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Polyphasic identification of *Sepedonium microspermum* isolated from two genera of Boletales in Iran

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Abstract: Moldy *Boletus* sp. and *Xerocomus* sp. were collected from several locations at the campus of the University of Tabriz, Iran. Fungicolous fungal isolates were recovered and characterized by the combination of morphological traits and phylogenetic analyses of combined ITS and LSU sequence data. Fungal isolates were identified as *Sepedonium microspermum*. This is the first report of *S. microspermum* on *Xerocomus* sp. from Iran which is comprehensively described and illustrated.

Key words: Morphology, ITS–rDNA, *LSU* and *Hypomyces*

INTRODUCTION

The fungi that grow on macromycetes, rusts, powdery mildew, slime molds and etc., are called fungicolous fungi, even when the nature of the fungus–fungus association and its trophic relationships are obscure (Jeffries 1995). In nature, most of the fungicolous fungi are known as parasites (necrotrophs or biotrophs), commensals or saprobes (Hawksworth et al. 1995). Most of the mycologists such as Gilman and Tiffany (1952) and Barnett (1963, 1964), used the term for the fungi related to other fungi (Sun et al. 2019).

The taxonomy of fungicolous fungi has considerably progressed from the nineteenth century, especially with the findings of anamorph–teleomorph relations in the Hypocreales, as well as the reports of new mycoparasitic heterobasidiomycetous fungi (Sun et al. 2019). Regional surveys have mostly been limited to the taxa of sporocarp inhibiting fungi (e.g., Helfer 1991). Most of the fungicolous fungi that are related to mushrooms and plant soil–borne pathogens are scattered in temperate and sub–tropical regions (Sun et al. 2019). Rudakov (1978) study on fungicolous fungi resulted in the identification of about 1,700 (nonlichenicolous) species of this fungus. Moreover, he indexed fungicolous fungi occurring in the former Soviet Union, but many of his identifications need to be revised (Rudakov 1981). In a revision of the conidial fungicolous fungi, Hawksworth (1979, 1981) reported the number of 1,100 species grown on approximately 2,500 species of host fungi (including lichenized taxa).

The Hypocreaceae (Hypocreales) are the most important fungicolous fungi grown on fruit body of other fungi while Bionecteriaceae and Nectriaceae belonging to the Hypocreales, include mycoparasitic or mycosaprobic species (Rossman et al. 1999). Six genera of the Hypocreaceae include fungicolous fungi such as *Trichoderma* (=*Hypocrea)* and *Hypomyces* whose species are identified by morphological characteristics of teleomorph and anamorph (Põldmaa 2000).

Different genera of fungicolous fungi such as *Cladobotryum*, *Mycogone*, *Stephanoma* and *Sepedonium* are asexual forms of *Hypomyces* (Gams & Hoozemans 1970, de Hoog 1978, Rogerson & Samuels 1993, 1994, Rossman et al. 1999). *Cladobotryum* is one of the most important anamorphic fungicolous fungi of *Hypomyces* (Rogerson & Samuels 1993, Põldmaa 2000). Rogerson & Samuels (1985, 1989, 1993 and 1994) and Põldmaa (2000), classified the species of *Hypomyces* in the four fungal host groups including species that grow on Discomycetes, *Boletus* species (boleticolous species), Agaricales (agaricicolous species) and Aphyllophorales (aphyllophoricolous species). Other fungal groups that were previously reported before such as Discomycetes (Leotiales, Pezizales), Agarics (Russulales), Boletes and Aphylophorales are also the hosts of Hypomyces species (Zare & Asef 2008). One of the asexual forms of *Hypomyces* is the genus *Sepedonium* Link 1809 based on *S. mycophilum* (Pers.) Link 1809 as the type. Until now, the number of 58 species has been reported and listed for *Sepedonium* in index fungorum (http://www.indexfungorum.org; accessed on 14th,

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January 2019). *Sepedonium* is characterized by the production of aleurioconida and phialoconidia and most of the species from this genus are parasites on Boletales. In addition to boletes, *Sepedonium* spp. have been reported on *Scleroderma*, *Rhizopogon*, agarics, air, soil, dung and etc. (Rogerson 1989).

