–2.8
	Slightly roughened	Smooth	Smooth	Smooth
	Subglobose to ellipsoidal	Ellipsoidal	Spheroidal	Ellipsoidal

CYA Czapek yeast extract agar, *MEA* malt extract agar, *G25N* 25% glycerol nitrate agar

[a] Data are derived from Pitt (1979)

[b] Data are derived from Peterson et al. (2004)

(subglobose to ellipsoidal) is different from that of *P. adametzii* (spheroidal) (Table 1). The growth rate of *P. viticola* on CYA at 37°C (no growth) is different from that of *P. decumbens* (5–20 mm) and the conidial texture of *P. viticola* (slightly roughened) differs from that of *P. decumbens* (smooth walled) (Pitt 1979). Moreover, *P. viticola* showed greater distance from *P. decumbens* in the phylogenetic tree (Fig. 1).

Penicillium viticola is phylogenetically close to *P. angulare*, *P. brocae* S.W. Peterson, Jeann. Pérez, F.E. Vega & Infante, and *P. adametzii* (Fig. 1). The growth rate and colonial margin on CYA of *P. viticola* (more than 30 mm; entire) are different from those of *P. angulare* (less than 20 mm; irregular), and the stipes of *P. viticola* (20–85 μm) are shorter than those of *P. angulare* (100–150 μm) (Table 1). The stipe of *P. viticola* (nonvesiculate) is different from that of *P. brocae* (vesiculate), and the conidial shape of *P. viticola* (subglobose to ellipsoidal) is different from that of *P. brocae* (spheroidal) (Peterson et al. 2003). Moreover, FKI-4410 produced notably puberulic acid. The results of our studies showed that *P. viticola* was a distinct species, and it is one example of the production of novel compounds by novel species.

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