

Eupenicillium saturniforme, a New Species Discovered from Northeast China

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Abstract A new *Eupenicillium* species, *E. saturniforme* was isolated from soil in Jilin Province, northeast China. Morphologically, it resembled *E. shearii* and *E. tropicum*, but is distinguished from them by slowly maturing cleistothecia, lenticular ascospores with nearly smooth-walled convex surfaces, strictly velutinous colony texture with abundant conidiogenesis, robust biverticillate penicilli, apically vesiculate metulae and rough-walled stipes and conidia. The partial β -tubulin gene sequence of the new species (EU644080) showed relationship with *Penicillium glabrum* in the BLAST search in GenBank. Further analyses of partial calmodulin and ribosomal DNA internally transcribed spacer 1-5.8S-internally transcribed spacer 2 (rDNA ITS1-5.8S-ITS2) sequence data confirmed that *E. saturniforme* fell in the clade with *P. glabrum*, *P. lividum*, *P. purpurascens*, *P. spinulosum* and *P. thomii* of Subgenus *Aspergilloides*. However, *E. saturniforme* is a distinctive species lacking close relatives among described species of penicillia.

Keywords Biverticillate penicilli · Calmodulin gene · Holomorphic penicillia · Phylogenetics

Introduction

Environmental factors such as temperature, light, pH, nutrients, etc. regulate fungal development [1, 2]. In penicillia, high temperature has been confirmed to facilitate teleomorphic development. It has been argued that heat shock of 80°C could induce teleomorphs of penicillia [3] and heat treatment of 60°C could recover ascospore penicillia [4]. In the case of a new species reported here, low temperature seems to be a cue for teleomorphic development. The type culture of the new species was isolated from a soil sample collected from Dunhua, Jilin Province, which is located in the chilly area of moderate-temperate zone of China. The average altitude of that area is 756 m with an atmospheric pressure of 955 hPa; the annual average temperature is 2.6°C, with the monthly average of -17.4°C in January and 19.8°C in July; the period of frozen soil is from late October to mid-May; the frost-free period is about 120 days from late-May to late September; the annual precipitation is 620 mm [5]. That culture had long been regarded as one special isolate of *P. citrinum* for the presence of sclerotia and typical biverticillate penicilli with apically vesiculate metulae, and no teleomorphs was discovered. It was kept on malt

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extract autolysate agar (MEA) slants in cotton-plugged tubes at 4°C. In an occasional transference, the sclerotia were found to develop into cleistothecia, in which lenticular ascospores with nearly smooth convex surfaces were generated. Combining the morphological and molecular characters, we reported it here as a new member of *Eupenicillium*.

There are 47 species reported in *Eupenicillium* [6–9]. Ten of them produce lenticular ascospores with two appressed equatorial flanges, predominantly biverticillate penicilli, and moderate to fast growth, namely, *E. lapidosum* and *E. terrenum* in Series *Lapidososa* (except *E. reticulisporum*); and *E. baarnense*, *E. bovisimosum*, *E. crustaceum*, *E. egyptiacum*, *E. molle*, *E. sinaicum*, *E. shearii* and *E. tropicum* in Series *Crustacea* [10]. This species is a new member of this group. Morphologically, it resembles *E. shearii* and *E. tropicum*, but molecular phylogenetic analyses indicate that it is a new teleomorphic penicillia with no close relatives in *Eupenicillium*. Although there is only one isolate discovered hitherto, the status of this species is validated by both morphological and molecular studies.

Materials and Methods

Isolation

Dilution plating method was used in the isolation of the fungus [10, 11]. About 1 g of the soil sample was dispersed in 9 ml of sterile water by drastically shaking. Immediately, 1 ml of the suspension was transferred to a second tube containing 9 ml of sterile water; this process was repeated four times to yield dilution to 0.00001. One millilitre of each dilution was pipetted into two Petri dishes and 15 ml melted MEA with chlortetracycline and dichloran rose bengal chlortetracycline agar of about 45°C were poured, respectively, into each dish, then the plates were moved gently to make the media spread evenly. The plates were incubated at 25°C for several days and single colonies were selected and transferred into slants.

