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Molecular and morphological characterization of *Endoconidioma populi* from Kurdistan Province, Iran

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Abstract: Black meristematic fungi are cosmopolitan and able to adapt to extreme fluctuations of ultraviolet radiation, temperature, and moisture and grow in exposed habitats such as stone surfaces. In a survey on fungi associated with fruit rots, leaf spots, canker, gummosis, dieback and trunk diseases of grapevine and walnut trees in Kurdistan Province (Iran), some black meristematic fungal isolates resemble to *Endoconidioma/Hormonema* were found on necrotic twigs. Based on morphology and sequence data (28S rDNA and ITS) the isolates were characterized as *Endoconidioma populi* as a first record for Iran mycobiota. This is a first report of this species from *Juglans regia* and *Vitis vinifera*.

Key words: *Coniozyma*, *Hormonema*, Dothideaceae, Grapevine, Walnut

INTRODUCTION

Endoconidioma Tsuneda, Hambleton & Currah (Ascomycota, Dothideales, Dothideaceae) is a melanized fungus belong to a group of fungi known as black meristematic fungi (BMFs) or black yeasts because of their yeast-like growth. BMFs are characterized by the slowly expanding dark and cauliflower-like colonies and meristematic growth via enlarging and repeated subdivisions of the isodiametric cells (de Hoog et al. 1999; Sterflinger et al. 1999). These fungi belong to a morphologically and phylogenetically diverse group of fungi encompasses some human, animal and plant pathogens (Matsumoto et al. 1993; Palencarova et al. 1995; Horré and de Hoog 1999; McGinnis et al. 1999; Tsuneda et al. 2001; Chabasse 2002). They are cosmopolitan and able to adapt to extreme fluctuations of ultraviolet radiation, temperature, and moisture and grow in exposed habitats such as stone surfaces (de Hoog et al. 1999; Sterflinger et al. 1999) and tree bark (Sigler et al. 1981; Tsuneda and Currah 2006; Tsuneda et al. 2008). *Endoconidioma* was typified by *E. populi* and is a monotypic genus. In a survey on fungi associated with fruit rot, leaf spots, canker, gummosis, dieback and trunk diseases of grapevine and walnut trees in Kurdistan Province (Iran), some black meristematic fungi (BMFs) with black fungal subicula were observed as epiphytic fungi on collected twigs of grapevine and walnut trees. The aim of this study was to characterize these isolates based on morphology and molecular data.

MATERIALS AND METHODS

Fungal isolation

In the survey during 2012-2014 on fruit rots, leaf spots, canker, gummosis, dieback and trunk diseases of grapevine and walnut trees in Kurdistan Province (Iran), some 20 necrotic twigs bearing black fungal subicula resemble to *Endoconidioma/Hormonema* were collected. Twigs were washed with tap water and surface sterilized with 70% ethanol for 1 min, rinsed with sterile distilled water and dried with sterile filter paper. To obtain pure cultures, the subicula were crushed in a drop of sterile distilled water and spread on water agar (2% WA) supplemented with chloramphenicol (100 mg/l). Streaked agar plates were incubated at 20-25 °C in the dark and then single germinated conidia were transferred to PDA plates. Representative isolates were deposited in the culture collection of the Iranian Research Institute of Plant Protection (IRAN, Tehran, Iran).

Morphology

To determine morphological features isolates were cultured on PDA at 25 °C in the dark. Structures were mounted in 100 % lactic acid and digital images were recorded with an Olympus DP72 camera on an Olympus BX51 microscope. Measurements were made with the Cell Sense Entry measurement module. For each isolate at least 50 fungal structures were measured. Dimensions are presented as a range with extremes in parentheses. Colony morphology, colour (Rayner 1970) and growth rate at 25°C were determined. Mycelial growth was measured by

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calculating the mean of two perpendicular colony diameters.

