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Short paper

First report of *Neosetophoma poaceicola* on apple leaf from Iran

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Abstract: Some saprophytic fungi were isolated during fungi isolation causing black spot or scab-like symptoms (genus Venturia) from Iran. One saprophytic isolate from apple leaf was identified as a member of the family *Phaeosphaericeae* based on morphological characteristics of pseudothecia, asci, and ascospores on oatmeal agar (OA). Mature pseudothecia were produced on OA after one-month incubation at 24 °C and continuous dark conditions; however, the pseudothecia produced on PDA were immature. Pseudothecia were dark brown and globose in shape. Numerous bitunicate asci formed in a broad hymenium. Asci were clavate, apically rounded, short pedicellate, with eight overlapping biseriate fusiform ascospores. Ascospores with the smooth wall, straight or slightly curved and slightly constricted at the second septum. Phylogenetic analysis based on sequence data of ITS and LSU regions of ribosomal DNA confirmed the morphological identification and specified the isolate as *Neosetophoma poaceicola*. The *N. poaceicola* is the first report from Iran and apple leaf in the world.

Keywords: *Phaeosphaericeae*, phylogeny, taxonomy, morphology, LSU

Introduction

Phaeosphaeriaceae is a large family of Pleosporales, including economically important plant pathogens (Phookamsak *et al.*, 2014). *Neosetophoma* Gruyter, Aveskamp & Verkley, with *N. samarorum* (Desm.) Gruyter, Aveskamp & Verkley, as type species, is a genus (Marin-Felix *et al.*, 2019) of the family *Phaeosphaeriaceae*. *Neosetophoma* was introduced as an asexual genus in 2010 (de Gruyter *et al.*, 2010) to accommodate isolates of *Phoma samarorum* Desm. a causal agent of leaf spots on grasses (Marin-Felix *et al.*, 2019). The genus was characterized by having solitary to confluent pycnidia, on the upper surface of the agar, globose to irregular, with papillate ostioles. Conidiogenous cells are phialides and discrete. Conidia slightly yellowish, aseptate, or septate, ellipsoidal to cylindrical (de Gruyter *et al.*, 2010). Since the establishment of the genus, 20 species have been described (Index Fungorum, 2020), most of which appear to be saprobes, except for some species such as *N. iranianum* that was isolated from soil (Karunarathna *et al.*, 2017), *N. lunariae* that is an endophytic species (Hernandez-Restrepo *et al.*, 2016) and *N. samarorum* causing leaf spots on the host (Marin-Felix *et al.*, 2019). *Neosetophoma poaceicola* was reported as a new saprobic species on dead grass in Thailand in 2017. The fungus is represented as the first record of the sexual morph of the genus and is characterized by globose to subglobose ascomata immersed under the epidermis,

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becoming erumpent through the host surface (Thambugala *et al.*, 2017). Asci bitunicate, fissitunicate, cylindric-clavate, round at the apex with short round pedicel. Ascospores overlapped 1–2-seriate, fusiform, with acute ends (Thambugala *et al.*, 2017).

In the present study, a saprophytic isolate of *Neosetophoma* was isolated from apple leaf accompanying apple scab symptoms caused by *Venturia inaequalis* and was characterized based on the morphological and molecular data of ITS and LSU regions of ribosomal DNA.

Materials and Methods

Fungal isolate and morphology

Fungal isolate was obtained from apple leaf with black spot or scab symptom. Fungal isolation was conducted by streaking out conidia and mycelia, from leaf spot surface, onto 2% water agar (WA), and pure fungal culture was obtained by transferring hyphal tips onto fresh potato dextrose agar (PDA). The dried specimen is maintained in the Fungarium, and the pure fungal colony is deposited in the Iranian Fungal Culture Collection (IRAN), respectively, at the Iranian Research Institute of Plant Protection, Tehran, Iran.

Colony color was assessed on oatmeal agar (OA) and PDA after seven days in the continuous dark condition at 24 °C, using Rayner's color charts (1970). Microscopic slides were prepared in lactophenol from four weeks old colony on OA incubated in continuous dark conditions at 24 °C. Measurement and microphotographs of fungal features such as pseudothecia, asci, and ascospores were taken from microscopic slides using an Olympus BH2 light microscope (Olympus, Japan).

