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Bark beetle galleries as natural habitat for *Scedosporium minutisporum* in Iran

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Abstract: Bark beetles dig galleries in bark tissue and can transmit fungi in the forest. Their association with the members of the orders Microascales and Ophiostomatales are among the most exciting examples of symbioses in nature. In the present study, several synnematous fungal isolates were recovered from bark beetle galleries on declining woody hosts in Arasbaran and Toskestan forests in the northwestern and north zone of Iran. By the integration of morphological features with sequence data of ITS-rDNA region and *TUB* gene, the isolates were identified as *Scedosporium minutisporum.* A phylogeny inferred based on combined data set of the ITS-rDNA and *TUB* sequences clustered our isolate together with reference stain of *S. minutisporum* in the family Microascaceae. To the best of our knowledge *S. minutisporum* is new to the mycobiota of Iran. The pathogenic relevance of this species on bark beetles remains to be studied.

KEYWORDS: *Microascaceae*, *Scedosporium*, *TUB*, ITS

INTRODUCTION

Bark beetles, belonging to the family Curculionidae, comprise a species-rich group of insects with a global distribution and significant social and economic impacts, in fact they are well-known forest pests. Bark beetles are can transmit mites, bacteria, and fungi and their association with fungi is among the most exciting examples of symbiosis in nature (Linnakoksi et al. 2012). They complete the life cycle inside tree tissues, where they excavate galleries in the inner bark with their strong mandibles (Biedermann and vega 2020). A large fungal flora, including ascomycetes, basidiomycetes, and zygomycetes, are associated with bark beetles and grow within their created galleries. Fungi are a good nutrition source for bark beetles, because they play a key role in the initial degradation of hard plant tissues and provide sterols, B vitamins, nitrogen, and readily digestible carbohydrates for them (Repe and Jurc 2010). Ascomycetes are the most abundant fungi found in association with bark beetles (Linnakoksi et al. 2012). Among them, the orders Microascales and Ophiostomatales belonging to the ophiostomatoid fungi are a diverse and polyphyletic group that are mainly present in these galleries. These orders often produce long stalks bearing sticky conidia (synnemata) that are suitable for dispersal with insects (Repe and Jurc 2010).

Microascaceae (order: Microascales, class: Sordariomycetes) which was originally erected by Malloch (1970), is a monophyletic family comprising morphologically heterogeneous fungi *viz.*, *Acaulium*, *Cephalotrichum*, *Fairmania*, *Fuscoannellis*, *Gamsia*, *Kernia*, *Lomentospora*, *Lophotrichus*, *Microascus*, *Parascedosporium*, *Petriella*, *Petriellopsis*, *Pithoascus*, *Pseudoscopulariopsis*, *Scedosporium*, *Scopulariopsis*, *Wardomyces*, *Wardomycopsis* and *Yunnania* (Abrantes et al. 2021). Some of these genera are saprobic, plant pathogenic, or human opportunistic pathogens (Jankowiak et al. 2019). Even though members of the family Microascaceae are morphologically diverse, two approximate groups can be distinguished within the family, including species with generally scedosporium-like anamorphs with slimy conidia, and species with prevalently scopulariopsis-like anamorphs with hydrophobic conidia (Lackner et al. 2014).

Scedosporium Sacc. ex Castell. & Chalm. (Microascaceae) was first introduced in 1911 with *S. apiospermum* as the type species. *Scedosporium* species are morphologically homogeneous, and in the past species delineation in this genus has been based on the size of the cleistothecia and ascospores (Cortez et al. 2008). *Scedosporium* spp. are widely known as opportunistic human pathogen, mostly causing mycetoma. They are able to live in extreme environments such as high temperature, very low oxygen pressure, high salt concentrations and high osmotic pressures (Rougeron et al. 2017). They are commonly found in the environment, where they can colonize soil, sewage and polluted water, but theyare also associated with wood in saline water for sexual

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sporulation (Issakainen et al. 1997). *Scedosporium* species have been also isolated from feces of poultry (such as chickens), bats, and blackbirds as well as burrows of bamboo rats (Rougeron et al. 2017).