DNA extraction, PCR and sequencing

Cultures were grown in YMA medium (0.3% Yeast extract, 0.3% Malt extract, 0.5% Peptone, 1.0% Dextrose, 2.0% Agar) and mycelium was collected with a loop. Genomic DNA was extracted using the phenol-chloroform method. Briefly, mycelium obtained from the growth on solid medium was transferred to lysis buffer (50 mM Tris; 250 mM NaCl; 50 mM EDTA; 0.3% w/v SDS; pH 8), phenol and chloroform (2:1:1). Samples were vortexed for 20 min in the presence of glass beads and then centrifuged at 13000 rpm for 25 min at 4 ºC. Genomic DNA was precipitated with ethanol and then resuspended in 20 µL of TE+RNase (100 mM Tris/HCl, pH 8, and 100 mM EDTA plus 50 µg/mL RNase A). The ITS region plus part of 28S rDNA was amplified using the primers ITS5 (White et al. 1990) and LR6 (Vilgalys and Hester 1990). The PCR reaction mixtures 50 µL contained 1X DreamTaq Buffer, 200 µM of dNTPs mix, 2mM of MgCl2, 0.4 µM of each primer, 1U of DreamTaq DNA Polymerase and ~25 ng of template DNA. The PCR reaction consisted of an initial denaturation for 5 min at 95ºC, followed by 35 cycles of 95ºC for 30 sec, 52ºC for 1 min and 72ºC for 2 min. The final extension step was carried out at 72ºC for 7 min. PCR products were visualized in 1% agarose gel running in 1xTAE buffer and purified with "illustra GFX PCR DNA Purification Kit" following manufacturer's instructions. Sequencing of ITS region and a part of 28S rDNA (D1/D2 domain) was performed by STAB VIDA (Portugal) using ITS1/ITS4 primer pairs and NL1 as internal sequencing primer.

Phylogenetic analyses

DNA sequences were checked and edition made where necessary with BioEdit v. 7.0.9.0 (Hall 2006). The sequences of 28S rDNA were compared against GenBank database using BLAST program on the NCBI website and the phylogenetic analyses performed based on ITS sequence data. The ITS sequences of additional isolates and two outgroups (*Phaeosclera dematioides* CBS157.81; *Sarcinomyces crustaceus* CBS156.89) were retrieved from GenBank. Alignment was done with ClustalX v. 1.83 (Thompson et al. 1997). Alignments were checked and improved manually where necessary. Phylogenetic analyses were carried out with PAUP v. 4.0b10 (Swofford 2003) for neighbour-joining (NJ) and maximum-parsimony (MP) analyses. The neighbour-joining analysis was performed as described by Abdollahzadeh et al. (2010). Maximum-parsimony analysis was performed using the heuristic search option with 1000 random taxon additions and tree bisection and reconnection (TBR) as the branch swapping algorithm. All characters were unordered and of equal weight and gaps were treated as fifth character. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications (Hillis & Bull 1993). Moreover, consistency index (CI), retention index (RI) and homoplasy index (HI) were calculated. New sequences were deposited in GenBank.

RESULTS

Isolates

In this study, 15 isolates morphologically resemble to black meristematic fungi; *Endoconidioma* and *Hormonema* were obtained from necrotic twigs of grapevine and walnut trees. In terms of morphology, all isolates were similar and two representatives were selected for molecular analyses.

Phylogeny

BLAST analysis of D1/D2 domain of 28S rDNA sequence data showed that our isolates (Accession no.: IRAN2350C: KX180153; IRAN2351C: KX180152) are belong to *Endoconidioma*. After alignment, ITS dataset of the two representative isolates sequenced in this study and 35 sequences of 24 taxa retrieved from GenBank contained 762 characters. Incomplete portions at the ends of the sequences were excluded from analyses. Of the 762 characters (including gaps) in the aligned dataset, 201 were excluded, 308 were constant and 83 were variable and parsimony uninformative. A heuristic search of the remaining 170 parsimony informative characters resulted in 564 most parsimonious trees of 539 steps (CI= 69, HI=31, RI=82), each with the same topology. NJ analysis produced a tree with similar topology to the MP trees. One of the MP trees is shown in Fig. 1 with bootstrap support values for MP above and NJ below the branches. Our isolates were placed in a group containing the type specimen of *Endoconidioma populi*. Isolates of *Hormonema carpetanum* placed outside *Hormonema* and constituted a subclade as a sister group with *E. populi* within *Endoconidioma*.

