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A new sexual morph for *Neosetophoma iranianum*, and a key to *Neosetophoma* species

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Abstract: Neosetophoma iranianum has so far only been described in its coelomycetous asexual morph. In this study, the sexual morph for this species was recovered for the first time, from dead branches of *Lonicera caprifolium*, in North Khorasan Province, Iran. The connection between the asexual and sexual morphs of the species is confirmed by phylogenetic analysis based on ITS rDNA sequence data, and morphological traits. The sexual and asexual morphs of *N. iranianum* are described and illustrated here, and compared with closely related species. A dichotomous key for the identification of all species assigned to the genus Neosetophoma is also provided.

Keywords: Biodiversity, *Phaeosphaeriaceae*, Phylogenetics, Ribosomal DNA, Saprobe, Taxonomy.

INTRODUCTION

Neosetophoma Gruyter, Aveskamp & Verkley, a member of the family Phaeosphaeriaceae (Pleosporales), was introduced by de Gruyter et al. (2010) and typified by N. samarorum (Desm.) Gruyter, Aveskamp & Verkley based on Phoma samarorum Desm. This genus currently contains 28 species (Index Fungorum; http://www.index fungorum.org/Names/Names.asp) which are saprobes, pathogens and endophytes on woody and herbaceous plants or in soil (Quaedvlieg et al. 2013, Phookamsak et al. 2014, Liu et al. 2015, Karunarathna et al. 2017, Wanasinghe et al. 2018, Marin-Felix et al. 2019, Hyde et al. 2020, Crous et al. 2021, Zhang et al. 2024). Neosetophoma species are mostly asexual morphs characterized by globose to irregular pycnidial conidiomata with papillate ostioles, and yellowish conidia that are attenuate at one end (de Gruyter et al. 2010, Marin-Felix et al. 2019).

Neosetophoma garethjonesii Tibpromma, E.B.G. Jones & K.D. Hyde was reported as the first sexual morph of this genus (Tibpromma et al. 2017). Later, other species with sexual morphs, i.e. N. camporesii Q. Tian & K.D. Hyde, N. guiyangensis J.F. Zhang, J.K. Liu, K.D. Hyde & Z.Y. Liu, N. miscanthi A. Karun., C.H. Kuo & K.D. Hyde, N. poaceicola Goonas., Thambug. & K.D. Hyde, N. shoemakeri Senwanna, Wanas., Bulgakov, E.B.G. Jones & K.D. Hyde, N. trachycarpi S.N. Zhang, K.D. Hyde & Jian K. Liu, and N. xingrenensis J.F. Zhang, J.K. Liu, K.D. Hyde & Z.Y. Liu were added to this genus (Hyde et al. 2018, 2019, 2020, Thambugala et al. 2017), from which only N. shoemakeri produces the asexual morph (Hyde et al. 2018). The sexual morph of Neosetophoma species is characterized by globose to subglobose, solitary, gregarious or scattered, unilocular, ostiolate ascomata; a carbonaceous peridium consisting of few layers of dark brown to brown pseudoparenchymatous cells of textura angularis; hamathecium comprising numerous, filiform, hyaline, pseudoparaphyses; bitunicate, fissitunicate, cylindrical, 8-spored asci; 0-3-seriately arranged ascospores, fusiform, smooth-walled, hyalin to brown, 1-3-septate, without gelatinous sheath (Thambugala et al. 2017, Hyde et al. 2018, 2019, 2020).

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In an investigation of the biodiversity of microfungi in the North Khorasan Province of Iran, we found an unknown sexual morph on dead branches of *Lonicera caprifolium* L. Further morphological and molecular studies enabled the recognition of *Neosetophoma iranianum* Papizadeh, Amoozegar, Wijayaw., Shahz. Faz. & K.D. Hyde (Karunarathna et al. 2017), for which no sexual morph has been reported yet. Therefore, a new sexual morph for *N. iranianum* is described here using morphological and molecular data, and *Lonicera caprifolium* is reported as a new host for this species. We also present an identification key to all described species of *Neosetophoma*.

