Phenotypic and molecular characterization of *Chaetopyrena penicillata* from Iran with description of a hyphomycete synanamorph

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During a survey of fungi associated with Russian olive fruit rot in Northern Iran, a coelomycete fungus with setose pycnidia was isolated from symptomatic fruits. The fungus was identified as *Chaetopyrena penicillata* based on morphological characteristics and sequence data of ITS and LSU rDNA. A hyphomycete synanamorph, which was observed in pure cultures of this species for the first time, is described. The morphology and phylogeny of *Chaetopyrena penicillata* is discussed.

Key words – Coelomycete – microcyclic conidiation – *Phoma* – *Pyrenochaeta* – setose pycnidia

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Introduction

Anamorphic ascomycetes with ostiolate pycnidia and hyaline conidia encompass a heterogeneous group of fungi, showing a diverse life style including economically important plant pathogens, saprobes, and pathogens of humans and other animals (Aveskamp et al. 2008, de Gruyter et al. 2010). These ubiquitous fungi, with wide distribution, are classified in several coelomycetous genera *viz.*, *Phoma* Sacc. (with nine sections), *Pyrenochaeta* De Not., *Pleurophoma* Höhn., and *Chaetopyrena* Pass. (Aveskamp et al. 2009, 2010, de Gruyter et al. 2010). The main morphological features for delineation of these genera are conidiomatal anatomy, conidiogenesis and conidial morphology (Boerema et al 2004). Recent molecular studies on phylogeny of *Phoma* and related genera have shown that morphological characters, used for delimitation of the sections in *Phoma* and other related genera, are not phylogenetically informative and overlapping characters can be seen amongst different entities (Boerema et al. 2004, Aveskamp et al. 2008, de Gruyter et al. 2009, 2010). Molecular phylogenies based on SSU and LSU rDNA sequence data have placed type species of all *Phoma* sections in different families of the order Pleosporales. Several new genera have been introduced to accommodate segregates from the genus *Phoma* and the other *Phoma*-like coleomycetes (Aveskamp et al. 2009, 2010, de Gruyter et al. 2009, 2010).

The genus *Chaetopyrena* was introduced in 1881 with *C. hesperidium* Pass. as the type species (Crous et al. 2004). The genus is characterized by setose pycnidia, flask-shaped conidiogenous cells and one-celled, hyaline conidia. Setose pycnidia are produced in other coelomycete genera, particularly

Pyrenochaeta, and in some sections of the genus *Phoma* such as *Phoma* sect. *Paraphoma* Morgan-Jones & J.F. White, *Phoma herbarum* Westend. and *Phoma glomerata* (Corda) Wollenw. & Hochapfel (de Gruyter et al. 2010). To date, 18 species have been described in *Chaetopyrena*, however, for many there is no living culture available. Phylogentic analyses have grouped *Chaetopyrena penicillata* (Fuckel) Höhn. with *Phoma herbarum* in the Didymellaceae (de Gruyter et al. 2010). In the present study we provide an emended description for *C. penicillata* and describe a hyphomycete synanamorph for the first time.

Methods

Isolates and morphology

Russian olive (*Elaeagnus angustifolia* L.) fruits showing dry rot symptoms were collected from trees growing at the campus of Tabriz University, Tabriz, Iran. Small pieces of tissue from the margins of the spots were immersed in 70% ethanol for 20 seconds, rinsed in sterile water and incubated on potato dextrose agar (PDA) at 25 ˚C. Pure isolates were obtained by single spore technique. Cultures were plated on 2% MEA, and oat meal agar (OA; 30 g oat meal, 15 g agar, water 1L), and potato dextrose agar (PDA; 200 g potato, 20 g dextrose, 15 g agar, 1 L water), and incubated at 25°C in dark. Morphological characteristics were described and growth rates recorded as maximum diameter of colony after 7 days incubation. Fungal structures were mounted on glass slides with lactic acid for microscopic examination. Measurements of all parameters were made at ×1000 or ×200 (pycnidia) magnification, with 30 measurements per structure. Drawings were made using a drawing tube attached to an Olympus BH-2 microscope.

