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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).

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.

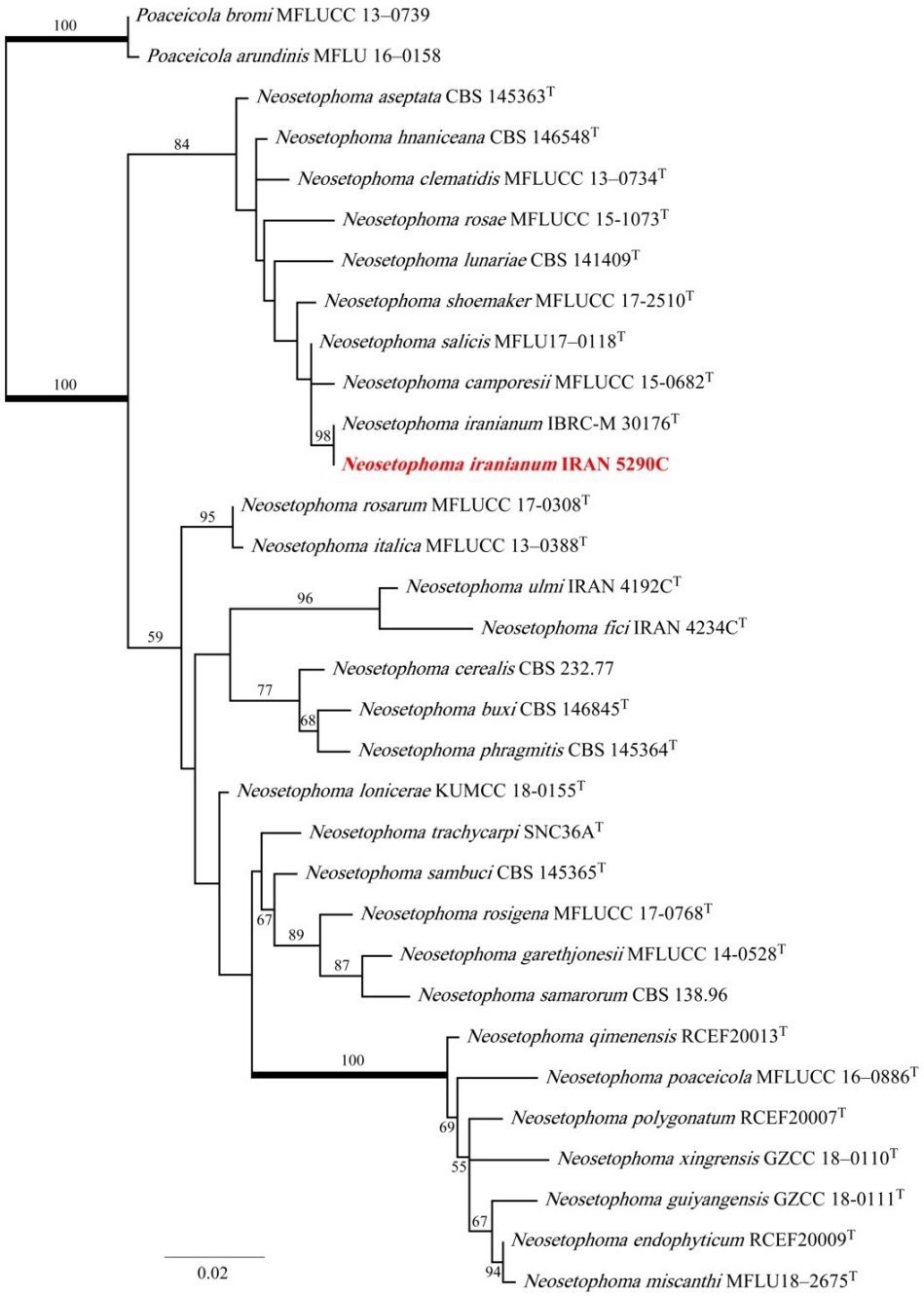

Fig. 1. RAxML tree based on the ITS sequence alignment of all *Neosetophoma* species. Maximum Likelihood bootstrap supports (≥50%) are shown above or below the branches. The branches with full statistical support (ML = 100%) are thickened. The scale bar shows the expected number of changes per site. 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 µm; (c-g) 20 µm; (h) 10 µm; (i-l) 5 µm.

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 µL) 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 $MgCl_2$ (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.