In Iran, there are limited studies on the species diversity of *Hypomyces* and related asexual forms in which species identification has relied solely on the morphological characteristics. Asef & Mohammadi Goltapeh (2002) listed four species of *Cladobotryum* including *C. dendroides*, *C. verticillatum*, *C. polypori*, and *C. varium*, but there was no sexual form in the investigated samples. Recently, Asef & Zare (2006) recorded three species of *Hypomyces* as a sexual form of fungicolous fungi and a species of *Cladobotryum* from Iran, as a sexual form of fungicolous fungi and a species of *Cladobotryum* from Iran. Anamorphic forms of *H. aurantius* and *H. rosellus* which are called *C. varium* and *C. dendroides* respectively were reported as fungicolous fungi from Iran (Asef & Mohammadi Goltapeh 2002). The occurrence of *S. microspermum* and *Sepedonium* sp. on *Boletus* sp. and *Leccinum* sp. were the only reported cases from *Sepedonium* species in Iran (Zare & Asef 2008). The hosts were identified according to the book written by Keizer (2004). In this study, we provide the first occurrence of *S. microspermum* on *Xerocomus* sp. in Iran. The identification of species was confirmed by a combination of morphological characteristics and sequence data of ITS–rDNA region and LSU gene.

MATERIALS AND METHODS

Sample collection and fungal isolation

During a field excursion at the campus of the University of Tabriz in East Azerbaijan, Iran in 2016, samples were collected from fresh, mature and moldy specimens of some infected Boletales (five specimens for each species) and were stored separately in the paper bags to keep them clean for culture work. Host specimens were identified at genus level following Keizer (2004) protocol. Morphological observations and fungal isolations were done according to the protocols of Gams et al. (2004). Fungicolous fungal isolates from specimens were all recovered from the caps and hymenium of the collected hosts. Single–spore isolations were conducted on 2 % malt extract agar, according to the protocol of Torbati et al. (2018); in brief, using a sterile inoculation needle, a mass of conidia was picked up from the grown fungus on the host with the aid of a dissection microscope and suspended on 2 % malt extract agar (MEA; Merck, Darmstadt, Germany) plates supplemented by streptomycin sulphate (100 mg/L) containing 10 ml sterile water. The suspension was evenly spread on the surface of the medium and plates were kept overnight in an oblique position. The plates were then checked under the dissection microscope and germinating conidia were transferred to the potato carrot agar plates (PCA; freshly prepared according to Crous et al. 2009). Single–spore cultures were preserved on PCA in 2 ml agar slants at 4 °C.

Morphological identification

Morphological characteristics were examined both on the natural substrate and *in vitro*, following the protocol of Sahr et al. (1999). For all of the isolates, the colony color (surface and reverse) and growth rates were recorded on MEA after plate incubation at 25°C in the darkness. Colour notations were conducted according to the Rayner (1970).

Microscopic characters were examined based on the shape and size of conidia and aleurioconidia on conidiophores and MEA medium, respectively (Sahr et al. 1999). Sample slides were prepared from agar cultures using a sterilized needle or with the oblique coverslip method (Nugent et al. 2006). All the microscopic characters were examined and measured using sterile water as a mounting medium. Whenever possible, a minimum of 25 measurements was made per structure with extreme values given in parentheses. Olympus digital camera system (DP 25) mounted on an Olympus BX41 light microscope was applied to take photographs of microscopic fungal structures. Adobe Photoshop CS6 (Adobe Systems Inc., USA) was used to edit the photos and prepare photo plates. Representative cultures were deposited in the culture collection of Tabriz University (CCTU), Tabriz, Iran and the culture collection of the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, Netherlands.