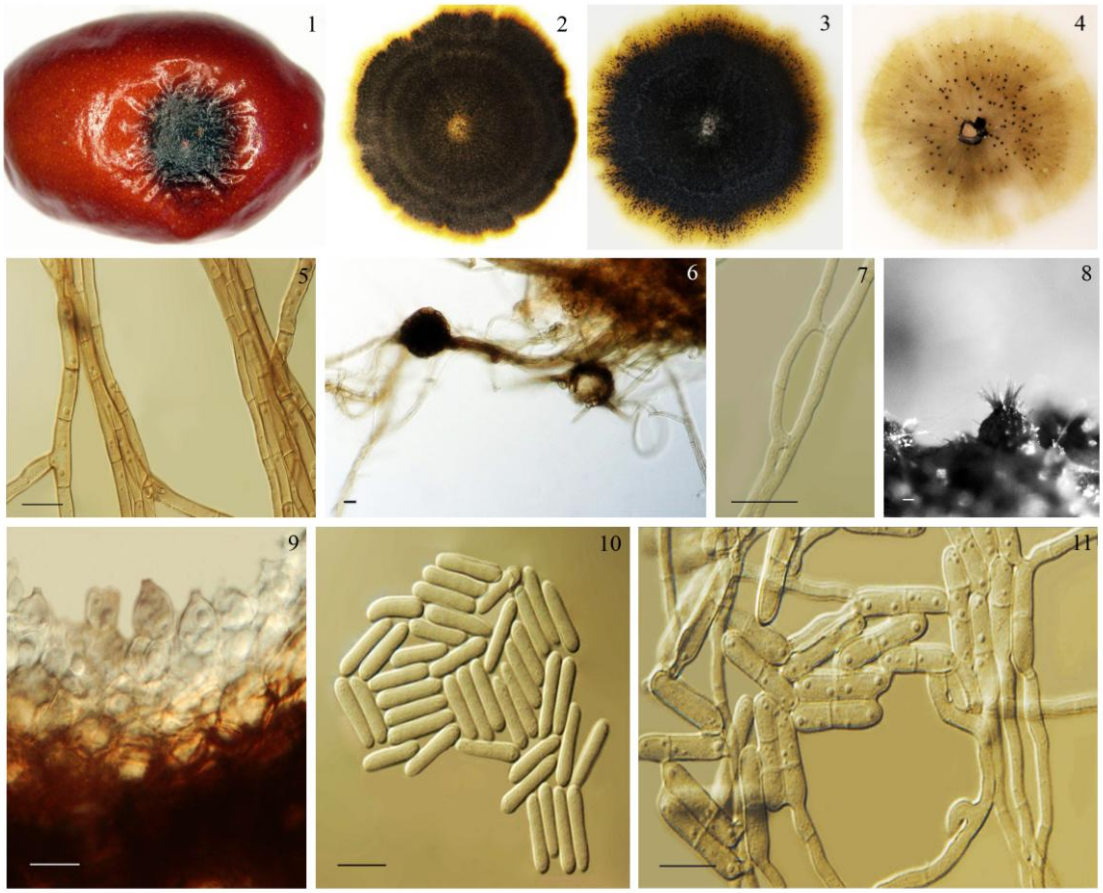
DNA Phylogeny

Genomic DNA was extracted from 8-day-old fungal isolates grown on 2% MEA at 25°C in dark using the method of Moller et al. (1992).The primers ITS1 and ITS4 (White et al. 1990) were used to amplify the internal transcribed spacer region (ITS) of the nuclear ribosomal RNA operon, including: the 3' end of the 18S rRNA gene, the first internal transcribed spacer region (ITS1), the 5.8S rRNA gene, the second internal transcribed spacer region (ITS2) and the 5' end of 28S rRNA gene. Part of the large subunit 28S rRNA (LSU) gene was amplified with primers LR0R (Rehner & Samuels 1994) and LR5 (Vilgalys & Hester 1990). The PCR reaction was performed in a mixture with 0.5 units Taq polymerase (Bioline, London, U.K.), 1× PCR buffer, 0.5 mM MgCl2, 0.2 mM of each dNTP, 5 pmol of each primer, approximately 10–15 ng of fungal genomic DNA, with the total volume adjusted to 25 µL with sterile water. Reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) with cycling conditions consisting of 5 min at 96 °C for primary denaturation, followed by 40 cycles at 96 °C (30 s), 52 °C (30 s), and 72 °C (60 s), with a final 7 min extension step at 72 °C to complete the reaction. The amplicons were sequenced using BigDye Terminator v. 3.1 (Applied Biosystems, Foster City, CA) or DYEnamicET Terminator (Amersham Biosciences, Freiburg, Germany) Cycle