MATERIALS AND METHODS

Morphological characterization

Samples were collected from dead branches of *Lonicera caprifolium* in the Shirvan Faculty of Agriculture in the North Khorasan Province of Iran

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(37° 26' 11.36" N, 57° 49' 24.49"). Single ascospore cultures were obtained on potato dextrose agar (PDA, Merck, Germany), following the method of Senanayake et al. (2020). Macroscopic observations were carried out using a Nikon (SMZ-1B) stereo microscope. Colony growth and characteristics were determined on PDA and potato carrot agar (PCA; Domsch et al. 2007) incubated at 25 °C. Color names and codes used for descriptions were based on Rayner (1970). Microscopic slides were prepared in water.

Free-hand sections of ascomata were prepared and examined under Olympus CX21 light microscope. Photographs were taken using an AmScope digital camera (MD500). Herbarium specimen was preserved at the Fungus Reference Collection (IRAN...F) of Herbarium Ministerii Iranici Agriculturae "IRAN," Iranian Research Institute of Plant Protection (Tehran, Iran), while living culture at the Iranian Fungal Culture Collection (IRAN...C) of the "IRAN" Herbarium.



Fig. 1. RAxML tree based on the ITS sequence alignment of all *Neosetophoma* species. Maximum Likelihood bootstrap supports (\geq 50%) are shown above or below the branches. The branches with full statistical support (ML = 100%) are thickened. The scale bar shows the expected number of changes per site. The tree is rooted to *Poaceicola arundinis* MFLU 16–0158 and *P. bromi* MFLUCC 13–0739 (*Phaeosphaeriaceae*). T: ex-type strain.

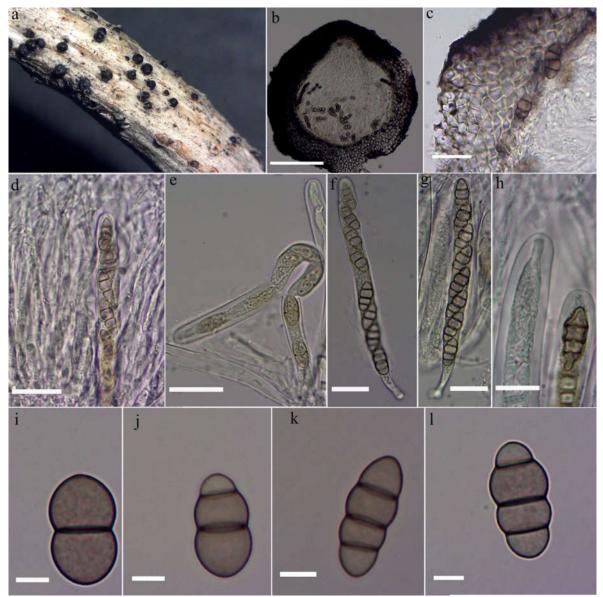


Fig. 2. *Neosetophoma iranianum* IRAN 18640F (sexual morph). a. Ascomata on *Lonicera caprifolium* branch; b. Vertical section through an ascoma; c. Peridium; d. Pseudoparaphyses; e-g. Asci; h. Apical apparatus of asci; i-l. Ascospores. Scale bars = (b) 100 μ m; (c-g) 20 μ m; (h) 10 μ m; (i-l) 5 μ m.