Cultures

Fifty-two isolates of which 32 penicillia were included in molecular phylogenetic analyses based on partial

calmodulin gene sequences, including 22 of which 14 species of *Eupenicillium* that possess lenticular ascospores with two appressed longitudinal flanges, 7 of which 5 species with apically vesiculate stipes from *Penicillium* subgenus *Aspergilloides*, 19 of which 10 species from subgenus *Furcatum* bearing typical biverticillate penicilli, 3 of which 2 species from subgenus *Penicillium* that produce sclerotia, and one from *Talaromyces*. Twenty-four strains were ex-type isolates. All strains were deposited in China General Microbiological Collection Center (CGMCC) (Table 1). Forty-nine species were included in the analyses of rDNA ITS1-5.8S-ITS2 sequences, including 27 in *Eupenicillium*, six in Subgenus *Aspergilloides*, 14 in Subgenus *Furcatum*, 1 each in Subgenus *Penicillium* and *T. bacillisporus* as the outgroup; 47 of them are ex-type isolates [12]. The ex-type strains were marked with ‘T’, and all the sequences newly generated in this study were submitted to GenBank.

Morphological Studies

Gross physiological characters were examined using the methods for identification proposed by Frisvad and Samson [13], Pitt [10] and Raper and Thom [14]. The names of colours were after Ridgway [15]. Wet mounts were prepared using materials from colonies on Czapek yeast autolysate agar (CYA) immersed in lactophenol solution without dye. Optical microscope examination and photography were done with an Axioplan2 imaging and Axiophot2 universal Microscope (Carl Zeiss Shanghai Co. Ltd., Shanghai, China). The scanning electron microscope (SEM) photographs were taken using a Hitachi SEM scanning electron microscope model S-570 (Hitachi Co. Ltd., Honsu, Japan).

DNA Extraction and Amplification

Genomic DNA extraction followed the methods of Scott et al. [16] with improvements for efficiency. For amplification of partial β -tubulin gene sequences, we used the primers by Glass and Donaldson [17]; for rDNA ITS1-5.8S-ITS2 sequences, the primers designed by White et al. [18] were used. Primers for amplification of partial calmodulin gene sequences, polymerase chain reaction (PCR) protocols for amplification of the above three gene segments, and the purification and sequencing of PCR products according to the methods of Wang and Zhuang [19, 20].

Table 1 Cultures included in molecular phylogenetic studies and the GenBank accession numbers for their partial calmodulin gene sequences