Sequencing Kits and analysed on an ABI Prism 3700 (Applied Biosystems, Foster City, CA) under conditions recommended by the manufacturer. Sequences were subjected to a Blast search at Q-Bank (http://www.q-bank.eu/), which provides a specific database on certain groups of fungi, specially *Phoma* and allied genera as well as NCBI-Blast tool. The identity of the isolates were further confirmed by the alignment of the sequences using NCBI Blast2 (Align two sequences) tool.

Results

DNA Phylogeny

Blast search of both ITS and LSU sequences on GenBank and Q-Bank showed that our sequences are identical to *Chaetopyrena penicillata* CBS 498.72 and CBS 199.89 (E-Value = 0.0; Query coverage and Maximal identity = 100%). Alignment of the sequences using Blast2 tool further confirmed the identity of the isolates as *C. penicillata*. Sequence data were deposited in GenBank (Accession Numbers: JQ663989, JQ663990).



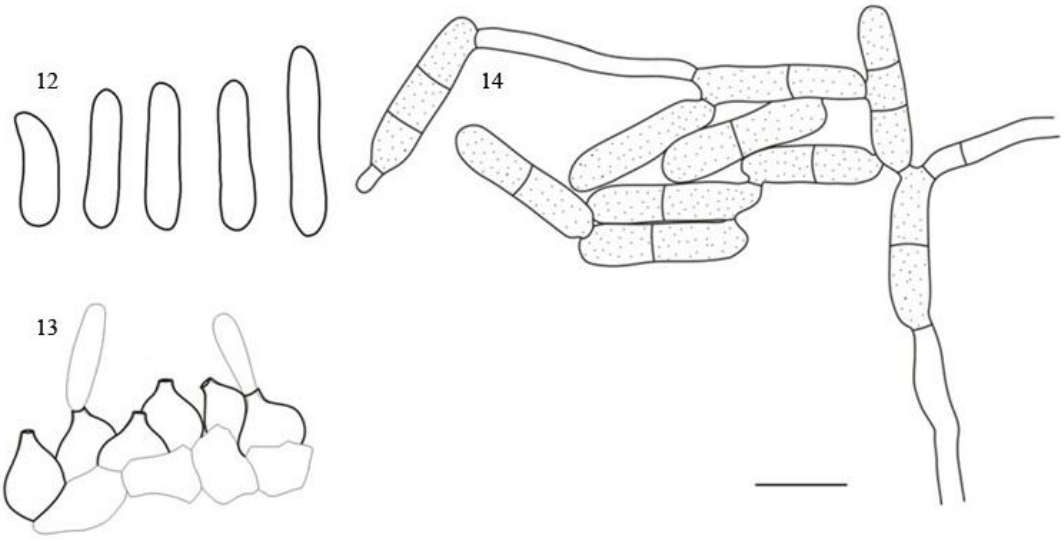
Figs 1–11 – *Chaetopyrena penicillata*. **1** Naturally infected Russian olive fruit. **2–4** Colony morphology on PDA, MEA, and, OA, respectively. **5** Hyphal bundles. **6** Microsclerotia. **7** Hyphal anastomosis. **8** Pycnidia with setae. **9** Conidiogenous cells. **10** Conidia. **11** Hyphomycete synanamorph with microcyclic conidiation in culture. – Scale Bars (5–7, 9–11 = 10 µm, 8 = 100 µm).