DNA extraction and sequencing

Fresh fungal mycelium was scraped from the surface of a PDA plate incubated at 25 °C for 7 d and transferred into a 1.5 mL centrifuge tube. Genomic DNA was extracted as described by Liu et al. (2000) with an initial step of grinding the mycelia in liquid nitrogen. The ITS region (ITS1-5.8S-ITS2) was amplified using primers ITS1/ITS4 (White et al. 1990). Amplification of D1–D2 of the 28S rDNA (LSU) was achieved using primers LROR/LR3 (Rehner and Samuels 1995). The PCR reaction (25

 μ L) contained 1 μL of each primer (10 pmol/μL, SinaClon Inc.), 1.0 μL of genomic DNA (30 ng/μL), 2.5 μL of 10× high yield PCR buffer (SinaClon, Iran), 0.3 μL of *Taq* polymerase (5 units/ μL, SinaClon, Iran), 1 μL of MgCl₂ (25 mM), 0.5 μL of dNTPs (10 mM), and 17.7 μL of sterile distilled water. PCR reactions were run on an Applied Biosystems 2720 Thermal cycler (Life Technologies, Singapore) with an initial denaturation step of 5 min at 95°C, followed by 35 cycles of 45 s at 94°C, 35 s at 58°C, 90 s at 72°C, and a final extension of 10 min at 72°C. PCR products were visualized in 1% agarose gel in 1×TBE buffer. The PCR products were purified by Microsynth Company, Switzerland. The purified DNA samples were submitted for sequencing to a capillary sequencing machine (ABI 3730XL, Applied Biosystem, Foster City, CA) of the same company.

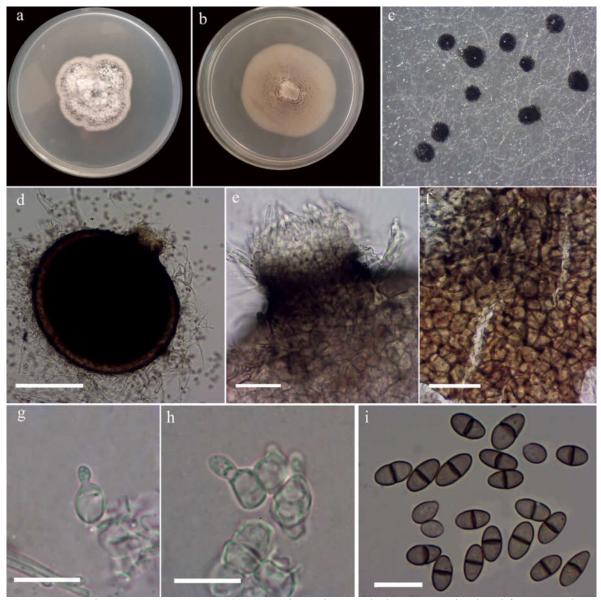


Fig. 3. *Neosetophoma iranianum* IRAN 5290C (asexul morph). a. Colonies on PDA, incubated for two weeks at 25° C; b. Colonies on PCA incubated for two weeks at 25° C; c. Pycnidia produced on PCA; d. Pycnidium; e. Ostiole; f. Pycnidial wall; g, h. Conidiogenous cells; i. Conidia. Scale bars = (d) 100 µm; (e, f) 20 µm; (g-i) 10 mm.

Phylogenetic analysis

The chromatograms of the obtained sequences were analyzed using Finch TV version 1.40v (Geospiza). The sequences of a strain recovered in this study (IRAN 5290C) were aligned against the ex-type or reference strains of all described species of *Neosetophoma* (Table S1). The alignments were obtained using MAFFT v. 7 at the web server (https://mafft.cbrc.jp/ alignment/server/) (Kuraku et al. 2013, Katoh et al. 2019), using default settings. The alignment was edited manually where necessary with MEGA X (Kumar et al. 2018). Maximum Likelihood (ML) analysis including 1000 bootstrap replicates was performed in RAxMLGUI v. 1.5b1 (Silvestro and Michalak 2012), and the best scoring tree was selected among suboptimal trees from each replicate by comparing likelihood scores under the GTR+GAMMA substitution model. The tree was rooted with *Poaceicola arundinis* W.J. Li, Camporesi,

D.J. Bhat & K.D. Hyde (MFLU 16–0158) and *P. bromi* Wijayaw., W.J. Li, Camporesi, D.J. Bhat & K.D. Hyde (MFLUCC 13–0739), the *Phaeosphaeriaceae* (*Pleosporales*). Trees were visualized in FigTree version 1.4.4 (Rambaut 2012). The sequences generated in this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank).