DNA extraction, amplification, sequencing

The whole genomic DNA has extracted from seven days old mycelia by Cenis (1992) method. ITS and LSU regions were amplified using ITS1 and ITS4 primers (White *et al.*, 1990) and LROR and LR5 primers (Vilgalys and Hester, 1990), respectively. Ebrahimi and Fotouhifar (2016) protocol for PCR amplification (annealing temperatures of 52 °C for both regions) and sequencing was followed.

Phylogenetic analysis

Sequences generated in this study were manually edited with Chromas 2.4 software (Technelysium, Australia). For species identification, sequences were subjected to MegaBLAST searches at GenBank (NCBI). Available reference sequences of the homologous regions of ITS and LSU rDNA for described *Neosetophoma* species were obtained from GenBank, NCBI (Table 1) to conduct phylogenetic analysis. It was then aligned using ClustalW (Thompson *et al.*, 1994), with *Didymella exigua* CBS 183.55 as an outgroup. Neighbor-joining (NJ) (Saitou and Nei, 1987) and maximum likelihood (ML) (Felsenstein, 1973) analyses were performed by heuristic search in MEGA7 (Kumar *et al.*, 2016). Character changes were unweighted and unordered, with gaps treated as missing data. The consistency of individual clades was assessed by ML bootstrap (Felsenstein, 1985) analyses with 1000 replicates.

Results and Discussion

Fungal isolate and morphology

During isolation of fungi from apple leaves with scab or scab-like symptoms, different fungal isolates were obtained, some of which have been reported by Ebrahimi and Fotouhifar (2016). One saprophytic isolate (IR8; IRAN 2429 C) was investigated based on morphological features, and molecular data in this research was identified as *Neosetophoma poaceicola*.

Taxonomy

Neosetophoma poaceicola

Specimen examined. IRAN, Hamadan Province, Hamadan, Heydareh (34º48.132′N – 48º27.853′E), on a leaf of apple [*Malus domestica* (Suckow) Borkh.] (accession number: IRAN 17250 F), 2 Oct 2014, *L. Ebrahimi* IR8 (accession number: IRAN 2429 C).

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Table 1 Sequences used in the phylogenetic analysis of *Neosetophoma* species.

Species	Culture accession number (s) [1]	Source	Origin	GeneBank accession numbers	
				ITS	LSU
Neosetophoma aseptata	CBS 145363^T	*Viburnum opulus*	Germany	NR_164449	MK540024
N. clematidis	MFLUCC 13-0734^T	-	Italy	NR_154228	KP684153
N. garethjonesii	MFLUCC:14-0528	Forest soil	United Kingdom	KY496758	KY496738
N. guiyangensis	GZCC 18-0111^T	Dead branch	China	MH018134	MH018132
N. lonicerae	CPC 26671^T	*Lonicera maackii*	Germany	NR_154242	KX306789
N. poaceicola	MFLUCC 16-0886^T	Dead grass	Thailand	NR_165861	KY550382
N. poaceicola	**IRAN 2429 C**	***Malus domestica***	**Iran**	**KT832078**	**MT102742**
N. phragmitis	CBS:145364^T	*Phragmites australis*	Germany	NR_164450	MK540025
N. rosarum	MFLU 17-0308^T	*Rosa canina*	Italy	NR_157524	MG829036
N. rosigena	MFLU 17-0626^T	*Rosa canina*	United Kingdom	NR_157525	NG_059870
N. rosigena	MFLUCC 17-0768	*Rosa canina*	United Kingdom	MG828928	MG829037
N. salicis	MFLU 17-0118	*Salix* sp.	Uzbekistan	MK608025	MK608026
N. samarorum	CBS:138.96^T	*Phlox paniculata*	Netherlands	NR_156263	NG_057836
N. sambuci	CBS:145365^T	*Sambucus nigra*	Germany	NR_164451	MK540026
N. shoemakeri	MFLUCC 17-0780	*Malva* sp.	United Kingdom	MG844346	MG844348
N. xingrensis	GZCC 18-0110^T	Decaying wood	China	MH018135	MH018133
Didymella exigua	CBS 183.55	-	France	MH857436	MH868977

[1]CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; MFLUCC: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; GZCC: Guizhou Culture Collection; KUMCC: Kunming Institute of Botany Culture Collection, Kunming, Yunnan, China; IRAN: Iranian Fungal Culture Collection; MFLU: Mae Fah Luang University herbarium, Thailand. ^{T} indicates type strains. The surveyed isolate in the current study is indicated in bold.

