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Two Novel *Eupenicillium* Species Isolated from Soil in China

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ABSTRACT

Two novel species of *Eupenicillium* isolated from the soil collected in China are described and illustrated. *Eupenicillium polygoniforme* is characterized by the lenticular ascospores with polygon edge of equatorial ridges, the convex surface of the ascospores resembling with reticulate anastomosed ribs and striking spinulose. *E. zhejiangensis* is characterized by the lenticular ascospores with distinct deeply equatorial ridges and anastomosed squamiform ornamentation of the convex surface. The phylogenetic relationship between the two new species and other species in *Eupenicillium* genus was investigated base on LSU-ITS combined sequences.

INTRODUCTION

Eupenicillium F. Ludw is frequently considered as the main teleomorphic stage of anamorphic genera of *Penicillium* [1]. *Penicillium* is one of the most common fungi worldwide distribution with important economic impact on human life, such as penicillin production, cheese food industry, production of novel enzyme, postharvest food pathogens on food crops, a range of mycotoxins production and so on [2,3]. There are 48 species reported in *Eupenicillium* Genus [4], many species isolated from soil [4-8], other substrate resources such as marine sediment [9], dry cow manure [10], two species colonize corn and other grains and seeds, even the conidial heads of *Aspergillus* [11].

In the course of investigation of filamentous fungi from soil of bamboo forest situated in south-east of China, 36 strains of *Penicillia* have been isolated. Surprisingly, among them, 12 isolates of *Penicillia* appeared ascospores within three weeks. They represent at least 2 new species in sufficiently different from all described species in the Genus *Eupenicillium* on the basis of morphology, and rDNA sequences [9,10].

MATERIALS AND METHODS

Isolation of strains from soil sample

Isolation of strains from soil sample were followed the methods described by Tuthill and Frisvad [8].

Observation of cultural characterization

Media and cultural conditions used for determination of growth characters performed according to the method of Visagie et al. [2]. Colony colors were determined by compare with Inter-Society Color Council (ISCC) and the National Bureau of Standards (NBS) color name charts [12,13].

Observation of morphological characters by microscope

Morphological features of penicillin, asci and Cleistothecia were observed using Nikon eclipse 80i microscope based on 6 to 10-d-old cultures grown on MEA at 25 °C according to the method used by Tuthill and Frisvad [10].

Morphological characters of ascospore determined by SEM

Material for observation was prepared by cultured at CA media for 4 weeks. Scanning electron microscope protocol was followed as used by Tuthill and Frisvad ^[10].

DNA extraction and rDNA fragment sequencing

Genomic DNA was extracted following the protocol of Peterson and Horn ^[14]. ITS region (primer pair ITS1/ITS4) and LSU region (primer pair LR5/LROR) of rDNA was obtained by PCR and sequenced. The primers and protocol used for PCR were followed as described by Wang and Zhuang ^[4]. PCR products were purified using the PCR cleaning Kit according to the manufacturer's protocols (Sangon Bio., CA). The primers mentioned above were used for bidirectional DNA sequencing using Applied Biosystem 3730 XL DNA analyzer.

Sequence alignment and phylogenetic analyses

Sequences of each gene fragment obtained were proofread manually using Bioedit software ^[4,15]. Additional sequences in Genus *Eupenicillium* used for phylogenetic analyses followed as Tuthill and Frisvad ^[10], Wang and Zhuang ^[4]. The accession numbers of sequences downloaded from NCBI GenBank are marked in **Figure 5**. One combined datasets (ITS + LSU rDNA) were used for parsimony analysis. All sequence datasets were aligned with Clustal X ^[16] and adjusted manually with Bioedit ^[4,15]. Gaps in all sequences were treated as missing data and parsimony analyses were carried out using PAUP version 4b10 ^[17]. The phylogenetic trees were inferred using the heuristic search with random sequence addition 100 replicates and tree branches of zero length were collapsed. All multiple parsimonious trees were saved. Branch support estimation of parsimonious trees were performed with bootstrap of 1000 replicates using PAUP version 4b10 ^[17].