Species	Strains	Origins	GenBank Accession nos. ^a
<i>Eupenicillium baarnense</i>	AS3.5700	Soil, Pingxiang, Guangxi Province	AY678594
<i>E. baarnense</i>	AS3.6595	Soil, Mount Donglingshan, Beijing	AY678598
<i>E. brefeldianum</i>	AS3.10044	IMI 216895 T	EU644078
<i>E. brefeldianum</i>	AS3.5698	Soil, Pingxiang, Guangxi Province	AY678592
<i>E. brefeldianum</i>	AS3.6689	Soil, Nanning, Guangxi Province	AY678593
<i>E. crustaceum</i>	AS3.5727	Soil, Dunhuang, Gansu Province	AY678595
<i>E. crustaceum</i>	AS3.6688	Soil, Lanzhou, Gansu Province	AY678597
<i>E. egyptiacum</i>	AS3.10045	IMI 40580 T	EU644063
<i>E. lapidosum</i>	AS3.10059	CBS 343.48 T	EU644070
<i>E. lassenii</i>	AS3.10050	UVIC JWP 69-26 T	EU644071
<i>E. meloforme</i>	AS3.10065	NHL 6468 T	EU644066
<i>E. molle</i>	AS3.10047	TRTC 45714 T	EU644064
<i>E. ochrosalmoneum</i>	AS3.10077	CBS 489.66 T	EU644067
<i>E. ochrosalmoneum</i>	AS3.5707	Soil, Nanning, Guangxi Province	AY678590
<i>E. saturniformae</i>	AS3.6886	Soil, Little Peony Forest Reserve, Dunhua, Jilin Province T	EU644062 EU644080 EU644081
<i>E. shearii</i>	AS3.7956	CBS 290.48 T	EU644068
<i>E. shearii</i>	AS3.5680	Soil, Beihai, Guangxi Province	AY678599
<i>E. sinaicum</i>	AS3.10049	NHL 2894 T	EU644069
<i>E. sinaicum</i>	AS3.5718	Soil, Nanning, Guangxi Province	AY678586
<i>E. sinaicum</i>	AS3.5738	Soil, Tianshui, Gansu Province	AY678596
<i>E. terrenum</i>	AS3.10051	CBS 313.67 T	EU644065
<i>E. tularense</i>	AS3.10064	UVIC JWP 68-31 T	EU644072
<i>Penicillium citrinum</i>	AS3.7960	IMI 92196ii T	EU644074
<i>P. citrinum</i>	AS3.6675	Soil, Sanlin Town, Shanghai	AY678554
<i>P. citrinum</i>	AS3.6577	Soil, Zhangjiajie, Hunan Province	AY678555
<i>P. citrinum</i>	AS3.6672	Soil, Changchun, Jilin Province	AY678556
<i>P. corylophilum</i>	AS3.6561	Soil, Hangzhou, Zhejiang Province	AY678548
<i>P. daleae</i>	AS3.4472	IMI 89338 T	AY678560
<i>P. glabrum</i>	AS3.5702	Eucalyptus oil, Nanning, Guangxi Province	AY678536
<i>P. gladioli</i>	AS3.6582	Soil, Wushan County, Chongqing	AY678582
<i>P. italicum</i>	AS3.7899	CBS 339.48 T	DQ911135
<i>P. italicum</i>	AS3.6587	Soil, suburb of Shanghai	AY678571
<i>P. janczewskii</i>	AS3.7967	IMI 191499 T	EU644079
<i>P. janczewskii</i>	AS3.6566	Soil, Hangzhou, Zhejiang Province	AY678553
<i>P. janthinellus</i>	AS3.6559	Soil, Sanlin Town, Shanghai	AY678549
<i>P. lividum</i>	AS3.7970	IMI 39736 T	DQ911124
<i>P. macrosclerotiorum</i>	AS3.6581	Soil, Wushan County, Chongqing T	AY678538
<i>P. macrosclerotiorum</i>	AS3.5681	Soil, Nanning, Guangxi Province	DQ911123
<i>P. madriti</i>	AS3.7971	CBS 86563 T	EU644076
<i>P. purpurascens</i>	AS3.7975	IMI 39745 T	DQ911125
<i>P. scabrosum</i>	AS3.7979	IMI 285533 T	EU644077
<i>P. scabrosum</i>	AS3.5754	Soil, Mount Tai, Shangdong Province	AY678545

Table 1 continued

Species	Strains	Origins	GenBank Accession nos. ^a
<i>P. simplicissimum</i>	AS3.5751	Soil, Qufu, Shandong Povice	EU644073
<i>P. simplicissimum</i>	AS3.6550	Soil, Nanjing, Jiangsu Province	AY678551
<i>P. simplicissimum</i>	AS3.7907	ATCC48681	DQ911128
<i>P. spinulosum</i>	AS3.7980	IMI 24316i T	DQ911126
<i>P. steckii</i>	AS3.7981	IMI 40583 T	EU644075
<i>P. steckii</i>	AS3.5684	Soil, Beihai, Guangxi Province	AY678547
<i>P. steckii</i>	AS3.6671	Soil, Mount Donglingshan, Beijing	AY678557
<i>P. thomii</i>	AS3.7982	IMI 189694 T	DQ911127
<i>P. thomii</i>	AS3.5978	Soil, Harbin, Heilongjiang Province	AY678544
<i>Talaromyces trachyspermus</i>	AS3.6568	Soil, Zhoushan, Zhejiang Province	AY678606

^a Sequences EU664062–EU664081 were obtained in this study and others from our former studies. EU644080 and EU644081 are the partial β -tubulin gene and rDNA ITS1-5.8S-ITS2 sequences of *E. saturniformae*, respectively. Ex-type strains are indicated with ‘T’

Data Processing and Statistical Analyses

The raw sequences were proofread manually and edited with BioEdit 5.0.9 [21]. For analyses of partial calmodulin gene and rDNA ITS1-5.8S-ITS2 sequences, the sequences were aligned using Clustal X 1.81 [22] with manual adjustments using BioEdit 5.0.9 [21]; *T. trachyspermus* and *T. bacillisporus* were considered as the outgroups, respectively, in the two data sets for their relationships with those species of *Dichlaenoideae* in *Trichocomaceae* [23, 24]. Both matrices were analysed, respectively, using the neighbour-joining (NJ) method with the Kimura model to calculate sequence divergence, and with 1000 bootstrap replicates. The two data sets were also converted into NEXUS format for maximum parsimony (MP) analyses. MP trees were obtained in a heuristic search with random sequence addition for 1000 replicates and gaps were treated as ‘missing data’, with 1000 bootstrap replicates using PAUP 4.0b8a [25].