The taxonomy of the genus *Scedosporium* is rather complex and has changed since the early 1910s, when the first species of the genus was described. The generic name was proposed by Saccardo (1911). He isolated a new fungus from a patient with mycetoma in Italy in 1911 (Cortez et al. 2008) and described it as *Monosporium apiospermum*. Castellani and Chalmers (1919), validated the generic name and accepted *S. apiospermum* instead of *M. apiospermum* (Abrantes et al. 2021). *Scedosporium* species develop different of asexual morphs (A) scedosporium-like: producing solitary conidia from annelidic conidiogenous cells (B) graphium-like synnematous synanamorph and also unicellular sessile conidia (Abrantes et al. 2021). Currently eleven *Scedosporium* species are accepted including *S. americanum*, *S. angustum*, *S. apiospermum*, *S. aurantiacum*, *S. boydii*, *S. cereisporum*, *S. desertorum*, *S. dehoogii*, *S. ellipsoideum*, *S. fusoideum*, *S. minutisporum*, and *S. apiospermum*. In the past, the identification was based on morphological features, biochemical assays, and host association, as the main criteria for fungal species delineation (Bhunjun et al. 2021). Identification of *Scedosporium* species solely based on morphological criteria has proven troublesome; hence, several studies were conducted employing DNA sequences. Sequence data of ITS-rDNA region is suitable to construct an overview of genera within the family Microascaceae (Lackner et al. 2014). *Scedosporium* species are easily distinguishable phylogenetically by comparing the sequences of *β-tubulin* gene and ITS region (Lackner et al. 2014). In Iran, only *S. apiospermum* and *S. dehoogii* have been reported from the soil of different parks (Pakshir et al. 2013). Recently *S. aurantiacum* has been isolated from 67-year-old woman lungs that the progression of the infection led to her death (Ghasemian et al. 2021).

The present study aimed to determine the diversity of *Scedosporium* species associated with bark beetle galleries in two undisturbed forest sites in Iran (Toskestan National Park and Arasbaran Protected Area; north and northwestern Iran). The fungal diagnosis is based on a combination of DNA sequencing and morphological characters.

MATERIAL AND METHODS

Sample collection and fungal isolation

In 2018, 32 tree outer bark samples were collected from bark beetle (Curculionidae) galleries on declining woody hosts (*Quercus* sp., *Umus* sp., *Juglans* sp.) in two sites in Iran: Toskestan National Park (Golestan province; 36.7819° N, 54.5828° E) and Arasbaran forest (East Azerbaijan province, 38.9079° N, 46.8652° E). Small pieces of tree outer bark with galleries, approximately 0.5-1 cm long, were surface-sterilized by soaking in 70% ethanol for 1 min and rinsed twice in sterile water and dried on sterile filter paper. Samples were subsequently transferred on potato dextrose agar (PDA, Merck, Darmstadt, Germany) supplemented with 100 mg l^{-1} streptomycin sulfate to prevent bacterial growth and incubated at 25 °C for 7 days. Fungal isolates were purified using the hyphal-tip technique (Torbati et al. 2016). Purified isolates were long-term stored on potato carrot agar (PCA; 40 g of each boiled and filtered carrots and potatoes, 15 g agar, 1 L distilled water), at 4 °C. The isolates were deposited in the Culture Collection of Tabriz University (CCTU; Tabriz, Iran) and the Iranian Fungal Culture Collection (IRAN; Iranian Research Institute of Plant Protection, Tehran, Iran).

Morphological identification

Morphological features of the isolates were determined following the protocol of Abrantes et al. (2021) and Meirelles (2020). Fungal isolates were grown on oatmeal agar (OA; Himedia, India), PDA, Sabouraud's dextrose agar (SDA; 40 g Glucose, 10 g Peptone, 15 g Agar, 1 L distilled water) and incubated at 25°C. The color description was made using Rayner's color nomenclature (1970). All fungal structures were mounted in 90% lactic acid and examined under a microscope, and photographed by Olympus digital camera system DP21 (Olympus Corporation, Japan) mounted on Olympus BX41 (Olympus Corporation). Thirty measurements were made for each microscopic element where possible. An average of measurements was made for each fungal structure, and after calculating the size of structures, 95th percentiles were counted for all measurements with the averages in between brackets.