Molecular identification

Genomic DNA was isolated from fungal mycelium grown on MEA using the Moller et al. (1992) protocols. The internal transcribed spacers 1 and 2. the intervening 5.8 S gene of the rDNA [ITS] and the large subunit gene of the rDNA [LSU] were amplified and sequenced using the following primer combinations: ITS1 plus ITS4 for ITS (White et al. 1990), and LR0R plus LR5 for LSU (Vilgalys & Hester 1990, Vilgalys & Sun 1994). Polymerase chain reaction amplifications were performed on the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) or a 2720 thermal cycler (Applied Biosystems, Foster City, CA). PCR amplification reactions were prepared with total volume of 12.5 µL and contained 0.1 µL Taq DNA polymerase (5 U/ µL BIOTAQ™ DNA Polymerase, BioLine, Germany), 1.25 µL PCR buffer (10X NH_4 reaction buffer, BioLine, Germany), 0.5 µL $MgCl_2$ (50 mM, BioLine, Germany), 0.5 µL dNTP mix (10 mM, BioLine, Germany), 0.7 µL dimethyl sulfoxide (DMSO, Sigma–Aldrich, Germany), 0.25 µL of each primer (10 µM) and 1 µl of template DNA. The PCR reaction was carried out a 94 ºC for 180 s, 35 cycles of denaturation/extension at 94 ºC for 30 s, annealing for 60 s at 57 ºC for ITS and LSU, the 80 s at 72 ºC, and a final extension for 60 s at 72 ºC. The amplified products were purified using Sephadex® G–50 Fine (GE Healthcare, Sigma–Aldrich, Germany) and were

sequenced by the BigDye Terminator v. 3.1 (Applied Biosystems, Foster City, CA, USA) Cycle Sequencing Kits and subsequently analysed on an ABI Prism 3700 or an AB 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommended instructions. Raw sequence files were edited manually and consensus sequences were generated from each forward and reverse sequence using SeqMan™ II (DNASTAR, Madison, WI, USA). The sequences were subjected to BLAST search at GenBank and sequences with high similarity were downloaded and included in the alignment file. Sequence alignments were performed independently for each gene using MAFFT (Katoh & Standley 2013, Li et al. 2015) under the European Bioinformatics Institute (EMBL–EBI) webserver. Alignments were visually inspected and manually edited using MEGA v. 6.06 (Tamura et al. 2013). Additional sequences were obtained from GenBank.

Phylogenetic analysis

The phylogenetic analyses included Bayesian (B) were conducted using XSEDE platform on the CIPRES Science Gateway Portal (Miller et al. 2012). Evolutionary models were calculated using MrModelTest v. 2.3 (Nylander 2004) and the gaps were coded as missing data. Bayesian analyses included two Markov chains of four incrementally heated runs each and lasted for 5 M generations with the stoprule option on, a stopval value set to 0.01 and a sampling frequency of every 1000 generations. After runs conversion, 50 % majority rule consensus tree and posterior probabilities were calculated after discarding 25 % of initial trees as a burn–in fraction. Statistical support for the branches was evaluated using bootstrap analysis (BS) of 1000 replicates.

RESULTS AND DISCUSSION

A total number of ten specimens corresponding to two different fungal hosts i.e., *Boletus* sp. and *Xerocomus* sp. were collected. Ten *Sepedonium* isolates with similar cultural and morphological features were obtained from collected specimens. The isolates were identified as *S. microspermum* based on cultural and morphological characteristics.

***Sepedonium microspermum* Besl, Zeitschrift für Mykologie 64 (1): 46 (1998)**

Colonies on MEA medium reached 9–10 mm in diameter after seven days; colony surface was initially white, then became yellow after seven days with flat to velvet and zonate with abundant aleurioconidia, margin entire (Fig. 1). Conidiophores arising from aerial mycelium, macronematous, hyaline, septate, with single or 2–3 verticillate and slender phialides, (45–)59–66(–75) × (2–)3–4(–5) µm, apex 1–2 µm width; phialoconidia ovoid to fusiform, unicellular, smooth, (9–)11–13(–17) × (3–)4–6(–7) µm. Aleurioconidia on short side branches were yellow, globose, 8–14 µm diam., with angular tubercles; mycelium developing irregular spots and pustules; optimum growth at 25 ˚C.