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 &

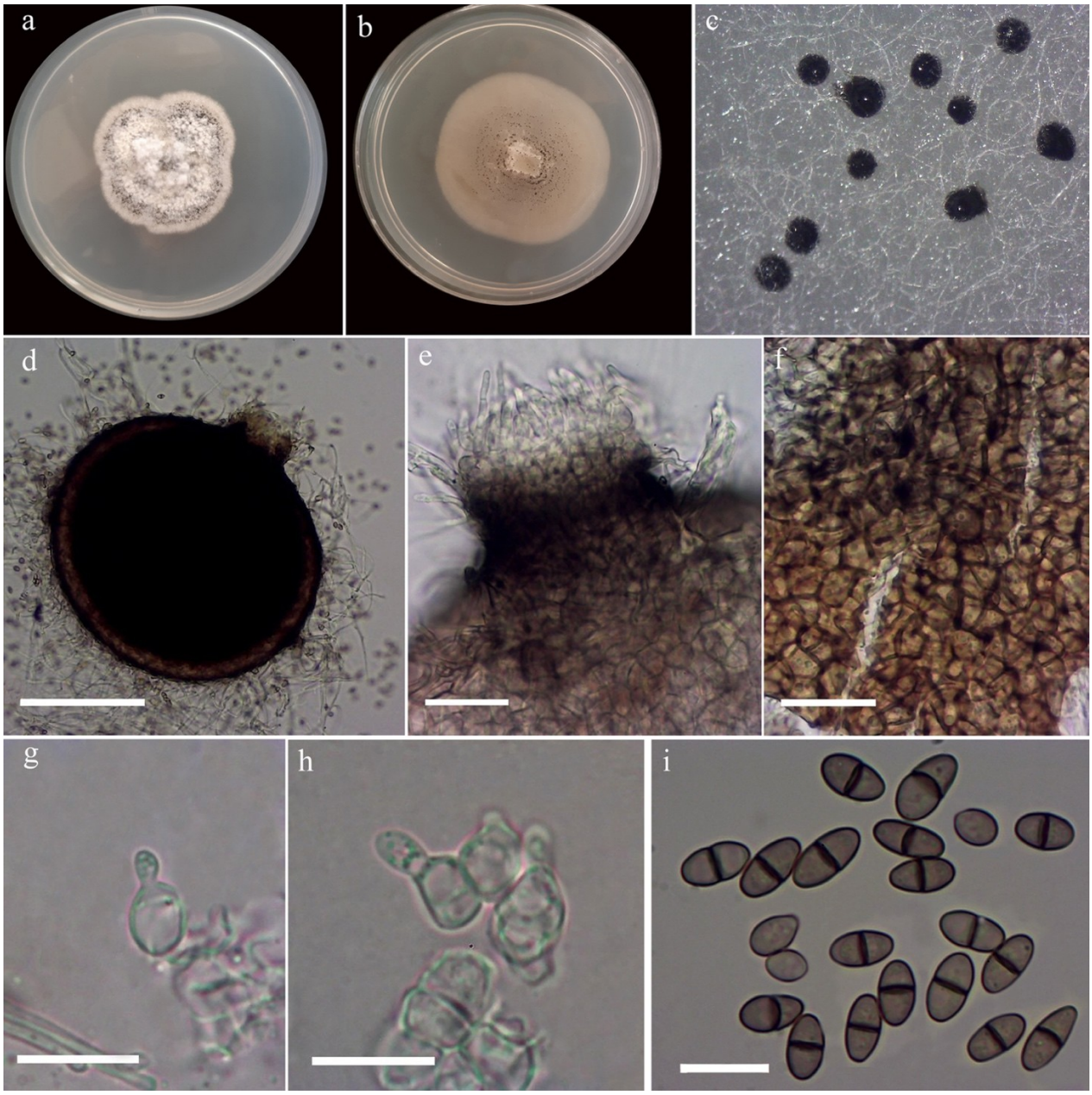

Fig. 3. *Neosetophoma iranianum* IRAN 5290C (asexul morph). a. Colonies on PDA, incubated for two weeks at 25°C; b. Colonies on PCA incubated for two weeks at 25°C; c. Pycnidia produced on PCA; d. Pycnidium; e. Ostiole; f. Pycnidial wall; g, h. Conidiogenous cells; i. Conidia. Scale bars = (d) 100 µm;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

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 best-scoring 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 α = 0.106773. Based on phylogenies of the ITS rDNA, our single ascospore culture (IRAN 5290C) was grouped with the ex-type strain of *Neosetophoma iranianum* (IBRC-M 30176), without known sexual morph, in a well-supported 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 µm high, 235–290 µm diam ($\overline{x}$ = 280 × 265 µm, 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 a gelatinous matrix. Asci 93–123 × 9–11 µm ($\overline{x}$ = 112 × 10.7 µm, n = 20), 8-spored, bitunicate, fissitunicate, cylindrical, short pedicellate, apically rounded with an ocular chamber. Ascospores 15–20 × 6–10 µm ($\overline{x}$ = 17.3 × 8.1 µm, 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 ($\overline{x}$ = 220 × 210 µm, n = 10), pycnidial, solitary, dark brown, globose to subglobose, immersed to semi-immersed, unilocular, ostiolate, 15–20 µm wide, with a 20–36 high × 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 µm high × 4–6 µm wide, enteroblastic, phialidic, ampulliform, determinate, hyaline, smooth-walled. Conidia 5–8.5 × 3–4 µm ($\overline{x}$ = 7.2 × 3.7 µm, 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 µm 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 × 186–296 µm vs 70–110 × 75–120 µm) and conidia (5–8.5 × 3–4 µm vs 4–6 × 2–4 µm) 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 × 7–10 µm) and ascospores size (20–32 × 3–5 µm), and from *N. shoemaker* (Hyde et al. 2018) in ascomata size (170–230 × 170–200 µm) and ascospores size (15–27 × 3–6 µm). 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 µm in *N. clematidis*, 8–9 × 3–4 µm in *N. hnaniceana*, 8–14 × 1.5–3 µm in *N. rosae*, 14–17 × 3 µm in *N. lunariae* and 7.5–10.5 × 2.5–3 µm in *N. shoemakeri*) and smaller pycnidia (120–180 × 120–160 µm 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 × 80–160 µm) and conidia size (9–12 × 4–5 µm).

