Mycologia Iranica 2(2): 119 – 126, 2015 Original Article

# Fungi associated with apple and pear sooty blotch and flyspeck diseases in Guilan province, Iran

**A. Heidari**
**S. A. Khodaparast** ✉
**S. Mousanejad**
*Department of Plant Protection, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran*

**Abstract**: Sooty blotch and flyspeck occur on fruit surfaces and result in economic losses due to less attractive appearance. Sooty blotch fungi form dark mycelial mats whereas flyspeck fungal agents are well characterized with black, sclerotium-like bodies on fruit surface. Whilst more than 60 species have been reported in association with these two fungal diseases, thus far they have not been studied in Iran. In this study specimens showing the symptoms related to sooty blotch and flyspeck were collected from different regions of Guilan province during 2012-14. Morphological characteristics and sequences of the internal transcribed spacer (ITS) regions of rDNA were generated for the isolates and compared to describe species. In this study *Microcyclosporella mali*, *Zasmidium* sp. and *Zygophiala jamaicensis* were identified based on morphological and molecular characteristics. *Microcyclosporella mali* and *Zygophiala jamaicensis* are described and illustrated for the first time from Iran.

**Key words**: Biodiversity, *Microcyclosporella*, *Zasmidium*, *Zygophiala*.

## INTRODUCTION

Sooty blotch and flyspeck (SBFS) are complex diseases caused by diverse fungi which grow superficially and colonize fruit, stem, twig and leaf surfaces of a wide range of cultivated and non-cultivated crops such as apple, pear, and banana, however, most research has focused on apple (Gleason et al. 2011). Due to production of pigmented hyphae and sclerotium-like bodies, infected fruits are blemished and often resulting in downgrading of crops and marketing value especially in humid regions. Sooty blotch and fly speck fungi usually appear together and encompass at least 60 putative species (Gleason et al. 2011; Li et al. 2011).

Most of these species belong to diverse anamorphic genera of Dothideomycetes including *Colletogloeopsis*-like fungi, *Devriesia*, *Diatractium*-like fungi, *Dissoconium*, *Geastrumia*, *Houjia*, *Leptodontidium*, *Microcyclosporella*, *Microcyclospora*, *Passalora*-like fungi, *Peltaster*, *Phaeothecoidiella*, *Phialophora*, *Pseudocercospora*, *Ramichloridium*, *Ramularia*, *Schizothyrium*, *Scleroramularia*, *Scolecobasidium*, *Sporidesmajora*, *Stomiopeltis*, *Strelitziana*, *Uwebraunia*, *Zasmidium*, *Zygophiala*, (Batzer et al. 2005; Frank et al. 2010; Gao et al. 2014; Gleason et al. 2011; Ivanović et al. 2010; Kwon et al. 2012; Li et al. 2011, 2012; Mirzwa-Mróz 2008; Sun et al. 2008; Yang et al. 2010).

Because of epiphytic habitat, isolation of these fungi on pure culture is the first challenge for morphological, biological and molecular studies. This is because surface disinfection of the fruit will remove the fungi. Moreover, slow growth, sparsely or no sporulations on agar media or fruits make the study of these fungi more complicated. However, during recent years several researchers deal with these fungi and have overcome these problems, hence, publications concerning these fungi have been increased. There are no comprehensive studies related to these fungi in Iran. According to literature there is only one report of *Schizothyrium pomi* on *Malus pumila* from Iran (Ershad 2009). The goal of this study was to identify fungi associated to SBFS in Guilan province, Iran.

## MATERIALS AND METHODS

### Fungal isolation

In this study, specimens showing the sooty blotch and flyspeck symptoms (Fig. 1) were collected from different regions of Guilan province during July to October 2012–14 and transferred to the laboratory of the Department of Plant Protection, Faculty of Agricultural Sciences, University of Guilan. For fungal isolation, infected fruits were rinsed for 30 min in tap water and vegetative structures of accompanying fungi were transferred from colonies on the fruit surface to PDA, MEA 2%, SNA and OA and incubated at 25 °C in darkness (Sun et al. 2003; Yang et al. 2010). All isolates were deposited in the Iranian Fungal Culture Collection (IRAN…C) of the Iranian Research Institute of Plant Protection.

### Morphological studies

To examine morphology, fungal material mounted in a solution consisting of equal amounts of glycerol and lactic acid, lactic acid (50 %) or cotton blue-lactic

Submitted 21 Sept. 2015, accepted for Publication 29 Nov. 2015
✉ Corresponding Author: E-mail: khodaparast@guilan.ac.ir

http://mi.iranjournals.ir

acid, and were studied using a light microscope. To observe the hyphae, conidiophores, and conidia produced on fruit surfaces, clear adhesive tapes was used to strip off these structures from the fruit surface. Measurements were taken in lactic acid (50 %), based on 30 fungal structures. For photography, an Olympus light microscope equipped with a Sony digital camera was used. Fungal species were determined according to keys and descriptions provided in Frank et al. (2010), Li et al. (2010) and Batzer et al. (2008).