Taxonomy

Endoconidioma populi Tsuneda, Hambleton & Currah, Mycologia 96 (5): 1129. 2004. MycoBank MB487733. Fig. 2.

On twigs of *Juglans regia* (Fig. 1-a) and *Vitis vinifera*: Conidiomata forming on black subicula, globose to subglobose, nonostiolate, brown to black. Endoconidia formed endogenously, smooth, hyaline, unicellular, mostly ovoid to ellipsoid. Hyphae smooth, dark, melanized, septate. Blastic conidia produced on hyphae, unicellular or two-celled, brown, ovoid to ellipsoid.

Cultural characteristics on PDA. Colonies initially mucoid and yeast-like, becoming a hemispherical, raised, carbonaceous mass of thick-walled cells, with a thin peripheral area, consisting mostly of submerged

hyphae, Grey Olivaceous (21""b) to Olivaceous Black (27""m) at the surface and reverse after 2 weeks in the dark at 25°C. Colonies reaching 25–30 mm on PDA after 2 wk in the dark at 25°C. Conidiomata brown to black, subglobose to flask-shaped, nonostiolate, 30–95 (–180) × 30–60(–110) µm, with a peridium consisting of pigmented and thick-walled cells. Endoconidia ovoid to ellipsoid, hyaline or brown, unicellular, (3.5–) 5.7–8.3 (–9) × 2–5 µm, released by dissolution of the peridial cells. Hyphae smooth, subhyaline to brown, septate, cylindrical, becoming moniliform with age, forming holoblastic conidia. Blastic conidia arising from sides of hyphae, hyaline or brown; mostly unicellular, rarely two-celled, unicellular conidia ovoid or ellipsoid to cylindrical, mostly 4–8 × 3–4.5 µm; two-celled conidia ovoid to ellipsoid, constricted at the septum, 8–20 × 5–10 µm.

Specimens examined. IRAN, Kurdistan Province, Sanandaj, Arendan village, on *Vitis vinifera*, Sept. 2011, J. Nahvi Moghadam (IRAN 2350 C); Kurdistan Province, on *Juglans regia*, Aug. 2011, S. Mirzaei (IRAN 2351 C, IRAN 2352 C).