Morphology

Chaetopyrena penicillata (Fuckel) Höhnel, Hedwigia 60: 132, 1918. Figs 2–14

In culture aerial mycelium consisting of subhyaline, branched, septate, smooth, 2–5 µm wide hyphae, with numerous anastomosis occurring in single or in bundles of hyphae; bundled hyphae pale brown, consisting of up to 15 hyphal strands. Pycnidia, globose to subglobose, dark brown to black, with 1–2 ostiole, up to 700 µm diameter, well-necked with setae around; setae brown, thick-walled, septate, unbranched or rarely branched, gradually tapering upward, up to 150 µm long, 5–7 µm wide basally, 2–4 µm wide apically; wall consisting of 3-4 layers of brown textura angularis. Conidiophores hyaline, simple, smooth, pyriform with abruptly sharpened or narrowed tips from the median, 8–12 × 7–10 µm, 1–3 µm wide apically, with a single, terminal conidiogenous locus. Conidia hyaline, smooth to finely punctate, 1-celled, cylindrical to sub cylindrical, straight, occasionally sigmoid or slightly curved with rounded apex and truncate base, 12–22 × 3-5 µm. Chlamydospores single or 2–4 in short chains, pale brown, up to 20 µm in diameter. subglobose, dark brown microsclerotia develop from bundled hyphae, reaching up to 140 µm diam.

In culture a synanamorph also can be seen – Conidiophores undifferentiated, reduced to conidiogenous cells, thin-walled, smooth, hyaline. Conidiogenous cells holoblastic, terminal, loci slightly thickened, not darkened. Conidia hyaline, at first aseptate, becoming 2–3-celled as they mature, cylindrical, conspicuously punctuate, both end rounded, 14–19 × 3–4 µm; conidia proliferate to form branched chains of secondary conidia. Both primary and



Figs 12–14 – *Chaetopyrena penicillata*. **12** Conidia. **13** Conidiogenous cells. **14** Hyphomycete synanamorph with microcyclic conidiation in culture. – Scale Bar = 10 µm.

secondary conidia germinate from both ends while they are attached to conidiophores, and produce a terminal conidiogenous cell and a conidium (microcyclic conidiation). Anastomoses between primary and secondary conidia frequently occur in culture (Figs 5–14).

Cultural characteristics – Colonies on PDA reaching 35 mm diam after 7 days at 25 ˚C in dark, flat, black, margins orange, irregular, reverse greenish black, folded, aerial mycelium sparse. Colonies on OA reaching 30 mm diam after 7 days at 25 ˚C in dark, circular, entire, olivaceous-green, mycelium submerged, conidiomata superficial (Figs 2–4).

Discussion

Chaetopyrena penicillata has been reported from Russia, Turkey, South Africa and Romaina on different substrates such as dead twigs, soil and stubble. Phylogenetic analysis based on sequence data of LSU rDNA has grouped this species in Didymellaceae together with *P. herbarum* and *P. gardeniae* (de Gruyter et al. 2010). *C. penicillata* is characterised by setose pycnidia and *Phoma*-like conidiogenous cells. *P. gardeniae* also produces short setae and setae-like structures have been recorded in older cultures of *P. herbarum* (de Gruyter et al. 2010). However, there is no culture yet available for *C. hesperidium*, the type species of the genus, to infer phylogenetic position of this genus. A few synanamorph relationships have been established in *Phoma*, and allied genera by means of molecular techniques. For example, by using ITS sequence data, *P. epicoccina* Punith., M.C. Tulloch & C.M. Leach and *Epicoccum nigrum* Link have been proven to be synanamorphs (Arenal et al. 2000, 2004). There is no report on the formation of a synanamorph in *Chaetopyrena penicillata* or for other species of this genus. Thus, a unique feature of *C. penicillata* is its synanamorph with generally 1-septate conidia that were observed to undergo microcyclic conidiation. The ecology of *C. penicillata* is still unclear, although it has been isolated from dark-colored, slightly sunken, circular or subcircular spots on the side of Russian olive fruits (Fig. 1). A teleomorph has not yet been observed, and its pathogenicity remains unproven.

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