**DNA sequencing**

Total DNA was isolated from a 3-4 days old fungal colony, using pieces of mycelia by the Chelex method (Walsh et al. 1991; Hirata and Takamatsu 1996; Khodaparast et al. 2001). A region spanning ITS1, 5.8S, and ITS2 of rDNA were amplified as described by Khodaparast et al. (2012) using the primers ITS1 (5'– TCCGTAGGTGAACCTGCGG–3') and ITS4 (5'–TCCTCCGCTTATTGATATGC–3') (White et al. 1990). The PCR products were purified using a USB® ExoSAP-IT® PCR Product cleaning kit (USB, USA). The nucleotide sequences of the PCR products were obtained using direct sequencing in an ABI 3730xl sequencer (Applied Biosystems, USA). DNA sequences determined in this study were deposited in GenBank accession nos. KX343019-KX343022. Sequences were compared with the sequences available in the NCBI GenBank nucleotide database using a BLASTN search method. Several sequences from GenBank were selected for phylogenetic analyses.

**Phylogenetic analysis**

Sequences were initially inspected visually and aligned using the Clustal X (Higgins et al. 1992). The data were analyzed using the minimum evolution (ME), neighbour-joining (NJ) and maximum likelihood (ML) methods with MEGA version 6.0 (Tamura et al. 2013). In ME, NJ and ML methods, the evolutionary distances were computed using Kimura 2-parameter. All ambiguous positions were removed for each sequence pairs. All nucleotide substitutions were equally weighted and unordered. The strength of the internal branches from the resulting trees was statistically tested by bootstrap analysis with 1000 pseudo-replicates (Felsenstein 1985).

## RESULT AND DISCUSSION

**Phylogenetic analysis**

The aligned sequences contained 388 and 417 bp for *Microcyclosporella* and *Zygophiala* receptively. The efficiencies of the different methods (ME, NJ, ML and MP) in obtaining the phylogenetic trees were nearly the same. The ME, NJ and ML using Kimura's Two-parameter Distance in MEGA 6.0 yielded trees with the similar topology for *Microcyclosporella* (Fig. 2) and *Zygophiala* (Fig. 3).