Genomic DNA extraction and PCR amplification

Genomic DNA was extracted from the fresh mycelium of colonies grown on PDA for 10 days at 25 °C in the dark, according to Moller et al. (1992). The DNA samples were stored at 4 °C for immediate use and stored at –20 °C for long-term storage. DNA sequencing data was obtained from the partial sequences of two loci, the internal transcribed spacers of nuclear ribosomal DNA (ITS) (White et al. 1990) and *beta-tubulin* (*TUB*) gene (Glass & Donaldson, 1995; O'Donnell & Cigelnik, 1997), respectively based on ITS1/ITS4 and Bt-2a/Bt-2b primer pairs. The amplification was performed by Bio RAD-Mj Mini thermal cycler in a total volume of 25 µL. PCR mixture contained 12.5 µL of Taq DNA Pol (2x) Master Mix (pishgam, Tehran), 0.2 µM of each forward and reverse primers and 50-60 ng of DNA template. PCR amplification condition consisted of a pre-denaturation step at 94 °C for 5 min, followed by 36 cycles of denaturation at 94 °C for 1 min,an annealing step at 52 °C and 50 °C, respectively, for ITS and *TUB*) for 1 min, primer extension at 72 °C

for 1 min and final extension of 7 min at 72 °C. PCR products were visualized on 1% agarose gel in 1 × TAE buffer containing 0.1 µg/mg ethidium bromide by ultraviolet gel imaging. PCR products were sent to Macrogen Company (South Korea) for sequencing. The obtained sequences were then analyzed using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and closely related phylogenetic sequences were obtained from the National Centre of Biological Information (NCBI) database.

Sequence alignment and phylogenetic analyses

The dataset for ITS and *TUB* sequences was selected for phylogenetic analyses from GenBank and recent publications (Gilgado et al. 2005; Crous et al. 2016; Abrantes et al. 2021). GenBank accession numbers for sequences used in the current research are presented in supplementary Table. 1. DNA Dragon v. 1.6.0 (Hepperle 2017) and BioEdit v. 5.0.6 (Hall 1999) software were used to create consensus sequences from the forward and reverse sequences. The collected sequences, together with sequences obtained in this study were aligned by MEGA 7 (Molecular Evolutionary Genetics Analysis) (Kumar et al. 2016). The aligned sequences were concatenated with Mesquite v. 3.10 (Maddison & Maddison 2015). The significant evolutionary models were achieved for each gene partition using MrModeltest v. 2.3 (Nylander 2004). To determine the position of our isolate in the genus, concatenated alignment was analyzed using Bayesian inference by MrBayes v. 3.2.1 software (Ronquist & Huelsenbeck 2003), using the same GTR+G model with heating parameter set at 0.15, and two MCMC running up to 1×10^7 generations and sampling trees every 1×10^3 generations. Satisfactory convergence was assessed using the standard deviation of split frequency. The first 25% of saved trees were discarded as the burn-in, and consensus trees and their posterior probabilities (PP) were determined from the remaining trees. The generated phylogenetic trees were examined using FIG TREE v. 1.3.1 (Rambaut 2009). *Petriellopsis africana* CBS 311.72 was used as an outgroup taxon.

RESULTS

Among a total number of 15 isolates, three of them, with similar morphology and growth patterns were obtained respectively from bark beetles galleries on oak, elm, and walnut trees in Arasbaran and Toskestan forests. Following a comprehensive morphological evaluation, one isolate was selected for further molecular studies.

Phylogeny

In the phylogenetic analysis of *Scedosporium* spp., ITS-rDNA alignment consisted of 14 samples and 536 sites (including alignment gaps), and the *TUB* alignment consisted of 14 samples and 441 sites (including alignment gaps). The final concatenated alignment comprised 14 taxa and 977 sites (locus boundaries: 1-536 and 537-977 for ITS-rDNA and *TUB*, respectively), of which 646 (ITS: 410 and *TUB*: 236) were constant. Bayesian analyses were performed using the best fitting substitution (GTR + G) model and lasted in 6000 generations and a total of 102 trees (Each file contained 51 trees). In the multigene phylogenetic assessments based on a combined ITS-rDNA and *TUB* sequence dataset, our isolates belonging to *S. minutisporum* along with the isolate FMR 4072 nested in a well-supported clade (Fig. 1).