RESULTS AND DISCUSSION

To elucidate the relationships of the newly discovered sexual morph, we conducted phylogenetic analyses using sequences of the ITS (483–563 bp) and partial LSU rDNA (526–857 bp) individually. Phylogenies of the LSU region (not shown) revealed that LSU sequences do not contain sufficient variation for distinguishing among species of *Neosetophoma*, and therefore this locus was excluded from our analyses. The ML analysis based on the ITS rDNA yielded a bestscoring tree (Fig. 1) with a final ML optimization likelihood value of -1917.529289. The matrix contained 154 distinct alignment patterns, with 4.98% of undetermined characters or gaps. Parameters for the GTR+GAMMA model were estimated as follows: base frequencies A = 0.225594, C = 0.243192, G = 0.228652, and T = 0.302562; substitution rates AC = 1.960977, AG = 5.029146, AT = 1.893325, CG = 0.611176, CT = 7.286687, and GT = 1.000000; gamma distribution shape parameter $\alpha = 0.106773$. Based on phylogenies of the ITS rDNA, our single ascospore culture (IRAN 5290C) was grouped with the extype strain of Neosetophoma iranianum (IBRC-M 30176), without known sexual morph, in a wellsupported clade (98% BS). The ITS and LSU sequence comparison between the two strains, as recommended by Jeewon and Hyde (2016), also revealed that these strains were the same (no base pair difference). Moreover, the asexual morph produced in culture by strain IRAN 5290C aligns with the morphology described by Karunarathna et al. (2017). Therefore, the relevant description of N. iranianum by Karunarathna et al. (2017) is here expanded to include the new sexual morph.

Taxonomy

Neosetophoma iranianum Papizadeh, Amoozegar, Wijayaw., Shahzadeh Fazeli & K.D. Hyde, Mycosphere 8(10): 1826 (2017)

Sexual morph: Ascomata 250–300 μ m high, 235–290 μ m diam ($\overline{x} = 280 \times 265 \mu$ m, n = 10), solitary, scattered, immersed under epidermis, becoming erumpent through host surface, uniloculate, globose to subglobose, black, glabrous,

ostiolate. Peridium 36-63 µm thick, consisting of several layers of dark brown to brown, pseudoparenchymatous cells of textura angularis, 3-8 µm diam. Hamathecium comprising up to 200 μm long, 2–3 μm wide, numerous, filamentous, septate, pseudoparaphyses embedded in gelatinous matrix. Asci 93–123 × 9–11 μ m (\overline{x} = $112 \times 10.7 \ \mu\text{m}, \ n = 20$), 8-spored, bitunicate, fissitunicate, cylindrical, short pedicellate, apically rounded with an ocular chamber. Ascospores 15- $20 \times 6-10 \ \mu m$ ($\overline{x} = 17.3 \times 8.1 \ \mu m$, n = 30), uniseriate, partially overlapping, fusiform to ellipsoidal, initially hyaline to subhyaline, becoming brown at maturity, 1-3-septate, mostly 3-septate, straight to slightly curved, deeply constricted at the septa, smooth-walled (Fig. 2, 3).