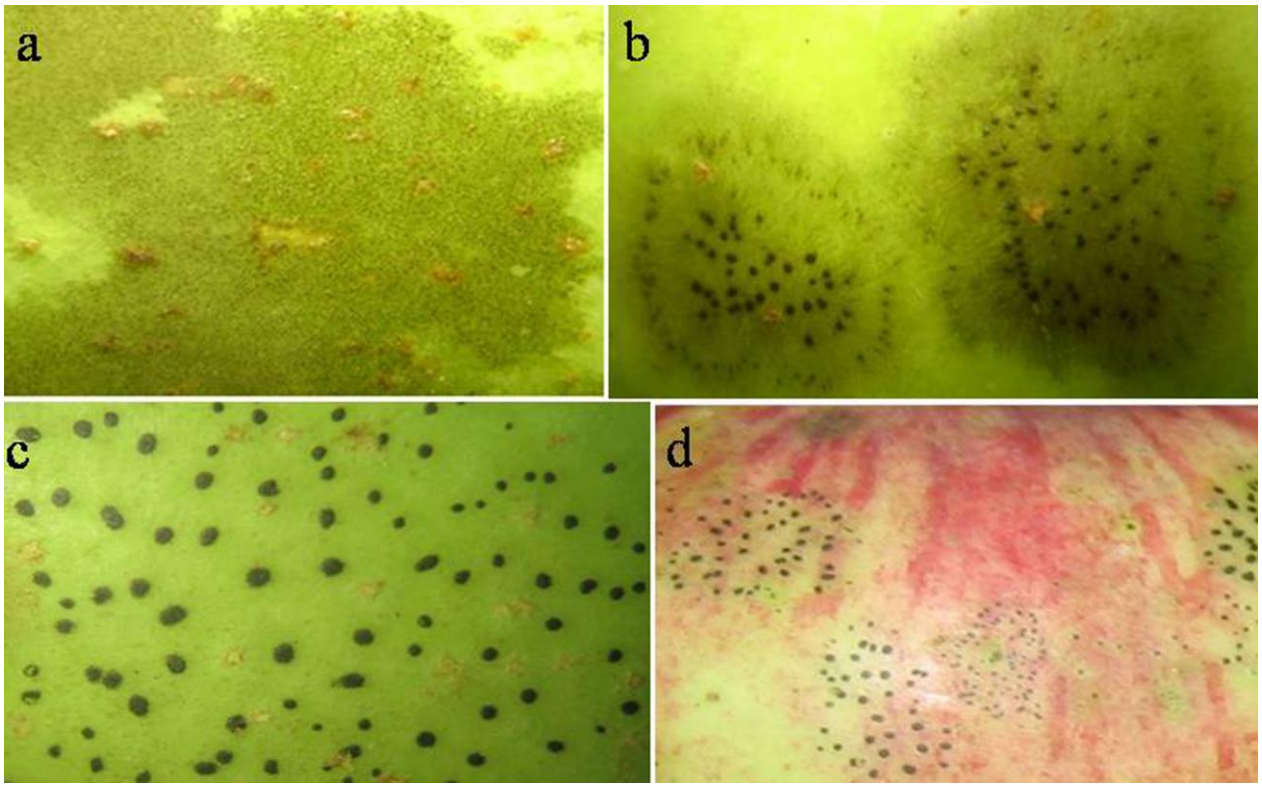


**Fig. 1.** Sooty blotch and flyspeck signs on fruit surface. **a.** *Microcyclosporella mali*; **b.** *Zasmidium* sp.; **c-d.** *Zygophiala jamaicensis.*

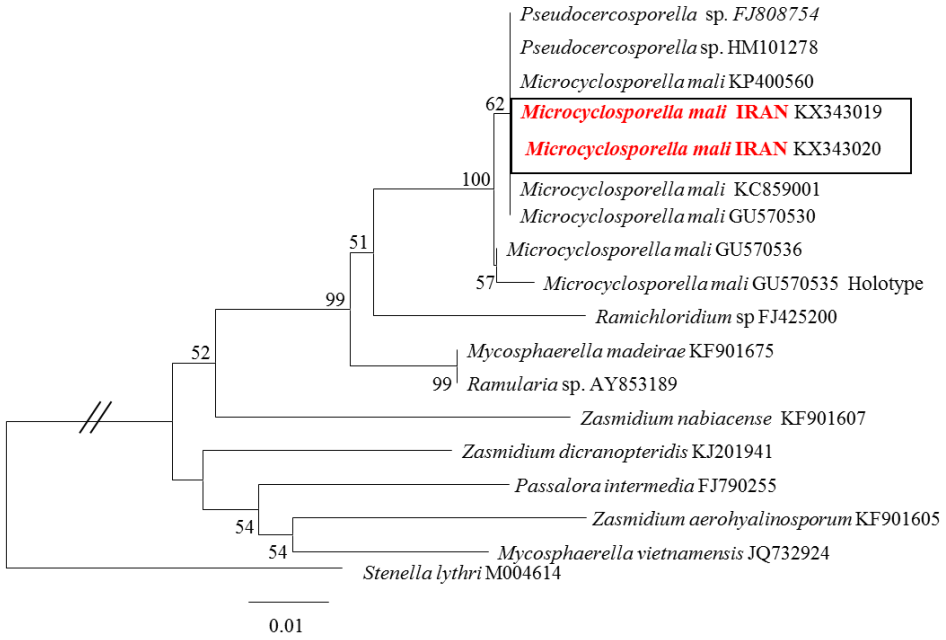


**Fig. 2.** The evolutionary history was inferred using the Minimum Evolution method. The optimal tree with the sum of branch length = 0.35728452 is shown. The numbers above the branches represent branch support using 1000 bootstrap replications (Bootstrap values below 50 % are not shown).