Key to the species of *Neosetophoma*

1a. Fruiting bodies absent 2
1b. Fruiting bodies present 3
2a. Colonies fluffy, with dense green spots in the center, hyphae 2–4.5 µm wide *N. endophyticum*[1]
2b. Colonies fluffy, later flocculent, hyphae 1.5–3.5 µm wide *N. polygonata*[1]/ *N. qimenensis*[1]
3a. Sexual morph determined; coelomycetous asexual morph rarely present 4
3b. Sexual morph undetermined; coelomycetous asexual morph always present 12
4a. Ascomata more than 250 µm high 5
4b. Ascomata less than 250 µm high 7
5a. Ascomata 235–360 × 170–280 µm, with ostioles having a reddish tinge at the pores, ascospores 20–32 × 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-septate 8
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 µm, on woody plants *N. guiyangensis*
9b. Ascospores 18.5–22.5 × 3.5–5 µm, on grasses and woody plants *N. poaceicola*
10a. Asci sessile, 52 × 9 µm, ascospores 22 × 5 µm *N. xingrensis*
10b. Asci pedicellate 11
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*
11b. Ascomata 90–130 × 110–120 µm, asci 48 × 10.5 µm, ascospores 18–21 × 4.7–5.3 µm; asexual morph absent *N. miscanthi*
12a. Conidia aseptate 13
12b. Conidia septate 19
13a. Conidia 3–4 × 1–1.5 µm, on *Ficus 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 × 2–3 µm, ellipsoidal to fusoid, on wood of *Buxus sempervirens* *N. buxi*
15b. Conidia 8–10 × 2–3 µm, allantoid, on twigs and branches of *Salix* sp. *N. salicis*
16a. Conidiogenous cells 2–3 × 1.5–2 µm, conidia 4–6 × 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 18
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× 220–270 µm, conidia 11–15 × 2–4µm, 3-septate *N. clematidis*
19b. Pycnidia less than 400 µm diam 20
20a. Conidia always 1-septate 21
20b. Conidia often multiseptate 22

21a. Conidia 7–10 × 3–4 µm, ellipsoidal to fusoid, on dead leaves and wood of *Buxus sempervirens* *N. hnaniceana*
21b. Conidia 5–10 × 2–3 µm, subcylindrical, on twigs of *Sambucus nigra* *N. sambuci*
22a. Conidia 5.5–8 × 1.5–2.5 µm, subcylindrical, 1–3-septate *N. rosarum*
22b. Conidia mostly longer than 8 µm 23
23a. Pycnidia more than 150 µm diam 24
23b. Pycnidia less than 150 µm diam 25
24a. Pycnidia 150–300 µm diam, conidia 10–22 × 2.5–3 µm, 1–4-septate *N. lunariae*
24b. Conidioma 250–280 µm diam, conidia 7–16 × 1–2 µm, 1–2-septate *N. samarorum*
25a. Pycnidia 100–120 µm diam, conidia 8–14 × 1.5–3 µm *N. rosae*
25b. Conidia wider than 3 µm 26
26a. Pycnidia 50–60 µm diam, conidia 6–11 × 3–4 µm, subcylindrical, fusiform, or ellipsoidal, 1–2-septate *N. italica*
26b. Pycnidia 80–160 µm 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 × 2 µm vs 6–8 × 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 *Neosetophoma 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 می‌شود. ارتباط بین شکل غیرجنسی و جنسی این گونه از طریق واکاوی‌های تبارشناختی بر اساس ناحیه ITS از DNA ریبوزومی و ویژگی‌های ریخت‌شناختی تأیید شده است. در این مطالعه، تصاویر و توصیف‌های مربوط به شکل‌های جنسی و غیرجنسی گونه *Neosetophoma iranianum* ارائه و با گونه‌های نزدیک مقایسه گردیده است. همچنین کلید شناسایی تمامی گونه‌های توصیف‌شده در جنس *Neosetophoma* ارائه شده است.

کلمات کلیدی: پوده‌زی، تاکسونومی، تبارشناسی، تنوع زیستی، DNA ریبوزومی، *Phaeosphaeriaceae*

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