RESULTS

Taxonomy

Eupenicillium polygoniforme H-K. Wang and F-C. Lin, sp. nov. (**Figures 1 and 2**)



A: Cleistothecium; B: asci; C-E: ascospore; F,G: sporulation structure; H: conidia. Bar:A,B,F-H, 10 µm; C-E: 1 µm

Figure 1. Morphology of *Eupenicillium polygoniforme*

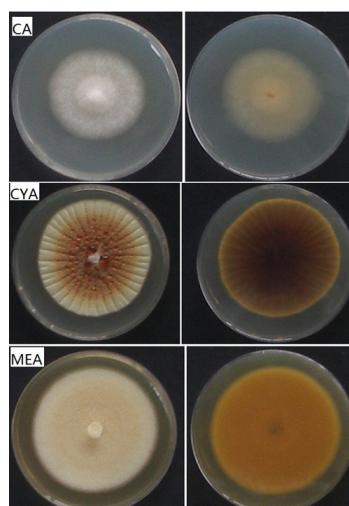


Figure 2. Cultural features of *Eupenicillium polygoniforme*.

Etymology: 'polygoniforme', referring to the ascospores having the polygon shape with edge of its rings.

Morphological description

Cleistothecia superficial, globose to subglobose, at first avellaneous but becoming dark-brown within 2 weeks, sclerotoid, 80–150 µm in diameter, solitary or clustered, maturing from center outwards margin after 3 weeks on CA, CYA and MEA. Asci 8-spored, pyriform when young but becoming subglobose to ellipsoidal at maturity, 8-13 × 5.5-8 µm; ascospores hyaline, lenticular, (3.0–4.0) 3.5 × 1.8(1.5–2.0) µm, with 2 distinct equatorial ridges with polygon edge. The structure of the convex surface of the ascospores finely roughened, producing reticulate anastomosed ribs near polygon equatorial ridges but with striking tetragonal pyramid spinulose at the central part of the convex surface.

Conidial state scanty on CA and CYA media, but produced on MEA media. Penicilli strictly monoverticillate; conidiophores variable, either arising from submerged hyphae or from aerial hyphae, 15-70 × 1.5–2.0 µm, with the apex occasionally enlarged to 4–6 µm width swollen apex; phialides lanceolate to cylindrical, (7–)8–10(–11) × 1.8–2.7 µm, 1–3 perverticil and uncrowded; conidia spherical, smooth-walled, 2.4–3.0 µm in diameter.

Cultural features

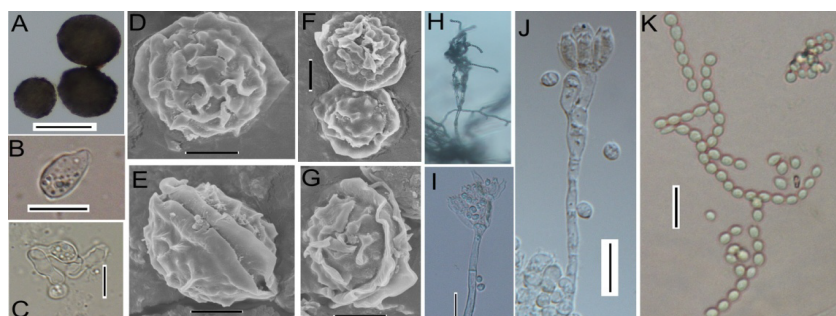
Colonies grown on MEA at 25 °C after 7 days with rapid growth rate and attaining 50-53 mm in diameter, consisting of a low felt with velutinous surface, greenish white (ISCC-NBS 153) at the central areas, pale yellowish green (ISCC-NBS 121) in periphery areas with 2-3 mm white margin. Sclerotia abundantly for the development of cleistothecia, sporulation structures abundantly produced at central areas but sparsely peripherally; exudates and soluble pigment lacking. Colony reverse strong orange yellow (ISCC-NBS 68).