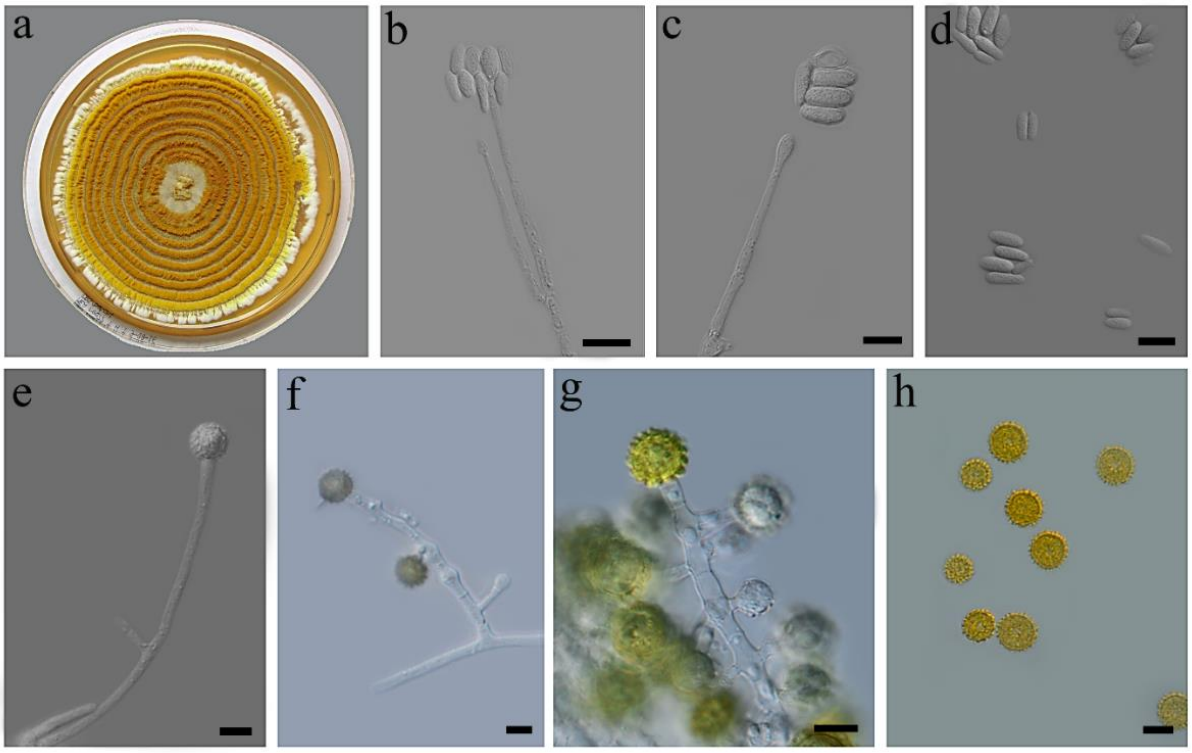

Fig. 1. *Sepedonium microspermum*. a. 7–d–old colony on MEA; b–d. Conidiophores and conidia; e–h. aleurioconidia. — Scale bar (b–h) = 10 µm.

Our BLAST search of ITS sequence data against the nucleotide sequences at GenBank showed high similarity with *S. microspermum*. Because of low LSU sequence data, we did not include our LSU sequence (MH878229) in the phylogenetic analysis. A phylogeny inferred based on ITS sequence data obtained in this study together with 90 sequences from GenBank grouped our sequence along with *S. microspermum* (AF054847) as a reference sequence chosen by Kadri Põldmaa in 2014 (Nilsson et al. 2018) in a clade composed of *S. microspermum* collections from different hosts that are available in GenBank. Based on ITS phylogeny, four species clades could be recognized within S. *microspermum* isolates which might represent additional cryptic species in *S. microspermum*. However, analysis of additional isolates and more genomic loci are required to address this question (Valdez & Douhan 2012). *Sepedonium microspermum* has been described only recently and is well characterized by smaller aleurioconidia and more distinctive tubercles than *S. chrysospermum* (Besl et al. 1998). *Sepedonium microspermum* has been linked to *Hypomyces microspermum*; however, in the present study, the sexual state was not observed. This species has a worldwide distribution and is currently known from a diverse range of substrates including soil, plant materials and other fungi (Arellano–Galindo et al. 2017).