Results

Eupenicillium saturniforme L. Wang and W-Y.
Zhuang, sp. nov. (Figs. 1, 2, 3, 4)

Etymology: ‘saturniforme’, referring to the shape of the ascospores, resembling Saturn with its ring.

In CYA 25°C post 7 dies: coloniae 27–29 mm diametro, tenues, planae; umbonatae in medio;

velutinae; sporulatio abundus, glauca; mycelium album; exsudatum praesens, incoloratum; pigmentum solubile absens; reversum eburneum.

Cleistothecia abundantia, prima eburnea, cinnamomeus in maturita; ascosporae lentiforme, leves, duabus costis, 4.5–5.0 × 3.0–3.5 μ m. Conidiophora ex hyphis submerses; stipites glabri ad spinosa, (80–) 100–180 (–200) × 3.5–4.5 μ m; penicilli praecipue biverticillati, interdum monoverticillati; metulae 2–4 (–5) per stipitem, 10–20 × 3.5–4.5 (–5.4) μ m, clavatae, sursum vesiculosae, ad 10–15 μ m diameter; phialides (4–) 8–12 verticillatae, ampulliformes, 7–11 × (2.0–) 2.5–3.5 (–4.0) μ m, collula distincta; conidia spherioidea, ovoidea vel ellipsoidea, (3.0–) 3.5–4.0 μ m, spinosa, in catenis inordinatis adhaerentia.

Holotypus hic designatus: HMAS 130355-1-4 (cultura viva AS3.6886), isolatus ex solo, Provincia Jilin sinica, in Instituto Microbiologica Academiae Sinicae, Beijing, conservatur.

On Czapek agar (CA) at 25°C after 7 days: Colonies 15–18 mm in diameter, low, plane, umbonate in central areas; velutinous; conidiogenesis moderate, from near-Pale Smoke Grey (R. Pl. XLVI) to Pea Green (R. Pl. XLVII); mycelia white at margins; cleistothecia abundant, Walnut Brown to Japan Rose (R. Pl. XXVIII) in central areas, and Pale Pinkish Buff to Pinkish Cinnamon (R. Pl. XXIX) in marginal areas; clear exudate limited; no soluble pigment; reverse Pale Pinkish Buff, but central areas near Sayal Brown (R. Pl. XXIX).

On CYA at 25°C after 7 days: Colonies 27–29 mm in diameter, low, umbonate in central areas,