Colonies grown on CYA at 25 °C after 7 days with moderate growth rate and attaining 47-50 mm in diameter, radically sulcate, consisting of a thin felt with strictly velutinous surface, deep orange (ISCC-NBS 51) at central areas, becoming pale greenish white (ISCC-NBS 153) to white towards the margin. Sclerotia abundantly for developing cleistothecia, conidial-bearing structures absent; no exudates and soluble pigment. Colony reverse light olive brown (ISCC-NBS 94) to deep yellowish brown (ISCC-NBS 75).

Colonies grown on CA at 25 °C after 7 days slowly and attaining 41-44 mm in diameter, consisting of a mycelial felt with dense floccose surface, white, sclerotia moderately on the surface of colony, unbonate in central areas; conidia-bearing structure absent; exudates and soluble pigment not present. Colony reverse white gray (ISCC-NBS 264).

Culture examined: The type strain ch17 was isolated from a soil sample collected in forest, Yixing City, Jiangsu Province, China (N: 31° 10' 34", E: 119° 40' 31"), June 2014. This strain was deposited in department of plant protection, Zhejiang University, P.R. China.

Eupenicillium zhejiangensis H-K. Wang and F-C. Lin, sp. nov. (Figures 3 and 4)



A: Cleistothecia; B,C: asci; D-G: ascospore; H-J: sporulation structure; K: conidia. Bar:A 100µm; B-C, H-K, 10µm; D-G: 1µm

Figure 3. Morphology of *Eupenicillium zhejiangensis*.

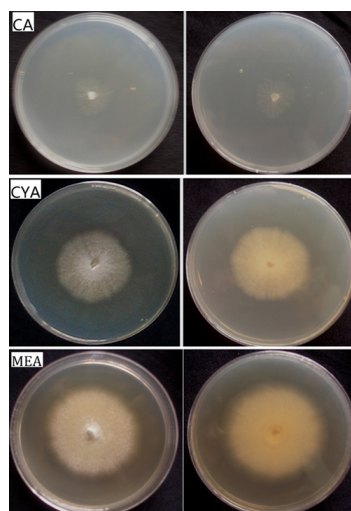


Figure 4. Cultural features of *Eupenicillium zhejiangensis*.

Etymology: 'zhejiangensis' describing the Geographic Location from which the species was isolated.

Morphological description

Vegetative hyphae hyaline, septate, 2-4 µm in diameter. Cleistothecia immersed or superficial, globose to subglobose, at first avellaneous but becoming dark-brown within 2 weeks, sclerotoid, 100–200 µm diameter, solitary or clustered, ripening from center outwards in four weeks on MEA. Asci 8-spored, borne singly or abundantly, pyriform at juvenility but becoming subglobose to ellipsoidal at maturity, 8-11 × 7-9 µm; ascospores hyaline, lenticular, 2.8–3.5 × 2–2.5 µm, with 2 distinct deeply equatorial ridges (ridges more than 0.5 µm). The structure of the convex surface of the ascospores finely roughened with anastomosed squamiform ornamentation.

Penicilli biverticillate, sometimes monoverticillate, bearing long conidial chains (>50 µm). Conidiophores variable, subterminal branching uncommon; arising from submerged hyphae and from aerial hyphae, sometimes arising from ascomata. Stipes smooth, 100–400 × 2.5–3.5 µm; metulae when present only 2 per stipe, 7–10 × 2.5–4.5 µm, upterminal swelling; phialides ampulliform, collula apparent, 2–6 per metula, 7–10 × 3.0–4.5 µm; conidia broadly ellipsoidal, smooth walled, (2.5–4.5)2.7 × (1.7–2.6)2.4 µm, average width/length= 0.91±0.05.

Cultural features

Colonies grown on MEA at 25 °C after 7 days with rapid growth rate and attaining 40-43 mm in diameter, consisting of a low plane with velutinous surface, pale orange yellow (ISCC-NBS 73), with 2-3 mm white margin. Sclerotia moderate at the periphery area but abundant near central area, sporulation structures abundantly produced throughout the surface of colony; exudates and soluble pigment lacking. Colony reverse pale yellow (ISCC-NBS 89) to light yellow brown (ISCC-NBS 76).