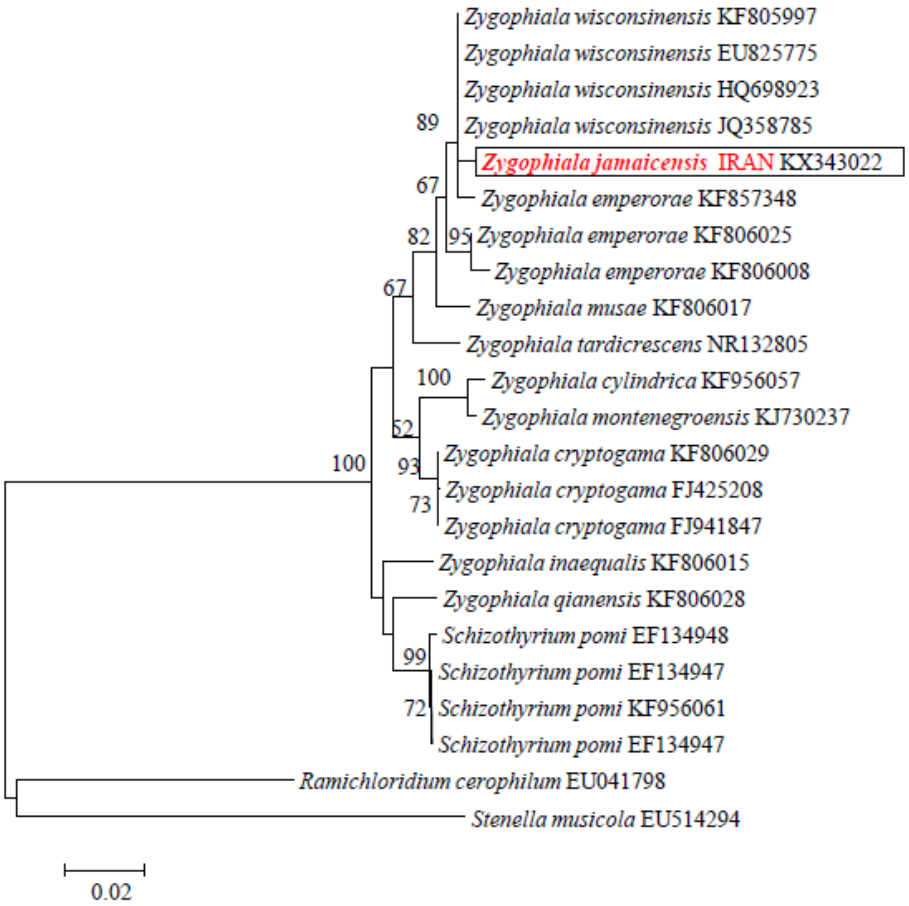


**Fig. 3.** The evolutionary history was inferred using the Minimum Evolution method. The optimal tree with the sum of branch length = 0.41122228 is shown. The numbers above the branches represent branch support using 1000 bootstrap replications (Bootstrap values below 50 % are not shown).

**Taxonomy**

According to the results, *Microcyclosporella mali*, *Zygophiala jamaicensis* and *Zasmidium* sp. were identified. *Microcyclosporella mali* and *Zygophiala jamaicensis* are new to Iran mycobiota and we provided here a description and illustration.

***Microcyclosporella mali*** J. Frank, Schroers & Crous, Persoonia 24: 101. 2010.

Two isolate of *Microcyclosporella mali* with similar ITS sequences were obtained. These ITS sequences of *M. mali* were 100% similar to those of several sequences of this fungus available in GenBank. However, they are differed in three ITS nucleotides from the holotype specimen (CPC16184, on *Malus domestica*, from Slovenia, accession number: GU570535). This genus is monotypic and has recently been introduced by Frank et al. (2010). According to our observations morphological characteristics of the Iranian isolates well agree to those of *M. mali* provided by Frank et al. (2010). Therefore, based on the current data we suggest that a few substitutions in ITS sequences could be related to intraspecific variation. A brief description of the *M. mali* is presented here as follows:

Colonies after one month at 25°C in the dark on SNA grey to olivaceous-grey, reaching 25–30 mm. On MEA 2% colonies reaching up to 20 mm, olivaceous-grey and surface folded, crumpled, similar to SNA, but on OA, surface not folded and crumpled. On SNA colonies developing and forming white and slimy drops in culture.

Mycelium consisting of branched, septate, finely verruculose, hyaline to pale brown hyphae, width 2–3 µm, sometimes with mucoid layer. Conidiophores simple, usually reduced to conidiogenous cells. Conidiogenous cells arising from hyphae, terminal and lateral, mostly terminal, 4–7.2 × 2–3.5 µm, cylindrical to doliiform, hyaline to pale brown, smooth, loci hyaline and inconspicuous, thin. Yeast-like colonies forming in culture that appear as white and slimy drops. Conidia smooth, hyaline, cylindrical to rarely obclavate or narrowly fusoid with rounded apex, 0–10 septate, 5–55 × 2–4 µm, conidial hila thin, hyaline. Microcyclic conidiation present (Fig. 4)

*Specimens examined.* IRAN, Guilan province, Sumaehsara, on *Malus pumila*, 19 Aug. 2013, A. Heidari (IRAN 2497 C); Rasht, Sangar, on *Pyrus communis*, 20 Sept. 2013, A. Heidari (IRAN 2496 C).