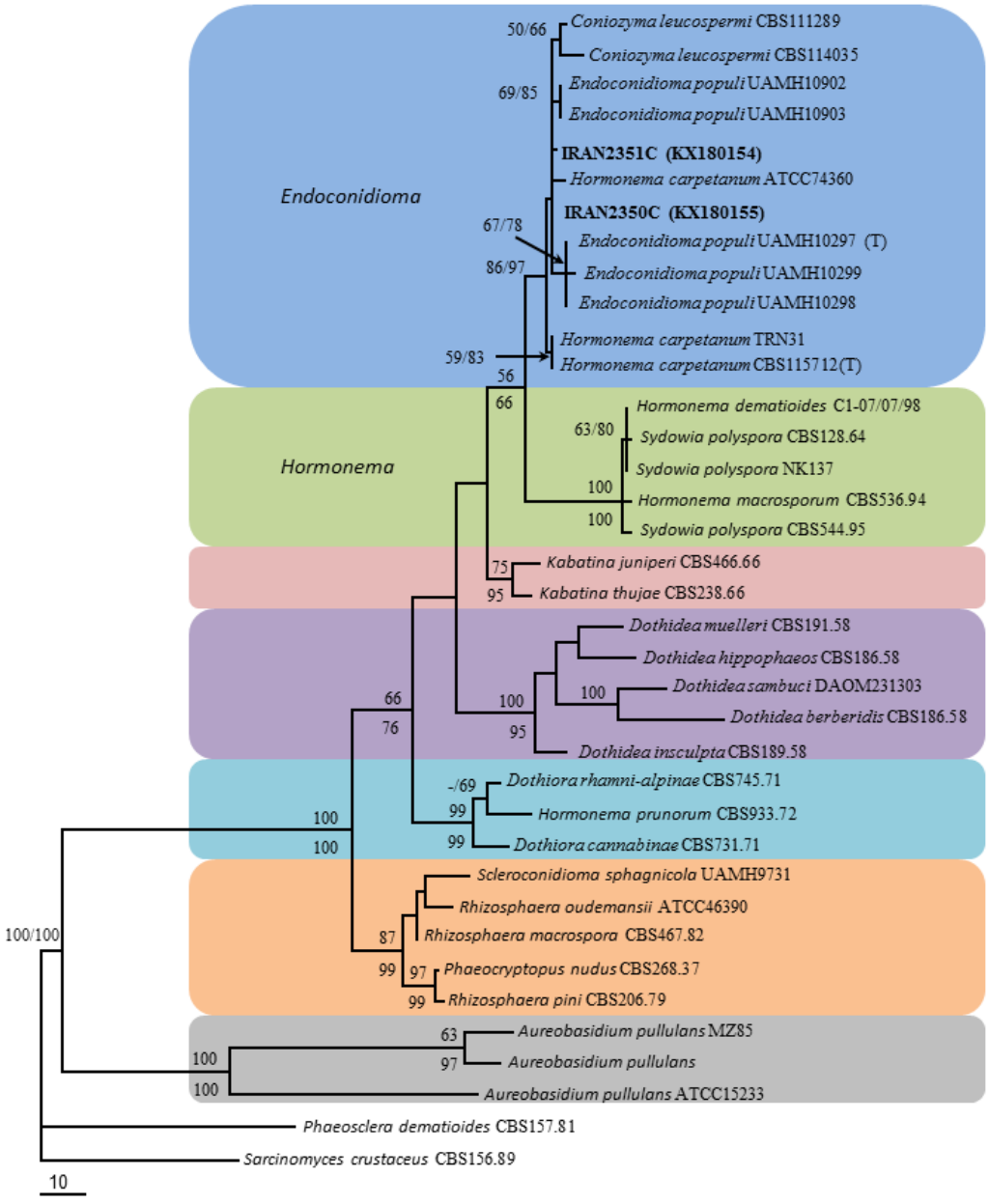

Fig. 1. One of 564 most parsimonious trees obtained from combined ITS sequence data. NJ and MP bootstrap values are given based on 1000 pseudoreplicates above and below the branches respectively. The tree is rooted to *Phaeosclera dematioides*

(CBS157.81) and *Sarcinomyces crustaceus* (CBS156.89). The bar represents 10 changes. Isolates characterized in this study are in bold typeface. (T): denotes a type strain.

