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The Genera of Fungi – G7: *Hirudinaria*

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Abstract: The current paper represents the seventh contribution in the Genera of Fungi series, linking type species of fungal genera to their morphology and DNA sequence data. This manuscript focuses on a genus of dematiaceous hyphomycetes, *Hirudinaria.* Two species of this genus are treated in this study. *Hirudinaria mespili*, the type species of the genus, as well as *Hirudinaria macrocarpa,* are epitypified and provided with DNA sequence data to resolve their phylogeny as members of *Mycosphaerellaceae* (*Mycosphaerellales*, *Dothideomycetes*).

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INTRODUCTION

The Genera of Fungi project (www.generaoffungi.org) is a series of publications introduced in 2014 (Crous *et al.* 2014a), which aims to revise the generic names of fungi, to provide DNA sequence data for them and to restudy or recollect their type species. In the present contribution, we focus on the poorly known and problematic genus *Hirudinaria.*

The genus *Hirudinaria* was originally established by Cesati (1856) and accommodates five species listed in MycoBank (accessed 12 September 2023) including *H. arundinariae*, *H. macrocarpa*, *H. macrospora* (current name = *H. macrocarpa*), *H. mespili*, and *H. oxyacanthae*. Since all these species were described before the DNA era, they lack living cultures and DNA sequence data, and as a result their phylogenetic status is unclear. In this study, we provide DNA sequence data for two species of this genus, namely *H. mespili* (type species), and *H. macrocarpa*. The two species treated are supplemented with recently collected epitypes which have been registered in MycoBank (Robert *et al.* 2013). Furthermore, the phylogenetic position of the genus *Hirudinaria* is clarified.

MATERIAL AND METHODS

Isolates

The diseased leaves of medlar (*Mespilus*) and hawthorn (*Crataegus*) trees were collected in Hyrcanian forests in north of Iran. Freshly collected leaves were immediately treated using a Zeiss Stemi 305 dissecting microscope (Carl Zeiss, Jena, Germany) and the fungi were isolated using the single conidial isolation method described by Bakhshi *et al.* (2021b). Single conidial colonies were sub-cultured onto malt extract agar (MEA; Merck, Darmstadt, Germany) and incubated at 25 °C for 14–21 d, in order to obtain axenic cultures. Reference isolates and specimens are maintained at the culture collection and fungarium of the Iranian Research Institute of Plant Protection (IRAN), Tehran, Iran, and also deposited in the CBS culture collection at the Westerdijk Fungal Biodiversity Institute, the Netherlands (WI-KNAW).

DNA isolation, amplification and analyses

Fungal mycelium was scraped from the agar surface of cultures with a sterile scalpel and the genomic DNA was extracted using the protocol of Möller *et al.* (1992). Four nuclear loci: 28S nrRNA gene (LSU), internal transcribed spacer regions and intervening 5.8S nrRNA gene (ITS) of the nrDNA operon, translation elongation factor 1-α (*tef1*), and DNA-directed RNA polymerase II second largest subunit (*rpb2*) were amplified using polymerase chain reaction (PCR) with LR0R/LR5 (Vilgalys & Hester 1990), V9G (De Hoog & Gerrits van den Ende 1998)/ITS4 (White *et al.* 1990), EF1-728F (Carbone & Kohn 1999) + EF-2 (O'Donnell *et al.* 1998), and RPB2-5F2 (Sung *et al.* 2007)/ RPB2-f5f + fRPB2-7cR (Liu *et al.* 1999) primers, respectively. Amplification reaction mixtures and conditions described by Bakhshi & Braun (2022) were followed for standard amplification and subsequent sequencing of the LSU and ITS loci, Bakhshi *et al.* (2021b) for *tef1*, and Bakhshi *et al.* (2021a) for *rpb2* loci. Subsequent sequencing was caried out