Additional specimen examined. IRAN, East Azerbaijan province, the campus of the University of Tabriz, on *Xerocomus* sp., April 2016, M. Torbati (CCTUMO14=TuXe1)

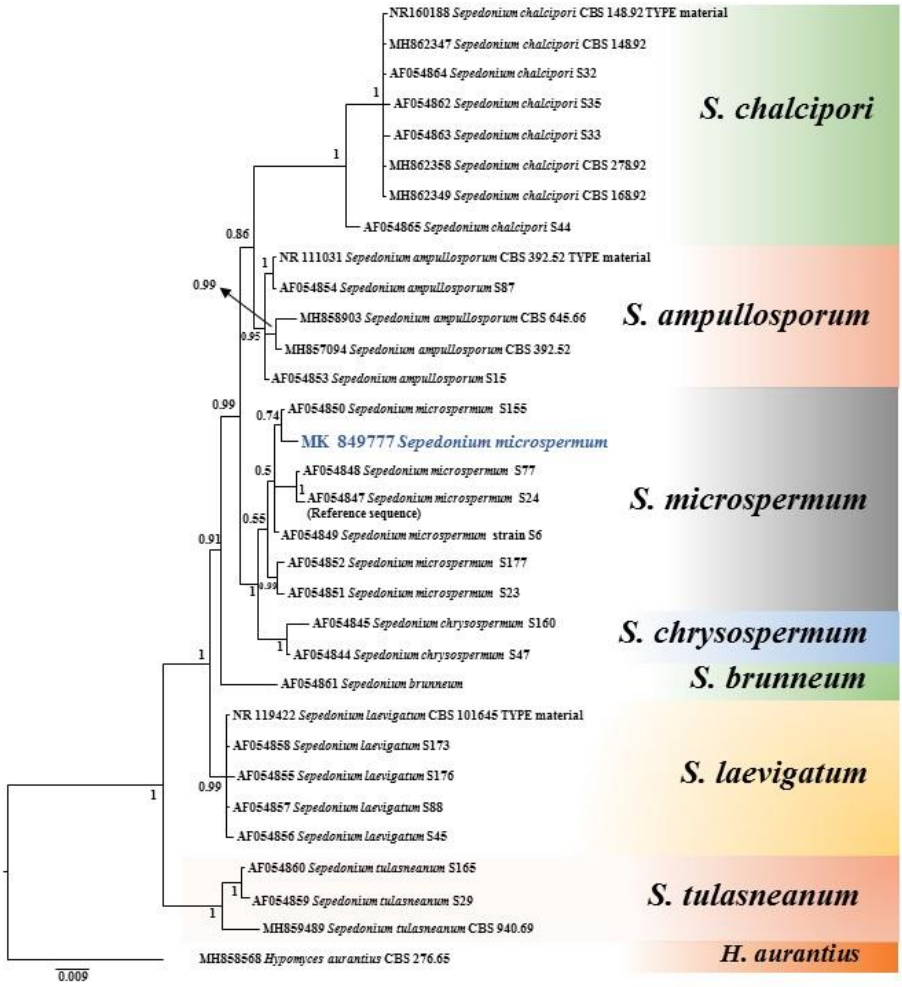

Fig. 2. Bayesian inference phylogenetic tree of *Sepedonium microspermum* generated using sequences of the internal transcribed spacer (ITS—rDNA). The representative strain CBS 141557 in this study is bolded in blue. The values above branches show Bayesian posterior probability. The scale bar indicates the number of expected substitutions per site. *Hypomyces aurantius* (MH858568) was used as the out–group

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شناسایی چند منظری گونه *Sepedonium microspermum* به دست آمده از دو جنس متعلق به راسته Boletales در ایران

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چکیده: در سال ۱۳۹۶ نمونه‌های متعددی از قارچ‌های کلاهکدار آلوده، متعلق به جنس‌های *Boletus* و *Xerocomus* از بخش‌های مختلفی از محوطه دانشگاه تبریز، استان آذربایجان‌شرقی‌تبریز جمع‌آوری شدند. گونه‌ای از قارچ قارچزی جداسازی شد که با استفاده از ویژگی‌های ریخت شناختی و داده‌های توالی نواحی ITS و LSU تعیین هویت گردیدند. جدایه‌های قارچی گونه *Sepedonium microspermum* تشخیص داده شدند. این اولین گزارش از قارچ *S. microspermum* روی *Xerocomus* sp. از ایران است. در این مقاله ما بطور کامل این گونه را توصیف و شرح داده‌ایم.

واژه های کلیدی: ریخت شناختی، ITS–rDNA، *LSU*، *Hypomyces*

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