Taxonomy

Scedosporium minutisporum (Gilgado, Gené, Cano & Guarro) Lackner & de Hoog, Fungal Diversity 67: 9 .2014. Fig. 2.

Colonies on OA, PDA and SDA attaining a diameter of 70, 51 and 48 mm, respectively after 2 weeks of incubation at 25 °C in darkness. Colonies on PDA dense and usually cottony to lanose, gray combined with white color that later becomes olive areas, with a whitish, irregular, and fimbriate margin; in reverse dark brown at the center and greenish olivaceous towards the periphery; on OA are creamy-white color, circular, flat, medium dense, entire, with granular at the center due to the abundance of ascomata and with aerial mycelium and colorless in reverse; on SDA dense, greyish-white cottony to woolly with concentric growth pattern white margins and brownish at the center and honey towards the periphery in reverse.

The isolates developed abundant ascomata on OA. Ascomata were solitary, nonostiolate, globose, yellowish brown and 70 to 180 µm in diameter. The asci were evanescent and ascus walls easily dissolved to release the ascospore. Ascospores were one-celled, subhyaline, smooth, ellipsoidal, and 3.8 to 8.1 µm high by 3 to 6.5 µm wide, with oil drops. Several types of asexual morphs were developed. The *Graphium* anamorph produced synnemata, which were septate, erect, flexuous, single or in clusters, stalk dark brown colored, becoming hyaline at the tip and 220-534 µm in length and 9.70-18.30 µm in wide and terminated in a slimy head of conidia by 35 to 80 µm wide. Conidiogenous cells annellidic. The conidia were predominantly cylindrical, 4.5 to 7.5 µm long by 2 to 3.5 µm wide. The sessile conidia holoblastic, light brown, one-celled, solitary, smooth and thick-walled, ovoid, 4.5–7 × 3–4.7 µm, attached directly to the hyphae or on short lateral branches.

The third type of asexual (*Scedosporium* anamorph) form included undifferentiated conidiophores from vegetative hyphae; branched, conidiogenesis anellidic, conidia subhyaline to light brown, smooth and thick-walled, obovoid, subclaviform, and 4.45 to 8.7 µm long by 1.80 to 4.8 µm wide.