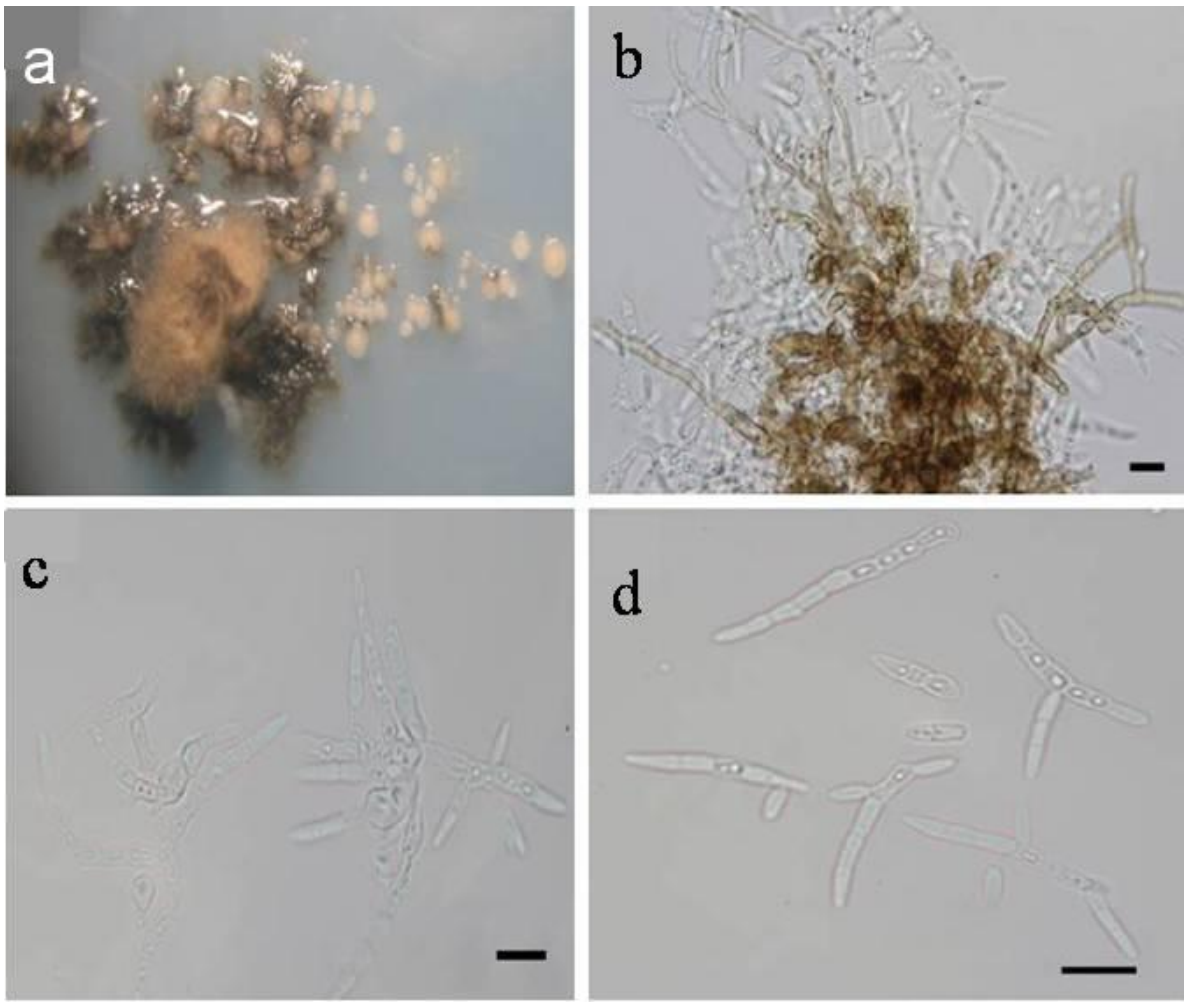


**Fig. 4.** *Microcyclosporella mali*. **a.** Colony on SNA; **b.** conidiophores; **c-d.** conidia — scale bars=10 µm.

***Zygophiala jamaicensis*** E.W. Mason, Mycological Papers 13: 5. 1945.

Colonies after 2 weeks on OA at 25 °C in the dark, spreading, without aerial mycelium, olivaceous-grey in the middle, regular dirty white to cream at the margins, reaching 20–25 mm on MEA 2% after 2 weeks at 25 °C. Conidiophores arising from superficial hyphae, 2–3 µm width, erect, scattered, 3–4 septate, 25.5–33(–36) × 5 µm, consisting of four parts: a modified of mycelial basal cell that is subhyaline, giving rise to a smooth dark brown, irregularly flexuous stipe, 17–20 × 4–5 µm, followed by a medium brown apical cells, about 4–5 × 3–4 µm, which finally giving rise to two divergent polyblastic conidiogenous cells, which are ovate to ampulliform, with prominent, darkened and thickened scars, somewhat refractive, about 6–8 × 4–5 µm. Conidia elliptical to obovate, smooth, hyaline, aseptate, 6–8 × 4–6 µm or 1-septate, 15–18 × 4–5 µm with thick-walled, with a darkened and thickened hilum, 1–2 µm width (Fig. 5).

The molecular analysis showed that our collection of *Zygophiala* is close to *Zygophiala wisconsinensis* Batzer & Crous but morphologically this taxon differs by having smaller conidia. Hence, we key out this species according to Batzer et al. (2008) as *Z. jamaicensis.* However, for exact confirmation of synonymy of these two taxa a molecular examination of *Z. jamaicensis* (holotype or an authentic strain) is needed.

*Specimens examined.* IRAN, Guilan province, Shaft, on *Malus pumila*, 13 Aug. 2013, A. Heidari (IRAN 2495 C).