Asexual morph coelomycetous. Conidiomata (On PCA) 180–290 µm high, 185–295 µm diam (x = $220 \times 210 \ \mu m$, n = 10), pycnidial, solitary, dark brown, globose to subglobose, immersed to semiimmersed, unilocular, ostiolate, 15-20 µm wide, with a 20–36 high \times 30–44 µm diam neck. Pycnidial wall 4-10 µm thick, composed of 2-3 layers of light brown to brown, thick-walled cells of textura angularis, 3-9 µm diam. Conidiophores reduced to conidiogenous cells. Conidiogenous cells 4–6 μ m high × 4–6 μ m wide, enteroblastic, phialidic, ampulliform, determinate, hyaline, smooth-walled. Conidia 5–8.5 × 3–4 um (\overline{x} = 7.2 × 3.7 μ m, n = 20), ellipsoidal to tear-drop shape, sometimes attenuated at one end, (0-)1(-2) septate, continuous or constricted at the septa, initially hyaline to subhyaline, becoming light brown at maturity, smooth-walled, exuding as a black mass.

Cultural characteristics (25 °C, 4 weeks): Ascospores germinate on PDA within 24–48 h. Mycelium consists of 1.5–3.5 μ m wide, branched, septate, smooth, subhyaline hyphae. Colonies on PDA reach 75 mm diam, circular to irregular, fluffy, surface rough with hyphal tufts, edge entire, at first week white to buff (45), with sparse aerial mycelium, becoming pale grey after one month; reverse grayish sepia (106) in margin and black in center. Colonies on PCA reaching 70 mm diam, circular, velvety buff (45); the reverse of the same obverse colony color.

Specimen examined: IRAN, North Khorasan Province, Shirvan Faculty of Agriculture, 37° 26' 11.36" N, 57° 49' 24.49", on dead branches of *Lonicera caprifolium* L., 2 October 2024, M. Mehrabi, IRAN 18640F, IRAN 5290C.

Notes: *Neosetophoma iranianum* was introduced by Karunarathna et al. (2017) from soil in the Golestan Province of Iran. The asexual morph of our strain has a pycnidium shape, a conidium shape and septation, peridial structures, and conidiogenous cells similar to those of N. iranianum. However, it produces larger pycnidia $(180-290 \times 186-296 \ \mu m \ vs \ 70-110 \times 75-120 \ \mu m)$ and conidia (5–8.5 \times 3–4 μ m vs 4–6 \times 2–4 μ m) than the ex-type strain of N. iranianum (IBRC-M 30176). Nevertheless, there are some contradictions between the scale bars and pycnidial and conidial dimensions presented in the original description of N. iranianum by Karunarathna et al. (2017). Further examination of the ex-type strain of N. iranianum will help to better resolve the asexual morph of this species.

Phylogenetically, N. iranianum strains were placed in a large, moderately supported clade (84% BS) comprising ex-type strains of N. aseptata, N. camporesii, N. clematidis, N. hnaniceana, N. lunariae, N. rosae, N. salicis and N. shoemaker (Fig. 1), from which only N. camporesii, lacks an asexual morph, and N. shoemaker have sexual morphs. The sexual morph of N. iranianum is different from N. camporesii (Hyde et al. 2020) in asci size (62–90 \times 7–10 μ m) and ascospores size $(20-32 \times 3-5 \mu m)$, and from N. shoemaker (Hyde et al. 2018) in ascomata size (170-230 × 170-200 μ m) and ascospores size (15–27 \times 3–6 μ m). The asexual morph of N. iranianum can be distinguished from all the above-mentioned species by its septate conidia (aseptate in N. aseptata and N. salicis), shorter conidia (11–15 × 2–4 μ m in N. clematidis, $8-9 \times 3-4 \mu m$ in N. hnaniceana, $8-14 \times$ 1.5–3 μ m in N. rosae, 14–17 × 3 μ m in N. lunariae and 7.5–10.5 \times 2.5–3 μ m in N. shoemakeri) and smaller pycnidia (120–180 \times 120–160 μ m in N. shoemakeri). Phookamsak et al. (2019) described N. lonicerae Phookamsak, Wanas. & K.D. Hyde as a coelomycetous fungus on dead hanging branches of Lonicera maackii in China. Morphologically, N. lonicerae is different from N. iranianum in conidiomata size (110–160 \times 80–160 μ m) and conidia size $(9-12 \times 4-5 \mu m)$.