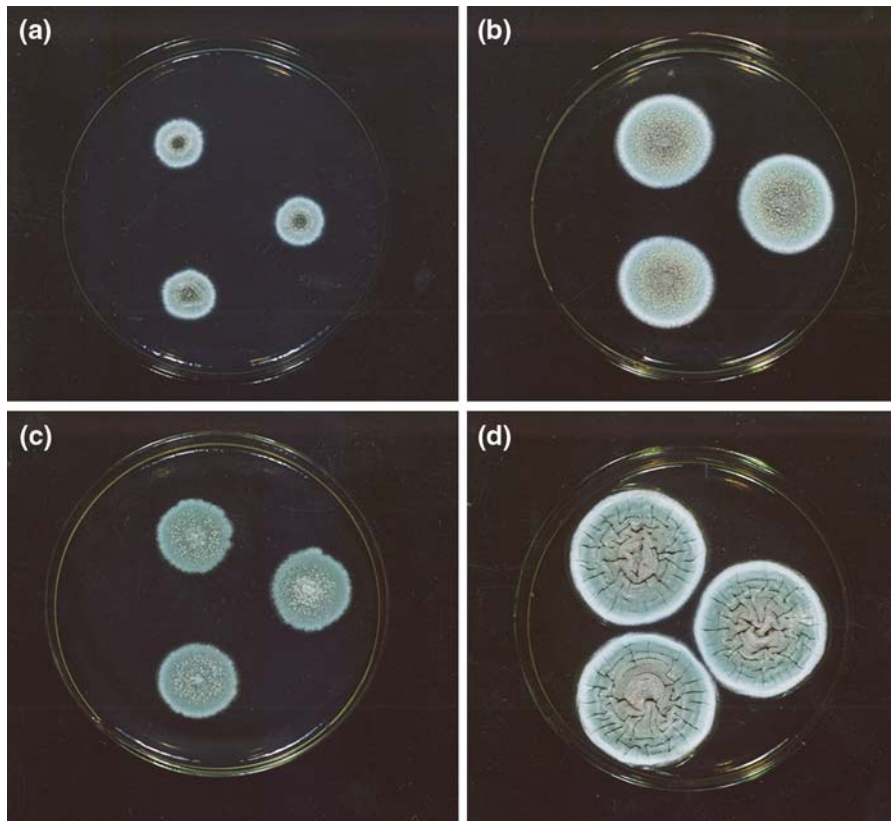


Fig. 1 *E. saturniforme* sp. nov. AS3.6886. Colonies on (a) CA, (b) CYA, (c) MEA and (d) YES at 25°C, after 7 days

radially sulcate and slightly annually plicate; velvety; conidiogenesis moderate, from near-Pale Smoke Green (R. Pl. XLVI) to Light Greyish Olive (R. Pl. XLVI); mycelia white at margins; cleistothecia abundantly produced, Walnut Brown to Rood's Brown (R. Pl. XXVIII), white to Pale Pinkish Buff in marginal areas (R. Pl. XXIX); clear exudate produced in central areas; no soluble pigment; reverse Light Buff (R. Pl. XV) to ivory yellow.

On MEA at 25°C after 7 days: Colonies 21–24 mm in diameter, low, plane, slightly umbonate in central areas; velutinous; conidiogenesis abundant, from near-Pea Green (R. Pl. XLVII) to Light Greyish Olive (R. Pl. XLVI); mycelia white, cleistothecia moderate, Pale Pinkish Buff to Light Pinkish Cinnamon (R. Pl. XXIX) when young then Vinaceous Pink (R. Pl. XXVIII) when mature; no exudate and soluble pigment; reverse Cream Buff to Chamois (R. Pl. XXX).

On yeast extract sucrose agar (YES) at 25°C after 7 days: Colonies 38–42 mm in diameter, low, radially and irregularly sulcate; velutinous; conidiogenesis

abundant, Pea Green (R. Pl. XLVII); cleistothecia abundant in central areas, Cacao Brown to Vinaceous-Russet (R. Pl. XXVIII); no exudate and soluble pigment; reverse Orange Cinnamon to Pinkish Cinnamon (R. Pl. XXIX).

On 25% glycerol nitrate agar (G25 N) at 25°C in 7 days: Colonies 5–7 mm, deep, radially sulcate, velutinous, conidiogenesis absent; mycelium white; no exudate and pigment; reverse Pinkish Buff (R. Pl. XXIX).

On CYA at 37°C in 7 days: No growth.

On CYA at 5°C in 7 days: No growth.

Conidiophores arising from surface hyphae, stipes (80–) 100–180 (–200) × 3.5–4.5 μm, finely rough-walled, commonly with swollen apices; penicilli typically biverticillate, occasionally monoverticillate; metulae 2–4 (–5), 10–20 × 3.5–4.5 (–5.4) μm; usually clavate to apically vesiculate up to 10–15 μm, sometimes cylindrical; phialides (4–) 8–12, ampulliform with short collula, 7–11 × (2.0–) 2.5–3.5