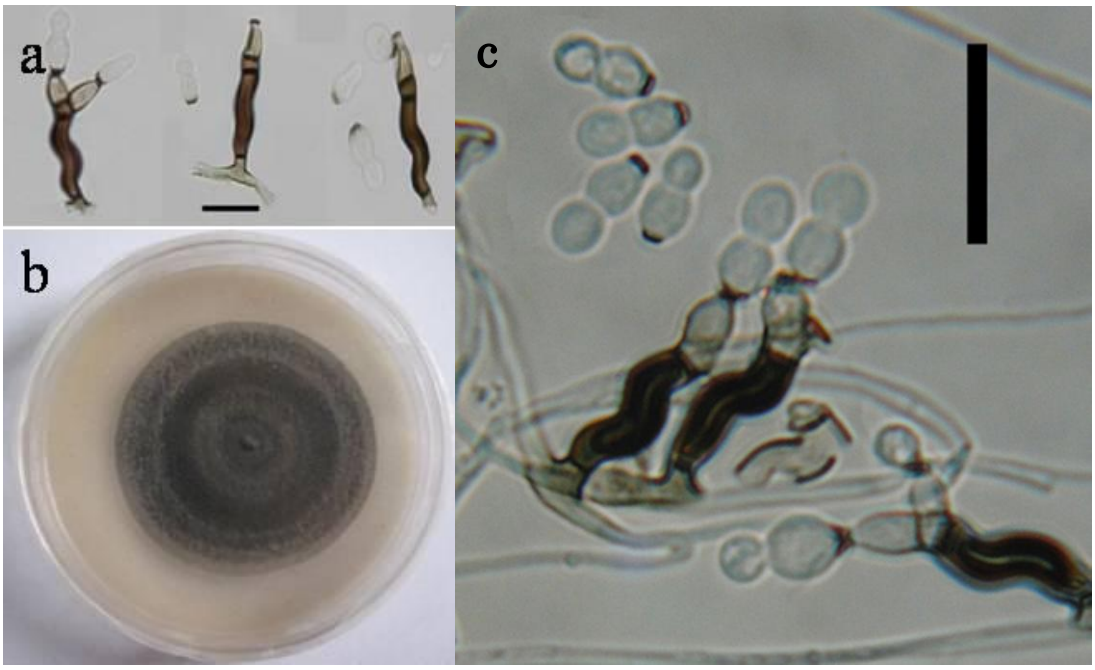


**Fig. 5.** *Zygophiala jamaicensis*. **a.** Conidiophores and conidia; **b.** Colony on Oat Meal Agar; **c.** Conidiophores and conidia. — Scale bars (a, b) = 10 µm, (c) = 20 µm.

***Zasmidium* sp.**

Colonies after 1 month at 25 °C in the darkness, spreading, with moderate aerial mycelium and on SNA olivaceous, reaching 35 mm, on MEA 2% dark olivaceous-grey, reaching 35–40 mm, on PDA olivaceous-grey on surface.

Mycelium consisting of septate, branched, brown, verruculose, 2–3 µm width hyphae. Conidiophores erect, solitary, arising as lateral branches on superficial hyphae, mostly straight to slightly curved, 2–12 septate, subcylindrical, dark brown, smooth to verruculose, unbranched, (-30)40–100 × 3 µm. Conidiogenous cells terminal, integrated, brown, smooth to verruculose, subcylindrical, straight, 5.2–20.8 × 2.5–3.5 µm, proliferating sympodially, with scars aggregated at clavate apex; conidiogenous loci, dark or refractive scars on lateral and terminal denticles, 0.5–1 µm width. Conidia solitary or catenulate, more variable in length and shape than those from fruit, small conidia ellipsoid-ovoid to subcylindrical, but most conidia longer and subcylindrical to subcylindrical-obclavate, 4–55 (-65) × 2–5.2 µm, hilum darkened and thickened, somewhat refractive, 0.5–1 µm diam (Fig. 6).

*Specimens examined*. IRAN, Guilan province Sumaehsara, on *Malus pumila*, 20 Aug. 2013, S. A. Khodaparast (IRAN 2494 C); Lahijan, on *Pyrus communis*, 10 Sept. 2013, A. Heidari (IRAN 2492 C).

Note — In this study we sequenced ITS rDNA for three isolates of *Zasmidium* sp. All isolates showed 100 % similarity in ITS sequences. However, based on our phylogenetic analysis (data not shown) and morphological examination, these isolates are not placed in the species described so far in *Zasmidium*. For precise identification of these isolates more genes are needed.