Key to the species of Neosetophoma

- 1a. Fruiting bodies absent
- 1b. Fruiting bodies present
- 2a. Colonies fluffy, with dense green spots in the center, hyphae 2–4.5 µm wide N. endophyticum¹

2

3

- 2b. Colonies fluffy, later flocculent, hyphae 1.5–3.5 μm wide N. polygonata¹/N. gimenensis¹
- 3a. Sexual morph determined; coelomycetous asexual morph rarely present 4
- 3b. Sexual morph undetermined; coelomycetous
asexual morph always present12
- 4a. Ascomata more than 250 µm high 5
- 4b. Ascomata less than 250 µm high 7

- 5a. Ascomata 235–360 \times 170–280 µm, with ostioles having a reddish tinge at the pores, ascospores 20–32 \times 3–5 µm N. camporesii
- 5b. Ascomata with ostioles lacking a reddish tinge at the pores 6
- 6a. Ascomata 250–300 × 235–290 μm, ascospores 15–20 × 6–10 μm; pycnidia 70–110(–290) × 75–120(–295) μm, conidia 4–6(–8.5) × 2–4 μ *N. iranianum*
- 6b. Ascomata 403–444 × 332–363 μm, ascospores 16–26 × 3–5 μm; asexual morph absent *N. garethjonesii*
- 7a. Ascospores mostly 3–5-septate8
- 7b. Ascospores mostly 1–3-septate 10
- 8a. Ascomata with darkened and yellow pigments around the ostioles *N. trachycarpi*
- 8b. Ascomata without darkened area and yellow pigments around the ostioles 9
- 9a. Ascospores 20–25.5 × 3.5–4.5 μ m, on woody plants *N. guiyangensis*
- 9b. Ascospores $18.5-22.5 \times 3.5-5 \mu m$, on grasses and woody plants *N. poaceicola*
- 10a. Asci sessile, 52 × 9 μ m, ascospores 22 × 5 μ m *N. xingrensis*
- 10b. Asci pedicellate
- 11a. Ascomata 170–230 × 170–200 μm, asci 84 × 9.5 μm, ascospores 15–27 × 3–6 μm; pycnidia 120–180 × 120–160 μm, conidia 7.5–10.5 × 2.5–3 μm

N. shoemakeri

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- 11b. Ascomata 90–130 \times 110–120 μ m, asci 48 \times 10.5 μ m, ascospores 18–21 \times 4.7–5.3 μ m; asexual morph absent *N. miscanthi*
- 12a. Conidia aseptate13
- 12b. Conidia septate1913a. Conidia $3-4 \times 1-1.5 \mu m$, onFicus elastica
- N. fici
- 13b. Conidia wider than 1.5 µm, on other hosts 14
- 14a. Conidia longer than 8 μm 15
- 14b. Conidia shorter than 8 μm 16
- 15a. Conidia $8-9 \times 2-3 \mu m$, ellipsoidal to fusoid, on wood of *Buxus sempervirens N. buxi*
- 15b. Conidia $8-10 \times 2-3 \mu m$, allantoid, on twigs and branches of *Salix* sp. *N. salicis*
- 16a. Conidiogenous cells $2-3 \times 1.5-2 \mu m$, conidia $4-6 \times 1.5-2.5$, on *Rosa* sp. *N. rosigena*
- 16b. Conidiogenous cells longer than 3 μm, on other hosts 17
- 17a. Pycnidia 55–170 μm diam, conidiogenous cells 6– 13 × 7–12 μm, conidia 4–7 × 1–2 μm, on Ulmus sp. N. ulmi (inval. nam.)
- 17b. Pycnidia more than 170 µm diam
- 18a. Pycnidia 250–350 µm diam, conidiogenous cells 4– 8 × 4–5 µm, conidia 4–5 × (1.5–)2 µm, on Viburnum opulus N. aseptata
- 18b. Pycnidia 150–350 μm diam, conidiogenous cells 4– 6 × 4–6 μm, conidia 6–8 × 2(–2.5) μm, on Poaceae N. cerealis (= N. phragmitis)²
- 19a. Pycnidia $425-475 \times 220-270 \ \mu\text{m}$, conidia $11-15 \times 2-4\mu\text{m}$, 3-septate N. clematidis
- 19b. Pycnidia less than 400 µm diam 20