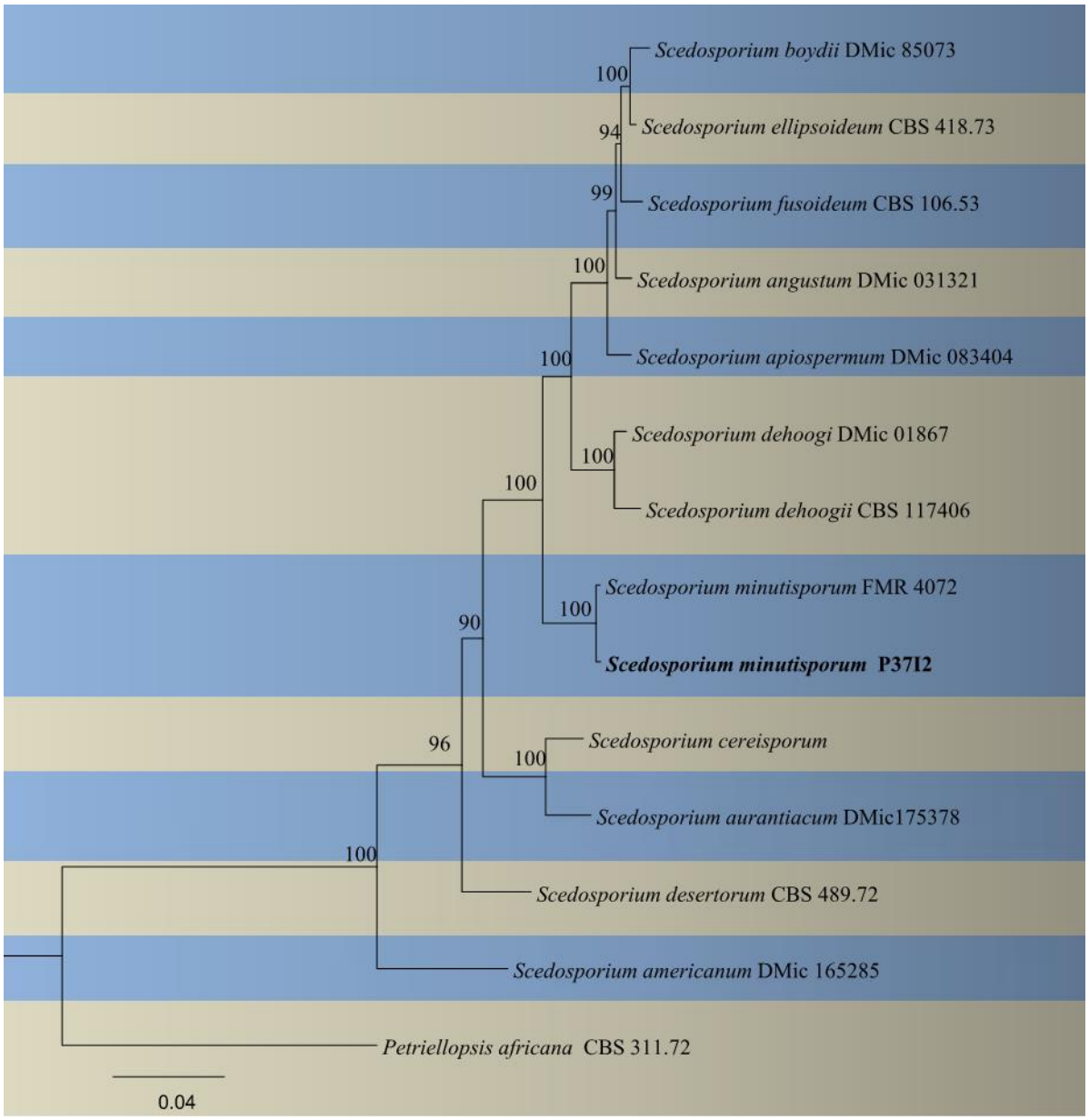

Fig. 1. Bayesian inference phylogenetic tree of *Scedosporium* species generated using concatenated sequences of ITS-rDNA and *TUB*. A GTR+G model was used for both loci. The values above branches show Bayesian posterior probability. The scale bar indicates the number of expected substitutions per site. *Petriellopsis africana* 311.72 was used as the out-group. The sequence generated in this study is shown in boldface.