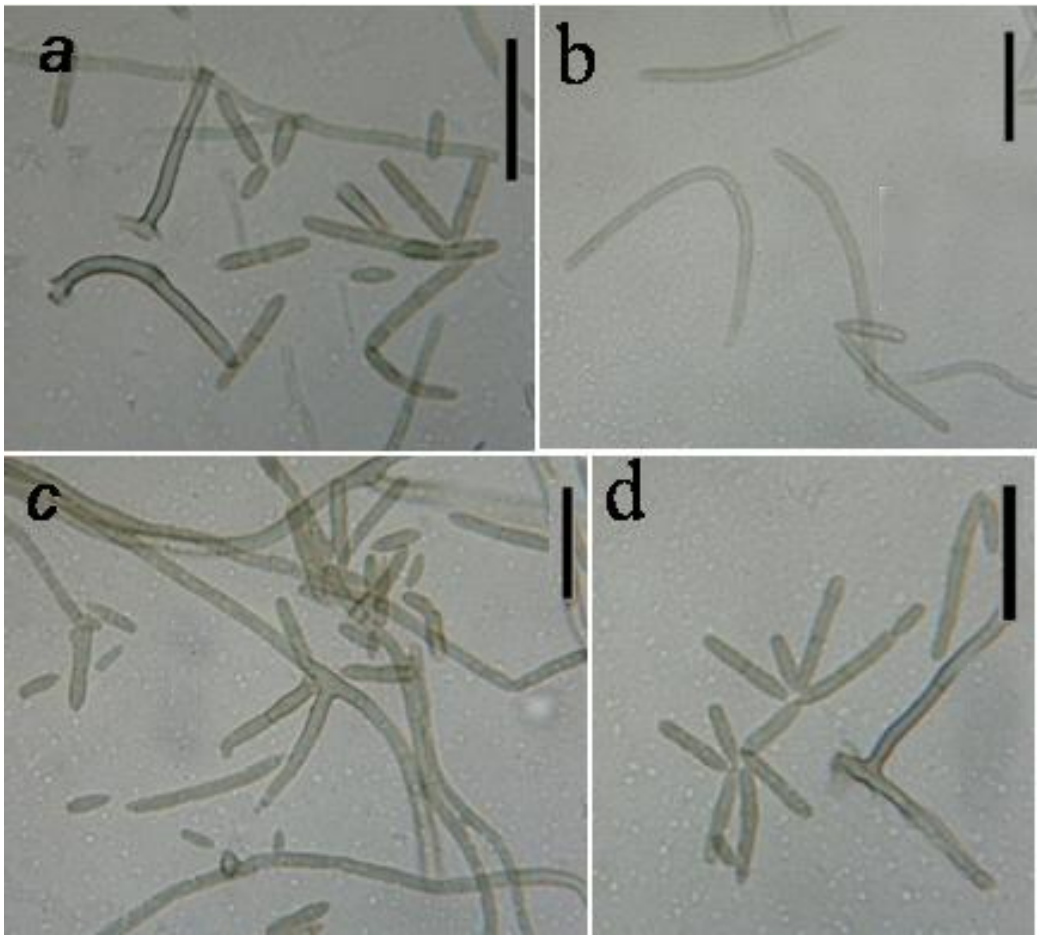


**Fig. 6.** *Zasmidium* sp. **a-d.** Conidia and conidiophores — scale bars = 20 μm.

## REFERENCES


Batzer JC, Gleason ML, Harrington TC, Tiffany LH. 2005. Expansion of the sooty blotch and flyspeck complex on apples based on analysis of ribosomal DNA gene sequences and morphology. Mycologia 97: 1268–1286.

Ershad D. 2009. Fungi of Iran. Iranian Research Institute of Plant Protection, Iran.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791.

Frank J, Crous PW, Groenewald JZ, Oertel B, Hyde KD, Phengsintham P, Schroers HJ. 2010. *Microcyclospora* and *Microcyclosporella*: novel genera accommodating epiphytic fungi causing sooty blotch on apple. Persoonia-Molecular Phylogeny and Evolution of Fungi 24: 93–105.

Gao L, Sun G, Zhang R, Gleason ML. 2014. Secondary spread of *Zygophiala wisconsinensis* on the surface of apple fruit. European Journal of Plant Pathology 139: 117–124.

Gleason ML, Batzer JC, Sun G, Zhang R, Arias MM, Sutton TB, Crous PW, Ivanovic M, McManus PS, Cooley DR, Mayr U. 2011. A new view of sooty blotch and flyspeck. Plant Disease 95: 368–383.

Higgins DG, Bleaby AJ, Fuchs R. 1992. CLUSTALV: improved software for multiple sequence alignment. Computer Applications in the Bioscience 8: 189–191.

Hirata T, Takamatsu S. 1996. Nucleotide sequence diversity of rDNA internal transcribed spacers extracted from conidia and cleistothecia of several powdery mildew fungi. Mycoscience 37: 265–270

Ivanović MM, Ivanović MS, Batzer JC, Tatalović N, Oertel B, Latinović J, Latinović N, Gleason ML. 2010. Fungi in the apple sooty blotch and flyspeck complex from Serbia and Montenegro. Journal of Plant Pathology 92: 65–72.