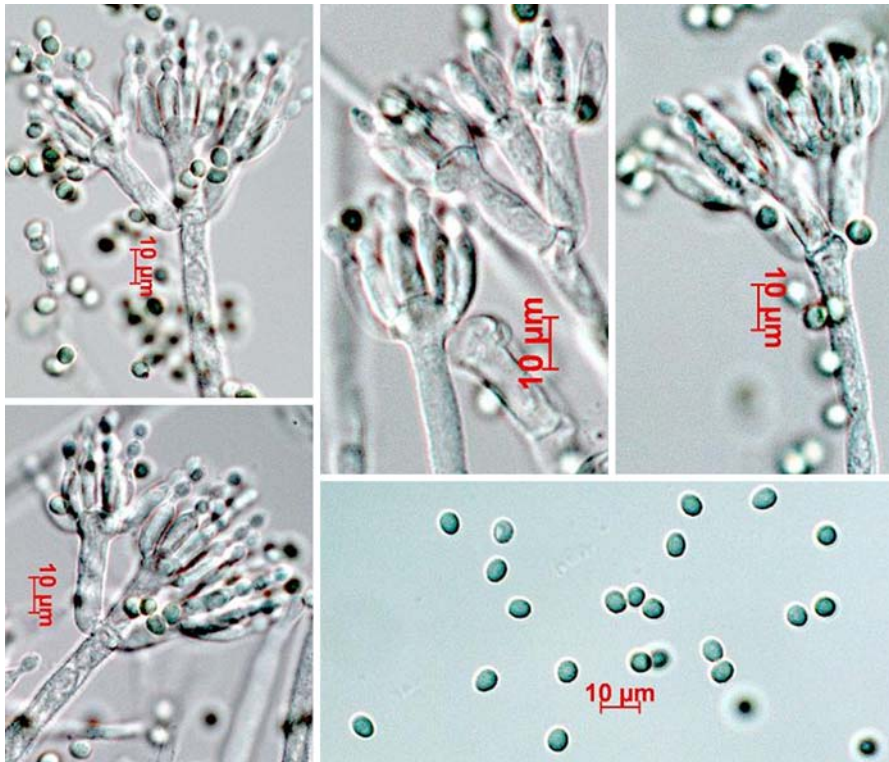


Fig. 2 Penicilli and conidia from CYA of *E. saturniforme* AS3.6886

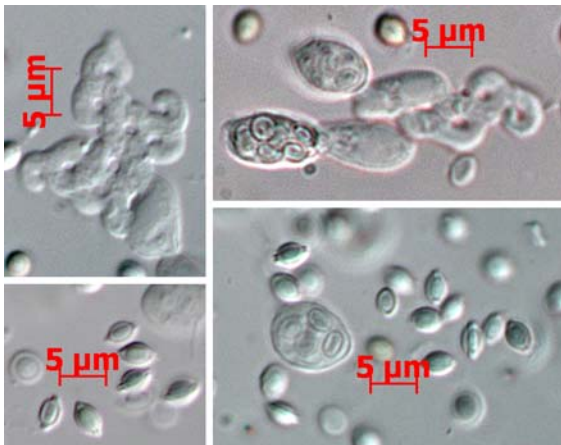


Fig. 3 Ascogenous hyphae, asci and ascospores of *E. saturniforme* AS3.6886

(4.0 – 5.0) μm ; conidia sphaeroidal, ovoid to ellipsoidal, (3.0 – 3.5)– 4.0 μm , finely rough-walled, borne in long tangled chains up to 120 μm .

Cleistothecia spheroidal to ellipsoidal, (300 – 400)– 480 μm diameter, white to yellow or ivory when young then pinkish brown when mature after at least

3 months; asci spheroidal to ellipsoidal, 8 – 12 μm , borne in short chains about 2–3 cells from short and strongly recurved branches of ascogenous hyphae, occasionally singly; ascospores lenticular, 4.5 – 5.0×3.0 – 3.5 μm , with two very closely appressed equatorial ridges about 0.2 μm , convex surfaces smooth with sparsely scattered fine warts or irregular ribs along the outer areas.

The type strain was isolated from a soil sample collected in Little Peony Forest Reserve, Dunhua, Jilin Province, China (N: $43^{\circ}13'12''$, E: $128^{\circ}7'48''$), Sept. 1998. This strain was deposited in China General Microbiological Culture Collection Center (CGMCC), Academia Sinica, Beijing, China, as AS3.6886^T (= CBS 122276).