Morphological features- immature ascocarps without ascus or ascospore were formed on PDA and immersed hyaline mycelium, later with a greyish olivaceous pigment and mature pseudothecia formed in OA after four weeks incubation at 24 °C and continuous dark condition. Ascocarps (pseudothecia) solitary, scattered and immersed in OA, 140–280 µm in diameter, dark brown, and globose in shape. Peridium thin-walled, composed of two layers of dark brown pseudoparenchymatous cells arranged in textura angularis. Asci numerous, in a broad hymenium, clavate, 60–132 × 10–14 µm, bitunicate, apically rounded, shortly pedicellate, with eight overlapping biseriate ascospores. Ascospores fusiform, straight or slightly curved, (19–)21–28 × 4–6 µm, yellowish-brown, smooth-walled, without a sheath, (3–)4–5(–6) septate, and constricted at the second septum (Fig. 1).

Culture characteristics: Colony on OA and PDA reached 32 mm in diameter after seven days at 24 °C in continuous dark condition. The colony was colorless on OA and greyish olivaceous on PDA after seven days.

Phylogenetic analysis

The NCBI blast of ITS and LSU sequences of our isolate showed 99.81% and 100% identity to *N. poaceicola* MFLUCC 16-0886 (GenBank accession no. NR_165861 and KY550382), respectively. To evaluate the relationships of our isolate with the species within the genus *Neosetophoma*, phylogenetic analyses of 16 isolates (Table 1), was done using sequences of two genomic regions, including LSU (582-583 bp) and ITS (454-462 bp). A combined dataset of sequences of these two genomic regions (ITS and LSU) was evaluated for phylogenetic inference. The length of the nucleotide sequence of an assembled dataset of two genomic regions (ITS and LSU) reached up to 1043 bp. The NJ and ML trees' topologies were almost similar concerning identified clades except for minor differences in bootstrap values, and only the NJ tree is presented here (Fig. 2).

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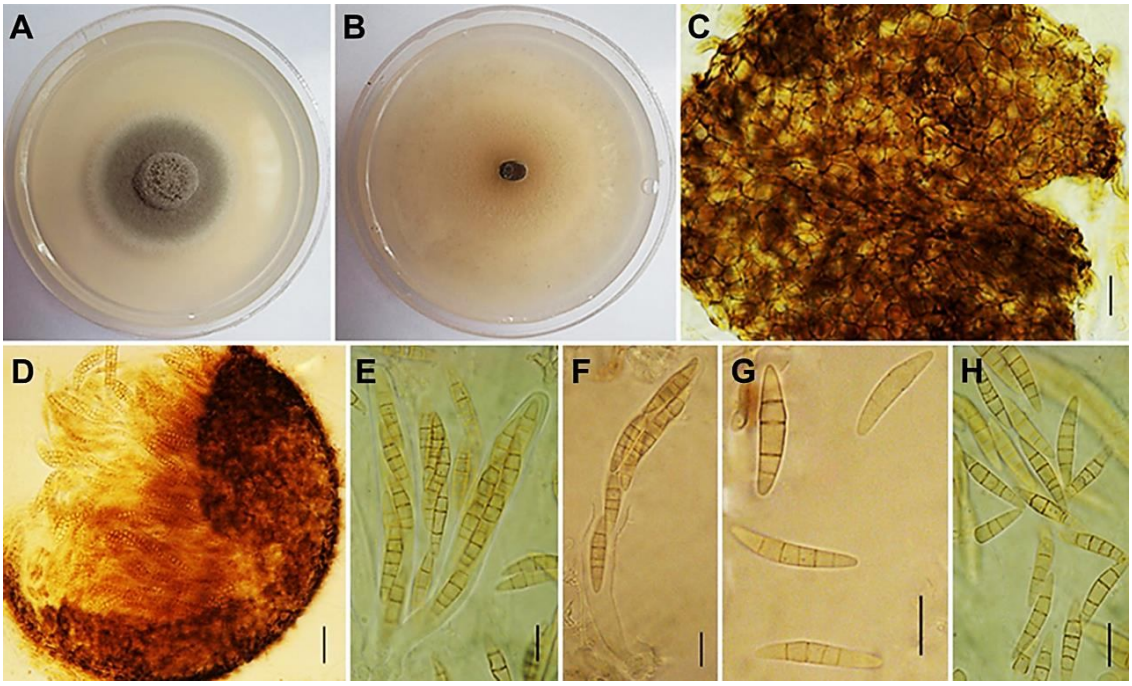