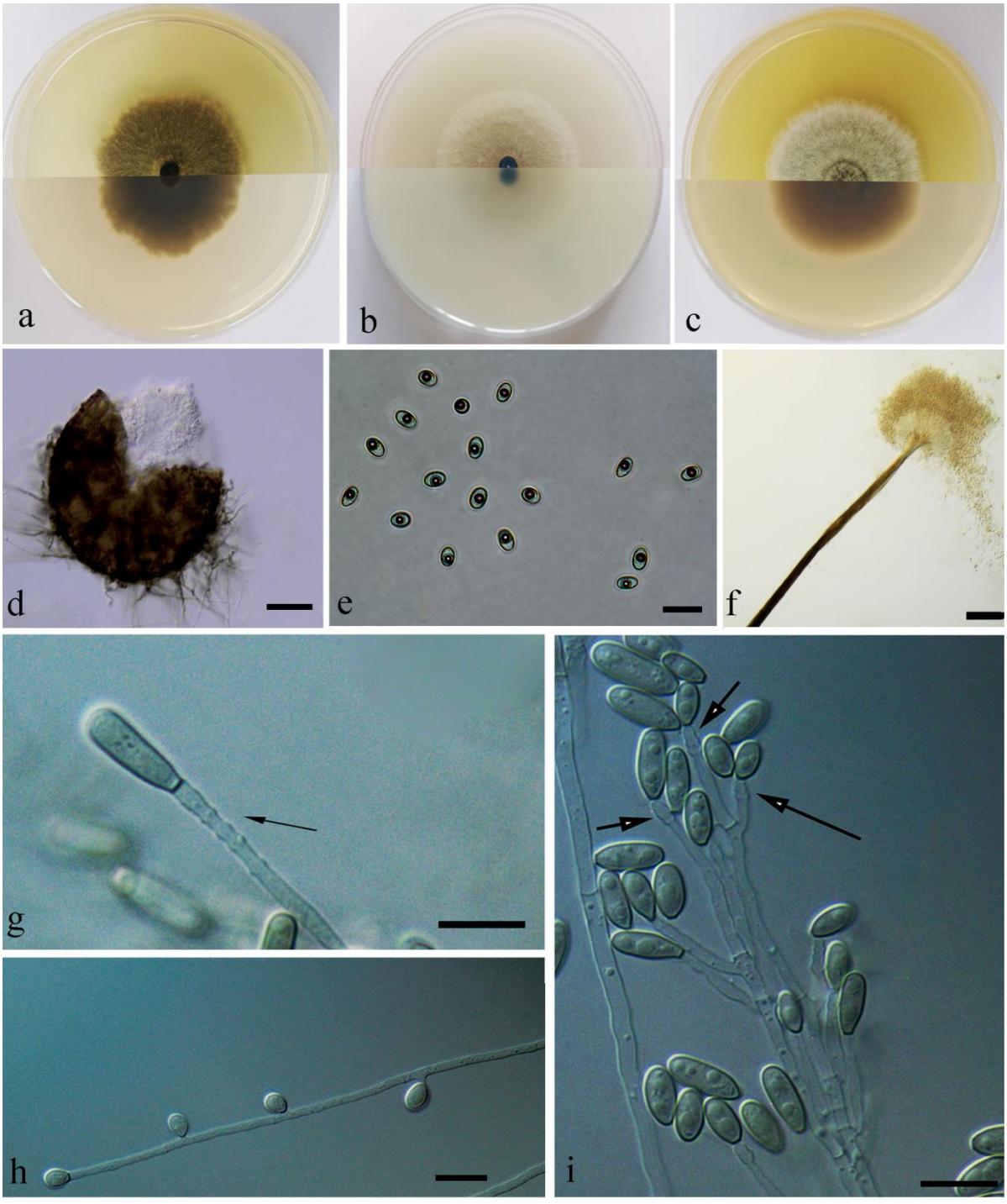

Fig. 2. *Scedosporium minutisporum*. a-c. Colony on PDA, OA and SDA after 14 days at 25 °C. d. Ascocarp. e. Ascospores. f. Synnematous conidiomata. g. Conidiogenous cell and conidia of *Graphium* anamorph. h. Sessile conidia. i. Conidiogenous cells and conidia of the *Scedosporium* anamorph. Arrows indicate annellations at the top of conidiogenous cells. Scale bars: d,f = 20 µm; e,g,h,i = 10 µm.

Tabe. 1. GenBank accession numbers of sequence data used in phylogenetic analysis**.**

Taxon	Strain	GenBank accession number		Reference
		ITS	BT2	
Petriellopsis africana	CBS 311.72	AJ888425	AJ889603	Gilgado et al. 2005
Scedosporium americanum	DMic 165285	MT803032	MT813171	Abrantes et al. 2021
Scedosporium angustum	DMic 031321	MT803009	MT813148	Abrantes et al. 2021
Scedosporium apiospermum	DMic 083404	MT803014	MT813153	Abrantes et al. 2021
Scedosporium aurantiacum	DMic 175378	MT803037	MT813176	Abrantes et al. 2021
Scedosporium boydii	DMic 85073	MT803004	MT813143	Abrantes et al. 2021
Scedosporium cereisporum	FMR 12995	KJ599660	KJ599659	Crous et al. 2016
Scedosporium dehoogii	CBS 117406	KT163400	KT163401	Abrantes et al. 2021
Scedosporium dehoogii	DMic 01867	MT803008	MT813147	Abrantes et al. 2021
Scedosporium desertorum	CBS 489.72	AM409106	AM409101	Abrantes et al. 2021
Scedosporium ellipsoideum	CBS 418.73	AJ888426	AJ889595	Abrantes et al. 2021
Scedosporium fusoideum	CBS 106.53	AJ888428	AJ889601	Abrantes et al. 2021
Scedosporium minutisporum	FMR 4072	AJ888384	AJ889592	Abrantes et al. 2021
Scedosporium minutisporum	P37I2; IRAN 4701C	OM914940	OP428980	Present study