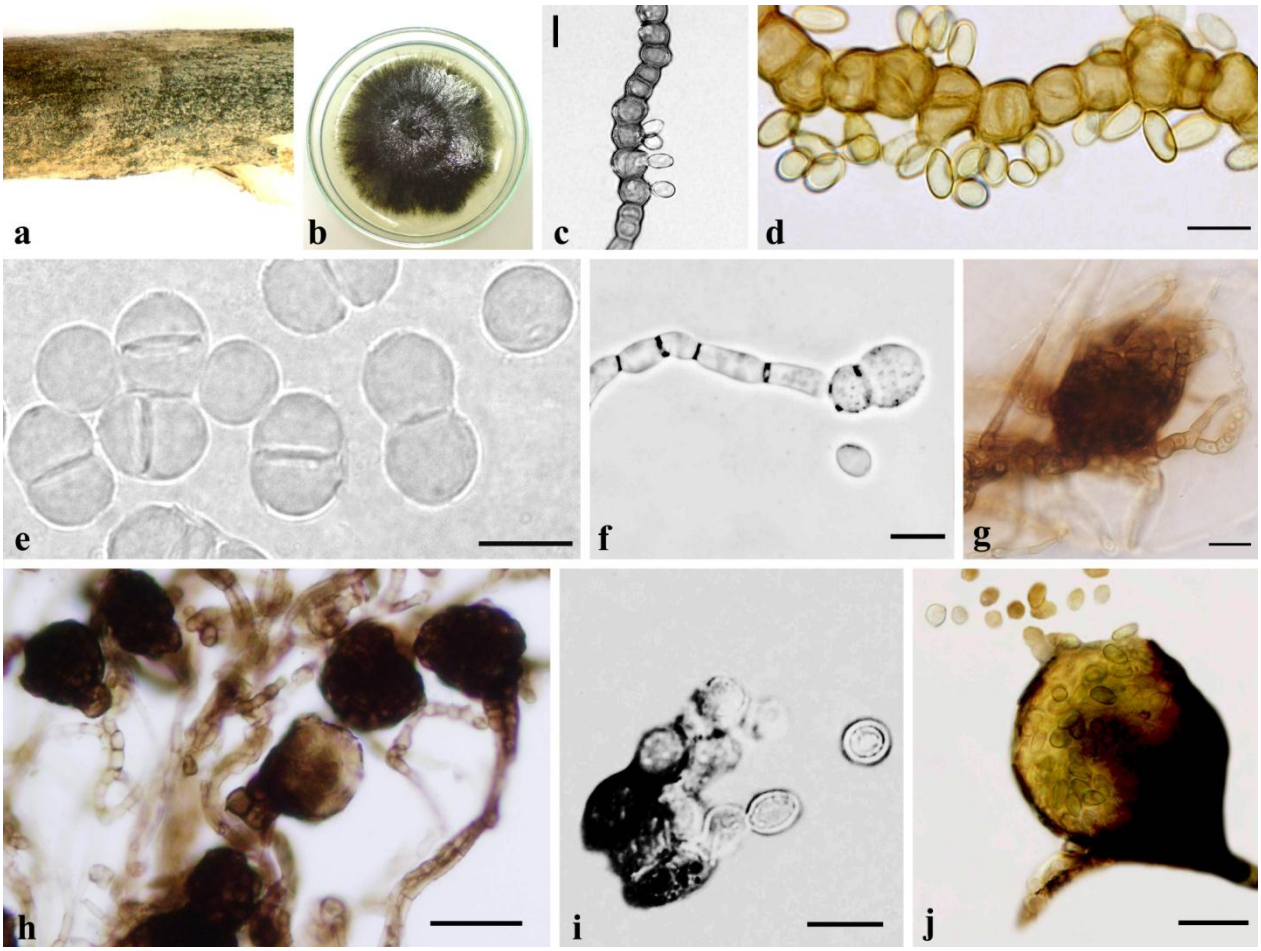

Fig. 2. *Endoconidioma populi*. **a.** Black subiculum formed on twigs of *Juglans regia*; **b.** Colony on PDA (30–day–old, IRAN 2350 C); **c., d.** Unicellular, blastic conidia on the hyphae; **e.** Two-celled blastic conidia; **f.** Germination of two-celled blastic conidia; **g.** Conidiomatal initials; **h.** Mature conidiomata; **i.** Endoconidia and conidiogenous cells; **j.** Conidiomata releasing endoconidia. — Scale bars. c–e, j = 10 µm; f–g = 20 µm; h = 50 µm; i = 5 µm.