Figure 1 *Neosetophoma poaceicola*, isolate IR8 (IRAN 2429 C). A: colony on PDA, and B: colony on OA after seven days at 24 °C in continuous dark condition, C-D: pseudothecium, E-F: asci and ascospores, G-H: ascospores. Bars = 10 µm.

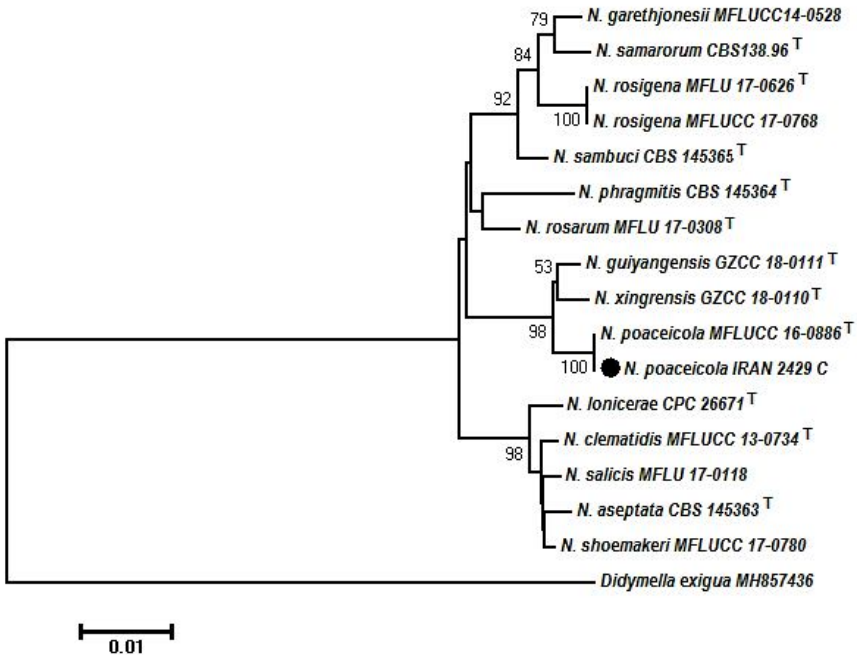

Figure 2 Neighbor-joining (NJ) phylogram obtained from the combined ITS and LSU sequence alignment of *Neosetophoma* species. The tree was rooted to *Didymella exigua* CBS 183.55. Bootstrap values (1000 replicates) indicated at the nodes. The scale bar indicates nucleotide substitution in NJ analysis, values ≥ 50 % are shown above/below the branches. ^{T} indicates type strains.

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In the phylogenetic analysis based on assembled sequences of ITS and LSU, our isolate (IRAN 2429 C) along with *N. poaceicola* MFLUCC 16-0886 was located in a well-supported clade (98%) together with *N. xingrensis* and *N. guiyangensis* (Fig. 2). *Neosetophoma xingrensis* is different from *N. poaceicola* by having ascospores overlapping 1–3-seriate, normally 3-septate, not constricted at the septum, and having sessile pedicel (Hyde *et al.*, 2018). *Neosetophoma guiyangensis* can be distinguished from *N. poaceicola* by producing ascospores with 1–3(–5)-septate, not constricted at the septum (Hyde *et al.*, 2018).