DISCUSSION

Species of *Scedosporium* are ubiquitous and can be isolated from a diverse range of environments, mainly nutrient-rich substrates, such as manure of livestock, poultry or cattle manure, bat and birds' guano, agricultural soils, or poorly aerated nutrient-rich substrates, such as muds from ditches or ponds (Mouhajir et al. 2020). The studies conducted so far indicate that these fungi commonly occur in human-impacted areas and human activities significantly influence *Scedosporium* ecology (Mouhajir et al. 2020). In addition, a large number of members of the *Scedosporium* genus are known as human pathogens and were predominantly isolated from the airways of patients with cystic fibrosis (CF), ranking the second among the filamentous fungi (Rougeron et al. 2017). Some *Scedosporium* species have been isolated from xylophagous or saproxylophagous insects. For example, *S. boydii* was isolated from an unspecified bark beetle and termites (Rougeron et al. 2017). This species in termites produces two antifungal metabolites that have a favorable antifungal effect on *Candida albicans* and *Trichophyton rubrum.* Today, many immunocompromised patients are at high risk for developing invasive candidiasis and filamentous fungi producing drugs with this compound can help them (Nirma et al. 2013). It has been shown that *S. boydii* residing in the gut of *Holotrichia parallela* larva produces four antibacterial metabolites active against the clinical strains (Wu et al. 2012). According to results of the latest taxonomic studies, *Scedosporium* includes 11 legitimate species (Abrantes et al. 2021). In fact, 11 species of *Scedosporium* can be differentiated phylogenetically by comparing the sequences of a fragment of the *TUB* gene rather than by phenotype (Abrantes et al. 2021). In this study, multigene phylogeny based on ITS-rDNA region and *TUB* gene showed that isolate P37I2 (IRAN 4701C) nested in a well-supported clade along with *S. minutisporum* (FMR 4072). The morphology of ascomata, ascospores, synnemata, and the cultural characteristics of our isolate also was in full

agreement with the original description of *S. minutisporum* species. *Scedosporium minutisporum* is an environmental and clinically relevant species which has been isolated from patients with cystic fibrosis in France and soil samples from France, Austria, the Netherlands, Australia and Thailand (Rougeron et al. 2017).

Contrary to previous studies, in which no *Scedosporium* species were isolated from the natural/intact area, this is the first time that *S. minutisporum* is consistently isolated from bark beetle galleries on declining oak, elm and walnut trees in forests in two sites in Iran located at approximately 1000 Km from each other, in fact, the previous study has shown that *Scedosporium* spp. are not present in natural habitats such as forests, although they can be found in agricultural soils, urban parks, playgrounds and industrial areas (Al-yasiri et al. 2017).

To the best of our knowledge this is the first record of *S. minutisporum* from Iran and the first report of the isolation of this fungus from bark beetle galleries in the world.

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دالان‌های سوسک‌های پوستخوار بعنوان زیستگاه طبیعی برای قارچ *Scedosporium minutisporum* در ایران

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چکیده: سوسک‌های پوست‌خوار در پوست درختان دالان‌هایی را حفر می‌کنند و از آن طریق باعث انتقال قارچ در جنگل می‌شوند. همزیستی آن‌ها با دو راسته‌ی Ophiostomatales و Microascales از هیجان انگیزترین مثال‌های همزیستی در طبیعت به شمار می رود. در تحقیق حاضر، چندین جدایه قارچی سینماتادار از دالان سوسک‌های پوستخوار روی میزبان‌های چوبی دارای علائم زوال از جنگل‌های توسکستان و ارسباران در شمال و شمالغرب ایران جداسازی شد. براساس تلفیق ویژگی‌های ریخت‌شناختی با داده‌های توالی ناحیه ITS-rDNA و ژن *TUB*، هویت جدایه‌ها گونه *Scedosporium minutisporum* تعیین گردید. آنالیز فیلوژنیتک توالی‌های داده ناحیه ITS-rDNA و ژن *TUB*، جدایه شناسایی شده در این تحقیق را همراه با جدایه مرجع *S. minutisporum* در یک خوشه و در خانواده Microascaceae قرار داد. بر اساس اطلاعات موجود *S. minutisporum* آرایه جدیدی برای مایکوبیوتای ایران به شمار می‌رود. پتانسیل بیماری زایی این گونه روی سوسک های پوستخوار نیاز به بررسی دارد.

کلمات کلیدی: Microascaceae، *Scedosporium*، *TUB*، ITS

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