Discussion

Morphologically, *E. saturniforme* resembles *E. shearii* and *E. tropicum*. All these three species regularly produce lenticular ascospores with two closely appressed equatorial flanges and biverticillate

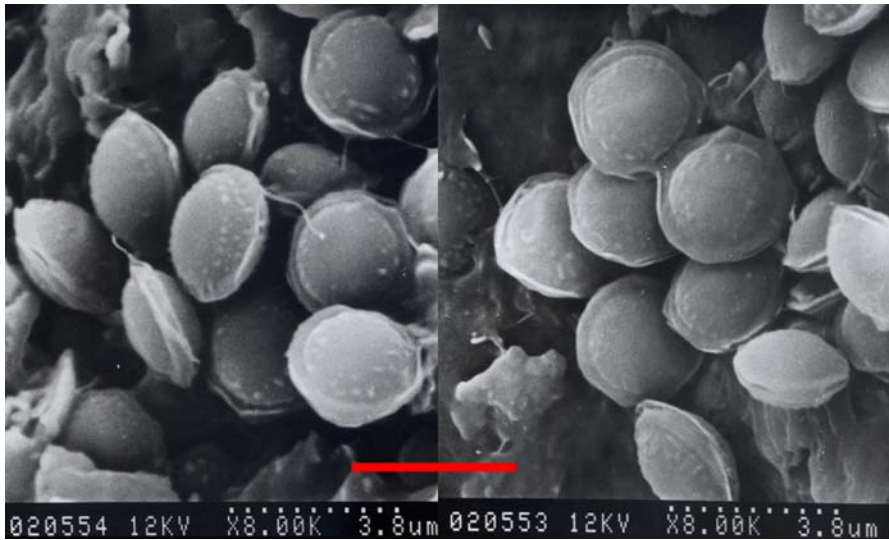


Fig. 4 SEM photographs of ascospores of *E. saturniforme* AS3.6886

penicilli. While the cleistothecia of *E. shearii* and *E. tropicum* ripen in a relatively short time and have a grey tint at maturity, while those of *E. saturniforme* mature after a long time (3 months or more) and are persistently pinkish brown at maturation. Moreover, asci of *E. shearii* and *E. tropicum* are borne singly, while those of the new taxon are borne in short chains. Although the shapes of their ascospores are much alike, ascospores of *E. shearii* and *E. tropicum* are finely rough-walled all over the convex surfaces when examined with SEM, and those of *E. saturniforme* are mostly smooth-walled, only appearing sparsely roughened along the outer marginal areas. The ascospores of *E. saturniforme* are bigger than those of the former two. Furthermore, the penicilli of the new species are larger and its metulae are clavate to characteristically vesiculate, but those of *E. shearii* and *E. tropicum* are cylindrical or only slightly inflated. In addition, *E. shearii* is able to grow at 37°C, sporulates poorly on standard media, and bears smooth-walled conidia; *E. tropicum* grows more slowly at 25°C on standard media. All these are different from the new taxon [3, 4, 9, 10].

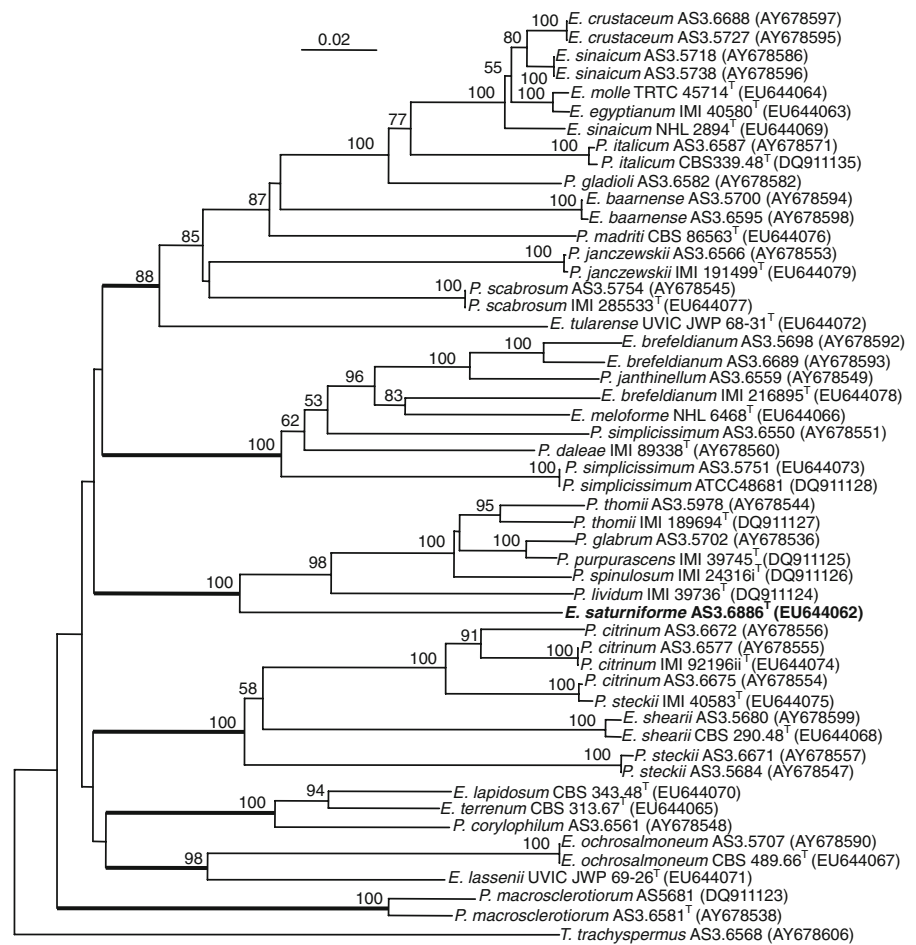
Eupenicillium saturniforme also resembles *E. lapidosum* in certain respects such as red-brown coloured and late-maturing cleistothecia, a mixture of biverticillate and monoverticillate penicilli, rough-walled stipes as well as apically swollen stipes and metulae. However, the dimensions and ornamentation of ascospores as well as the dimensions of conidiophore