21

22

20a. Conidia always 1	-septate
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- 20b. Conidia often multiseptate
- 21a. Conidia 7–10 × 3–4 μ m, ellipsoidal to fusoid, on dead leaves and wood of Buxus sempervirens

N. hnaniceana

- 21b. Conidia 5–10 \times 2–3 μ m, subcylindrical, on twigs of Sambucus nigra N. sambuci
- 22a. Conidia 5.5-8 × 1.5-2.5 µm, subcylindrical, 1-3-septate N. rosarum 23
- 22b. Conidia mostly longer than 8 µm
- 23a. Pycnidia more than 150 µm diam 24
- 23b. Pycnidia less than 150 µm diam 25
- 24a. Pycnidia 150–300 μ m diam, conidia 10–22 \times 2.5–3 µm, 1–4-septate N. lunariae
- 24b. Conidioma 250–280 μ m diam, conidia 7–16 \times $1-2 \mu m$, 1-2-septate N. samarorum
- 25a. Pycnidia 100–120 μ m diam, conidia 8–14 \times 1.5– 3 µm N. rosae
- 25b. Conidia wider than 3 µm 26
- 26a. Pycnidia 50–60 μ m diam, conidia 6–11 \times 3–4 µm, subcylindrical, fusiform, or ellipsoidal, 1-2septate N. italica
- 26b. Pycnidia 80–160 μ m diam, conidia 8.5–14 × 4–5 μm, ellipsoidal, 1–3-septate N. lonicerae

¹ It is difficult to distinguish these three species using morphological traits but they are phylogenetically distinct.

² Crous et al. (2020b) combined Coniothyrium cerealis E. Müll. into the genus Neosetophoma as N. cerealis (E. Müll.) Crous. They also, despite some variation regarding the conidial dimensions, synonymized N. phragmitis Crous, R.K. Schumach. & Y. Marín under N. cerealis based on phylogenies of the ITS rDNA. Neosetophoma phragmitis (Marin-Felix et al. 2019) has smaller conidia than N. cerealis (4–5 \times 2 μm vs 6–8 \times 2 μm). In this identification key, we follow Crous et al. (2020b) and treat N. phragmitis as a synonym of N. cerealis; nevertheless, based on the presented phylogeny in this study (Fig. 1) these species are phylogenetically distinct.

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شکل جنسی جدید برای Neosetophoma iranianum، همراه با یک کلید شناسایی برای گونههای Neosetophoma

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چکیده: گونه Meosetophoma iranianum تا کنون تنها در شکل غیرجنسی سلومیستی توصیف شده است. در مطالعه حاضر، شکل جنسی این گونه برای اولین بار از شاخههای مرده گیاه Lonicera caprifolium در استان خراسان شمالی گزارش می شود. ارتباط بین شکل غیرجنسی و جنسی این گونه از طریق واکاویهای تبارشناختی بر اساس ناحیه ITS از DNA ریبوزومی و ویژگیهای ریخت شناختی تأیید شده است. در این مطالعه، تصاویر و توصیفهای مربوط به شکلهای جنسی و غیرجنسی گونه ویژگیهای ریخت شناختی تأیید شده است. در این مطالعه، تصاویر و توصیفهای مربوط به شکلهای جنسی و غیرجنسی گونه شده در جنس Neosetophoma iranianum ارائه شده است.

كلمات كليدى: پودەزى، تاكسونومى، تبارشناسى، تنوع زيستى، DNA ريبوزومى، Phaeosphaeriaceae

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