Khodaparast SA, Takamatsu S, Hedjaroude GA. 2001. Phylogenetic structure of the genus *Leveillula* (Erysiphales: Erysiphaceae) inferred from the sequences of the rDNA ITS regions with special references to the *Leveillula taurica* species complex. Mycological Research 105: 909–918.

Khodaparast SA, Takamatsu S, Harada M, Abbasi M, Samadi S. 2012. Additional rDNA ITS sequences and its phylogenetic consequences for the genus *Leveillula* with emphasis conidium morphology. Mycological Progress 11: 741–752.

Kwon JH, Kim J, Choi O, Gang G, Kwak YS. 2012. Occurrence of sooty blotch and flyspeck disease

on sweet persimmon in Korea. Mycobiology 40: 210–213.

Li H, Sun GY, Zhai XR, Batzer JC, Mayfield DA, Crous PW, Groenewald JZ, Gleason ML. 2012. Dissoconiaceae associated with sooty blotch and flyspeck on fruits in China and the United States. Persoonia-Molecular Phylogeny and Evolution of Fungi 28: 113–125.

Li H, Sun G, Batzer JC, Crous PW, Groenewald JZ, Karakaya A, Gleason ML. 2011. *Scleroramularia* gen. nov. associated with sooty blotch and flyspeck of apple and pawpaw from the Northern Hemisphere. Fungal Diversity 46: 53–66.

Li H, Zhang R, Sun G, Batzer JC, Gleason ML. 2010. New species and record of *Zygophiala* on apple fruit from China. Mycological Progress 9: 245–251.

Mirzwa-Mróz E. 2008. Fungi from genera *Pseudocercospora* and *Pseudocercosporella* as the causal agents of sooty blotch in Poland. Phytopathologia Polonica 50: 81–84.

Sun GY, Zhang R, Li H, Gleason ML. 2008. Diversity of fungi causing flyspeck-like signs on apple in China. Phytopathology 98: S153.

Sun GY, Zhang R, Zhang Z, Zhang M. 2003. Isolation of sooty blotch and flyspeck fungi from apple surface by picking up the thalli. Acta Phytopathology Sinica 33: 479–480 [in Chinese].

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution 30: 2725–2729.

Yang HL, Sun GY, Batzer JC, Crous PW, Groenewald JZ, Gleason ML. 2010. Novel fungal genera and species associated with the sooty blotch and flyspeck complex on apple in China and the USA. Persoonia - Molecular Phylogeny and Evolution of Fungi 24: 29–37.

Walsh PS, Metzger DA, Higuchi R. 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 10: 506–513.

White TJ, Bruns TD, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal genes for phylogenetics. In: PCR Protocols: a guide to methods and applications. (MA Innis, DH Gelfand, JJ Sninsky & TJ White, eds): 315–322. Academic Press, USA.

# قارچ های همراه با لکه دوده ای و فضله مگسی در استان گیلان

**اکرم حیدری، سید اکبر خداپرست ✉ و صدیقه موسی نژاد**

گروه گیاه‌پزشکی دانشکده علوم کشاورزی دانشگاه گیلان، رشت

**چکیده:** لکه دوده‌ای و فضله مگسی روی میوه‌ها رخ می دهند و باعث کاهش ارزش اقتصادی آنها در اثر کاهش ارزش بازار پسندی می‌شوند. قارچ‌های مولد لکه دوده‌ای ریسه‌های تیره تولید می‌کنند در حالی که قارچ‌های مولد فضله مگسی با وجود اجسام اسکلرت مانند سیاه روی سطح میوه‌ها مشخص می‌شوند. در حالی که بیش از ۶۰ گونه قارچ همراه با این بیماری‌ها گزارش است، اما تاکنون این قارچ‌ها در ایران مطالعه نشده‌اند. در این مطالعه نمونه‌های مشکوک و دارای علائم مرتبط با این بیماری‌ها از نقاط مختلف استان گیلان طی سال‌های ۹۳-۹۲، جمع‌آوری و به آزمایشگاه منتقل شدند. ویژگی‌های ریخت‌شناسی و توالی ناحیه ITS برای این جدایه‌ها به دست آمد و با گونه‌های شرح داده شده مقایسه شد. در این مطالعه سه گونه *Zygophiala jamaicensis*، *Microcyclosporella mali* و *Zasmidium* sp. بر اساس خصوصیات مورفولوژیکی و مولکولی شناسایی شدند. گونه‌های *Microcyclosporella mali* و *Zygophiala jamaicensis* برای اولین بار از ایران توصیف و به تصویر کشیده شده‌اند.

**واژه‌های کلیدی:** تنوع زیستی، *Microcyclosporella*، *Zasmidium* و *Zygophiala*

مکاتبه کننده: سید اکبر خداپرست Email: khodaparast@guilan.ac.ir

تاریخ دریافت: ۱۳۹۴/۰۶/۳۰ تاریخ پذیرش: ۱۳۹۴/۰۹/۰۹