elements render the marked differences between the two species. In addition, the asci of the *E. saturniforme* are usually borne in short chains whereas those of *E. lapidosum* are always borne singly (rarely two in a chain *fide* Stolk and Samson [3]). The conidial walls of the new species are finely roughened but those of *E. lapidosum* are strictly smooth. *Eupenicillium lapidosum* grows more rapidly, giving a dense, floccose colonial texture with yellow to orange yellow mycelia and poor sporulation. All these characters can be readily recognized and used to differentiate *E. saturniforme* from *E. lapidosum*.

The biverticillate penicilli, vesiculate metulae, rough-walled conidia, velutinous colonies with abundant conidiogenesis, and moderate growth rate of *E. saturniforme* present many similarities to *P. citrinum*, but the latter species has never been reported to produce cleistothecia. Moreover, the conidiophore stipes of the new taxon are rough-walled and the conidial chains are loosely tangled, while those of *P. citrinum* are consistently smooth, with the conidial chains forming long, well-defined columns.

The occasional presence of monoverticillate penicilli with vesiculate stipes and rough-walled conidia of the new species suggests some relationships with *P. lividum*, *P. purpurascens*, *P. spinulosum*, and *P. thomii*. The latter four species all produce strictly monoverticillate penicilli, although *P. spinulosum* occasionally presented metulae, and none of these species has known teleomorphs.

Fig. 5 The NJ tree inferred from the partial sequences of calmodulin gene data set. The bootstrap percentages over 50% derived from 1000 replicates were indicated at the nodes



According to the classification of Pitt [10], *E. saturniforme* should be placed in Series *Crustacea*; according to Stolk and Samson [3], it would be placed in Section *Eupenicillium*.

Both NJ and MP analyses of the two data sets confirmed the placement of *E. saturniforme* in the clade with *Penicillium glabrum*, *P. lividum*, *P. purpurascens*, *P. spinulosum* and *P. thomii* rather than in the clades including the similar species discussed above, which justified the status of the new taxon. Only the NJ trees generated from partial calmodulin gene data set were presented in this article (Fig. 5). The NJ tree resulting from rDNA ITS1-5.8S-ITS2 sequence data set is presented as supplementary Fig. 1. In the MP analysis of partial calmodulin gene sequences, the aligned data matrix contained a total of 715 characters with 321 characters constant, 33 variable characters parsimony-uninformative and 361 characters parsimony-informative. Four

equally parsimonious trees were obtained and the topologies were basically identical; we selected the one that was more in accordance with morphological classification (Supplementary Fig. 2). In the MP analysis of rDNA ITS1-5.8S-ITS2 sequences, the aligned data matrix contained a total of 592 characters with 401 constant characters, 74 variable characters parsimony-uninformative and 117 characters parsimony-informative. There were 943 equally parsimonious trees generated, and we also selected the one which was more in accordance with morphological classification (Supplementary Fig. 3). Both matrices were supplied as supplementary data set 1 and data set 2.

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