DISCUSSION

Based on morphology and molecular sequence data we identified *Endoconidioma populi* as a new record for Iran mycobiota. Thus far it has been characterized on *Populus tremuloides* (Tsuneda et al., 2004) and *Alnus crispa* (Tsuneda et al., 2010), and here it is identified on *Vitis vinifera* and *Juglans regia*. The two isolates sequenced in this study were identical in 28S rDNA but differed in one nucleotide in ITS sequence data. Our isolates differed from the *E. populi* type specimen by three nucleotides in 28S rDNA (D1/D2 domain) and four nucleotides in ITS sequence data. Accordance with Tsuneda et al. (2010) in our phylogenetic analyses seven strain of *E. populi* including UAMH 10297 (type strain), UAMH 10298, UAMH 10299, UAMH 10902, UAMH 10903 and two Iranian isolates IRAN2350 and IRAN2351 along with two *Coniozyma leucospermi* strains (CBS 111298, CBS 114035) and three strains of *Hormonema carpetanum* ATCC 74360, TRN31 and CBS 115712 (type strain) constituted a monophyletic group with high bootstrap support from NJ (%97) and MP (%86) analyses. Phylogenetic analyses by Tsuneda et al. (2004, 2010) revealed a close relationship of *E. populi* with *Hormonema carpetanum*. In our study, the type strain of *Hormonema carpetanum* was also placed in a distinct subclade within *Endoconidioma* supported by NJ (%83) and MP (%59) analyses. Despite very small differences this species is morphologically similar with *E. populi*. The two strains of *Coniozyma leucospermi* constituted a subclade with low bootstrap support from both NJ (%66) and MP (%50) analyses. In phylogenetic study by Tusneda et al. (2010) this species formed a subclade with no support within a well-supported clade containing *E. populi* and *H. carpetanum*, but in terms of morphology *C. leucospermi* differs from *E. populi* in both conidioma morphology, conidiogenesis and the absence of blastic conidia. Generally based on our results and previous studies (Tsuneda et al., 2004, 2010) *C. leucospermi* and *H. carpetanum* belong to *Endoconidioma*, but we believe more sampling from various hosts around the world and combination of

protein-coding genes with ITS sequence data is necessary to clarify the extent of *E. populi* and its relationship with these two taxa. Hence at the time being we prefer to avoid recombine these two species in *Endoconidioma*. As it is deduced from phylogenetic studies, *Endoconidioma* is closely related to *Hormonema* and some isolates characterized as *Hormonema* phylogenetically belong to *E. populi*. On the other hand Enfumafungin an antifungal triterpenoid glycoside is produced by *Hormonema* sp. ((Liesch et al. 1998, Peláez et al. 2000, Schwartz et al. 2000). Therefore it is important to consider the bioactivity and antagonistic potential of *E. populi* against plant pathogenic fungi in future studies.

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شناسایی مولکولی و مورفولوژیکی گونه *Endoconidioma populi* در استان کردستان، ایران

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چکیده: قارچ های مریستماتیک سیاه قارچ هایی همه جازی بوده که قادر به تحمل نوسانات شدید اشعه ماوراء بنفش، دما و رطوبت هستند و در محیط های در معرض این نوسانات شدید مثل سطوح سنگ ها رشد می کنند. در یک بررسی روی قارچ های مرتبط با پوسیدگی های میوه، لکه برگی ها، شانکر، گموز، سرشاخه میری و بیماری های تنه گردو و مو در استان کردستان تعدادی جدایه قارچی مشابه جنسهای *Endoconidioma* و *Hormonema* موسوم به قارچ های مریستماتیک سیاه یا مخمر سیاه از شاخه های نکروتیک گردو و مو جمع‌آوری شد. براساس ویژگی های مورفولوژیک و داده های مولکولی (28S rDNA و ITS) جدایه ها به گونه *Endoconidioma populi* تعلق دارند که به عنوان یک رکورد جدید برای فلور قارچ های ایران ترسیم و توصیف می شود. این گونه در این مطالعه برای اولین بار از گردو و مو جداسازی و گزارش می‌شود.

واژه‌های کلیدی: *Coniozyma*، *Hormonema*، Dothideaceae، ، درخت مو، درخت گردو

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