Colonies grown on CYA at 25 °C after 7 days with moderate growth rate and attaining 31-33 mm in diameter, consisting of a thin felt with surface bearing granular, light gray (ISCC-NBS 264) to light greenish gray (ISCC-NBS 154), with 2-3 mm white margin. Sclerotia moderate at the periphery areas but abundant near central areas, sporulation structures moderately produced on the surface of colony; exudates and soluble pigment absent. Colony reverse pale yellowish green (ISCC-NBS 121) to yellowish white (ISCC-NBS 92).

Colonies grown on CA at 25 °C after 7 days slowly and attaining 18-20 mm in diameter, a rosy aggregations of hyphae with radially granular (sclerotia) towards the margin, sclerotia moderate on the surface of colony, greenish white (ISCC-NBS 153); conidia-bearing structure absent; exudates and soluble pigment not present. Colony reverse very pale green (ISCC-NBS 148).

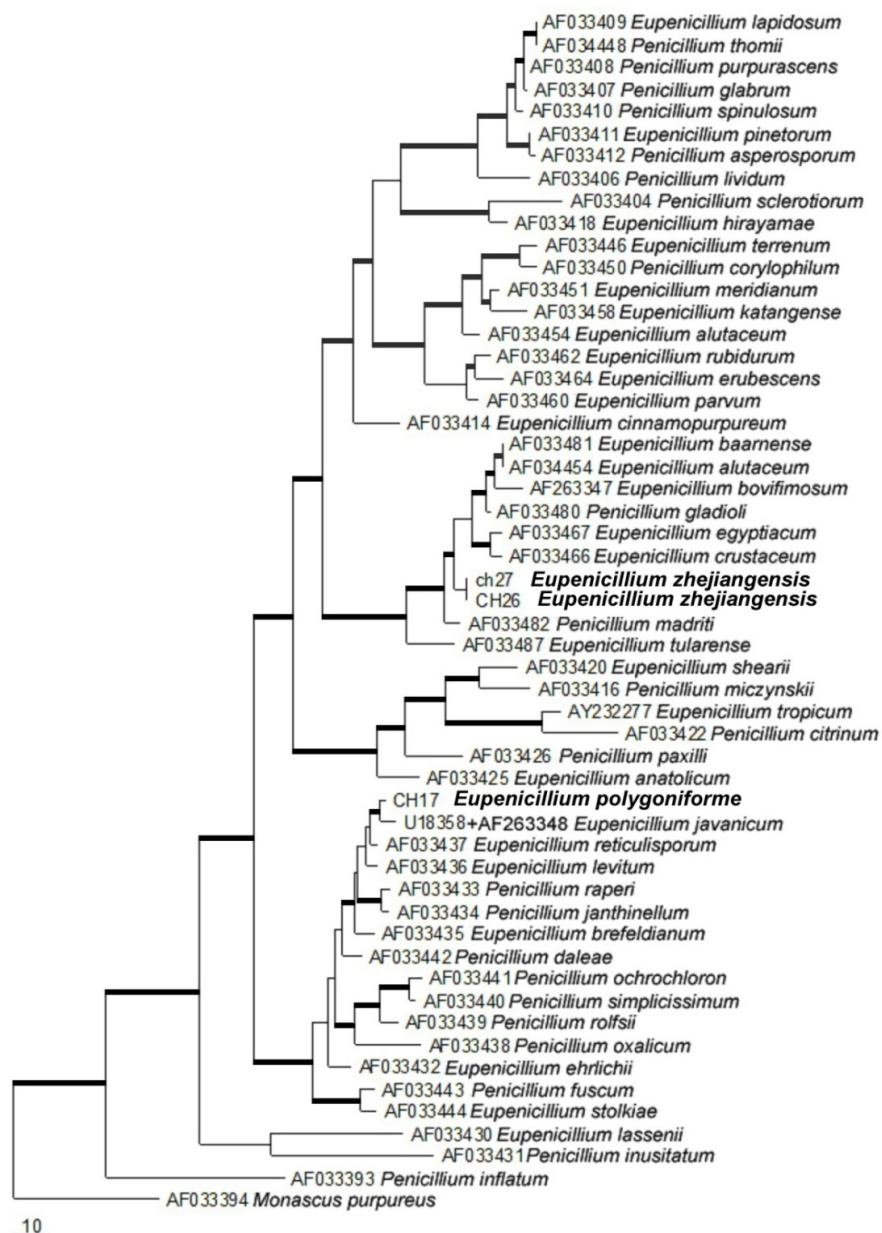
Culture examined: The type strain ch27 was isolated from a soil sample collected at forest of Hangzhou City, Zhejiang Province, P.R. China (N: 29°35'18", E: 119°31'52"), July 2013. This strain was deposited in department of plant protection, Zhejiang University, P.R. China.