Based on the molecular data and morphological investigation, the isolate IRAN 2429 C was confirmed as *N. poaceicola*. Some morphological features such as pseudothecia, asci, and ascospores size of our isolate were different from *N. poaceicola* MFLUCC 16-0886 [with pseudothecia 130–145 × 135–160 µm, asci 55–82 (70.5) × 7–9 (8.4) µm, ascospores 18.5–22.5 (20.5) × 3.5–5 (4.5) µm in diameter] (Thambugala *et al.* (2017). These differences may be due to the substrates of isolates. Other differences between our isolate and the type strain are in the color of ascospores and the number of septa described as hyaline with occasionally 1-septate, respectively, in type strain. Based on genus description, ascospores of this group are hyaline or subhyaline when young, becoming pale yellow, pale brown, or yellowish-brown to brown at maturity (Marin-Felix *et al.*, 2019). Also, in the published figure (Fig. 28 in Thambugala *et al.*, 2017) of the type strain, it is evident that septa of ascospores are forming, and they are more than one and constricted at the second septum. Moreover, it is mentioned in Thambugala *et al.*, 2017 that "cell near the septum slightly larger", showing the ascospores have more than two cells as well as more than one septum, which is not indicated in the description.

Thambugala *et al.* (2017) described the fungus morphological features on the host substrate, but our isolate did not have any reproductive fruiting bodies on apple leaf, and the description of the isolate IRAN 2429 C is based on the features of the isolate on OA culture. For the first time, *N. poaceicola* was reported as a saprobe on the dead grass of *Poaceae* from Thailand (Thambugala *et al.*, 2017). The *N. poaceicola* is the first report from Iran and apple leaf in the world.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Authors' Contributions

Leila Ebrahimi performed the project, analyzed the data, and wrote the manuscript.

Khalil-Berdi Fotouhifar revised the manuscript.

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اولین گزارش از گونه *Neosetophoma poaceicola* روی برگ سیب در ایران

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چکیده: طی جداسازی جدایه‌های قارچی عامل بیماری از علائم لکه سیاه یا اسکب روی میزبان‌های گیاهی متنوع جمع‌آوری شده از مناطق مختلف ایران، برخی جدایه‌های قارچی ساپروفیت نیز جداسازی شدند. یک جدایه ساپروفیت به‌دست آمده از برگ سیب براساس ویژگی‌های ریخت‌شناختی اندام‌های سودوتسیوم، آسک و آسکوسپورها روی محیط‌کشت عصاره یولاف-آگار (OA) در شرایط آزمایشگاهی به‌عنوان عضوی از خانواده Phaeosphaericeae شناسایی شد. سودوتسیوم‌های قارچ روی محیط‌کشت OA پس از یک ماه نگه‌داری در دمای ۲۴ درجه سلسیوس و شرایط تاریکی مطلق تشکیل شدند، ولی سودوتسیوم‌های تشکیل شده روی محیط‌کشت PDA نابالغ بودند. سودوتسیوم‌های قارچ روی محیط کشت OA به رنگ قهوه‌ای تیره و کروی شکل بودند. آسک‌های دوجداره چماقی شکل، با نوک گرد و پایه کوتاه در لایه‌های منیوم تشکیل شدند که آسکوسپورهای دوکی شکل در دو ردیف در داخل آن قرار گرفته بودند. آسکوسپورها دارای دیواره صاف، راست تا کمی خمیده و در محل دیواره عرضی دوم کمی فرورفته بودند. تجزیه‌وتحلیل فیلوژنتیکی براساس توالی‌های ناحیه ITS و LSU از DNA ریبوزومی به‌دست آمده در این پژوهش و توالی‌های به‌دست آمده از بانک ژن، نتایج بررسی‌های ریخت‌شناختی را تأیید کرد و جدایه موردنظر به‌عنوان *Neosetophoma poaceicola* شناسایی شد. این اولین گزارش از این گونه در ایران و روی برگ سیب در دنیا می‌باشد.

واژگان کلیدی: تاکسونومی، ریخت‌شناختی، فیلوژنی، LSU، *Phaeosphaericeae*

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