Phylogenetic analysis

E. polygoniforme strain ch17 and *E. zhejiangensis* strains ch26 and ch27 were used for phylogenetic analysis. The ITS of strain ch17 is 534 bp, ch26 is 520 bp and ch27 is 520 bp. The LSU of strain ch17 is 895 bp, ch26 is 884 bp and ch27 is 889 bp. The accession respectively. Parsimony analysis of LUS-ITS combined datasets was performed in this study. 54 taxa were contained in the data matrix including taxa used by other researchers^[4,10], *Monascus purpureus* as out group. The data matrix produced 1235 total characters, including 935 constant characters and 94 distinct characters, 206 parsimony-informative characters. Tree length is 1066 (CI=0.483, RI=0.742, HI=0.517, RC=0.358). The phylogenetic trees are very similar to the results of Tuthill and Frisvad^[10]. Phylogeny results showed that *Eupenicillium polygoniforme* (strain ch17) is closely related to *Penicillium javanicum* (**Figure 5**), supported by bootstrapping value 90; fallen in a monophyletic group of Clade 1A: subgenus *Aspergilloides*^[3], and well supported by bootstrapping value 91. *E. zhejiangensis* Strains ch26 and ch27 are clustered in to a monophyletic group containing *E. crustaceum*, *E. egyptiacum*, *E. gladioli*, *E. bovisimosum*, *E. alutaceum*, *E. baamense* (**Figure 5**). This clade defined as *Penicillium* sensu strict. by Houbraken and Samson. Clade 1B: subgenus *Penicillium*^[3] supported by bootstrapping value 100.

DISCUSSION

The predominate species of genus *Eupenicillium* isolated from soil^[18]. In the present research, two new species belonging to *Eupenicillium* were identified. The morphology of sporulation type of *E. zhejiangensis* is striking similar to *E. abidjanum* (Mycobank #330718)^[5], but differ it from growth rate, cultural characters and ornament of ascospores. According to the findings of Udagawa and Horie^[19], *E. zhejiangensis* can accommodate the group of ascospores with distinct equatorial ridges. The ornament features of ascospores of *E. zhejiangensis* are related to subgroup of ascospores reticulate, but quite distinct with the species of this group by the large and irregular ridges. *E. zhejiangensis* also differ these species with colonial morphology and sequence data. Phylogenetic analysis showed this species closely related to species in series *Crustacea*.^[4,18]



Phylogenetic tree based on the ITS and LSU rDNA. Extra thick branches indicate the bootstrap confidence value >50%

Figure 5. Phylogenetic analysis of *Eupenicillium polygoniforme* and *E. zhejiangensis* in genus *Eupenicillium*.

Eupenicillium polygoniforme has features of ascospores group with distinct equatorial ridges in *Eupenicillium* genus (lenticular ascospores)^[19], but it differs species in this group from the ridges appearance of polygon shape. The structure of the convex surface resembles the characters of ascospores ribbed group and ascospores spinulose group. Phylogenetic analysis inferred that this species is closely related to species *E. javanicum*, but *E. javanicum* has the pattern of globose ascospores without distinct equatorial ridges. They are separated to different taxa well supported by bootstrap value 90. Phylogenetic analysis showed that *E. polygoniforme* belongs to *Penicillium* section *Aspergilloides*, this is consistent with the observation of conidial-bearing type pattern.

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