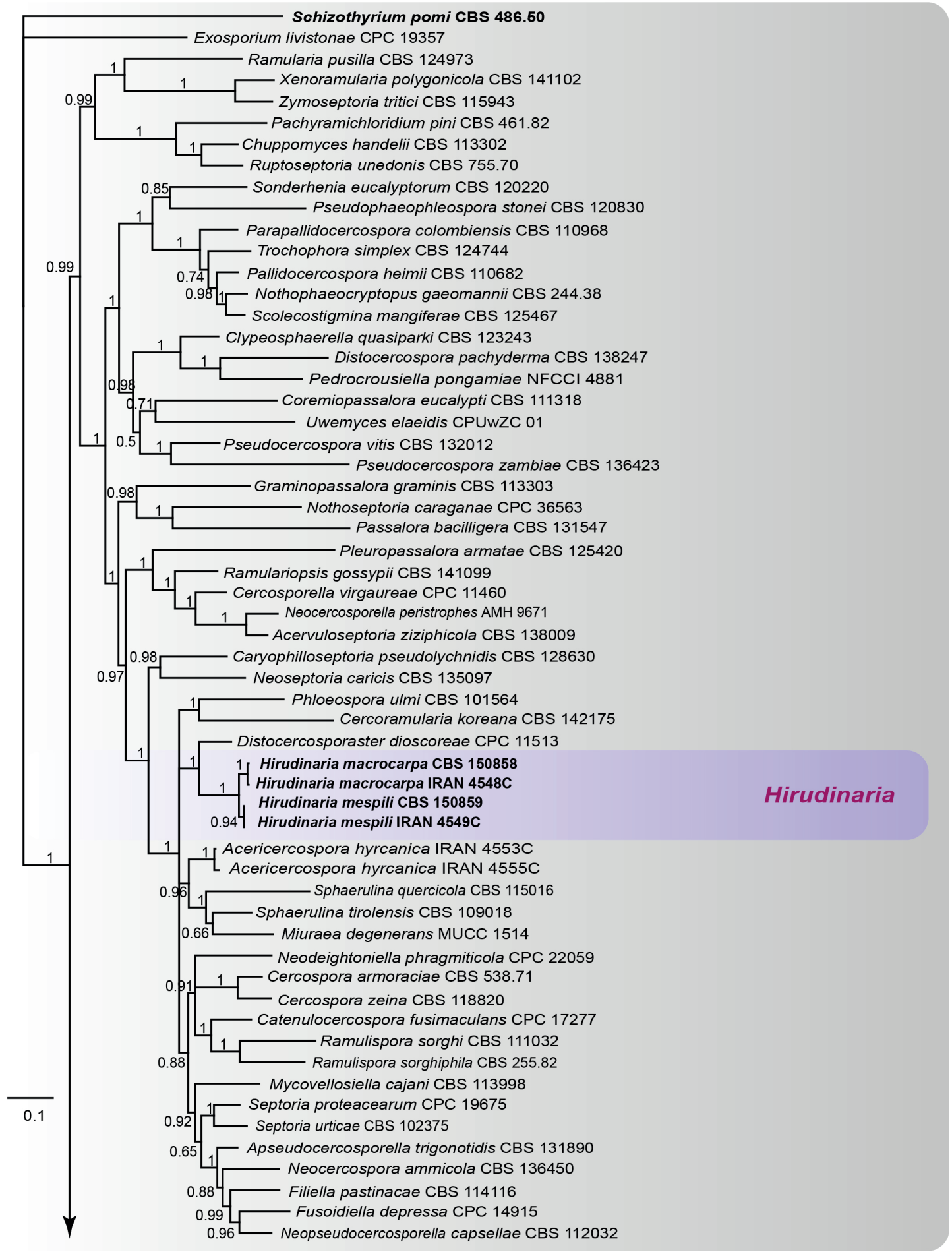

Fig. 1. Bayesian phylogram constructed from LSU, *rpb2* and ITS sequences of *Hirudinaria* spp. within *Mycosphaerellaceae*. The tree was rooted to *Schizothyrium pomi* (CBS 486.50). The scale bar indicates 0.1 expected changes per site.

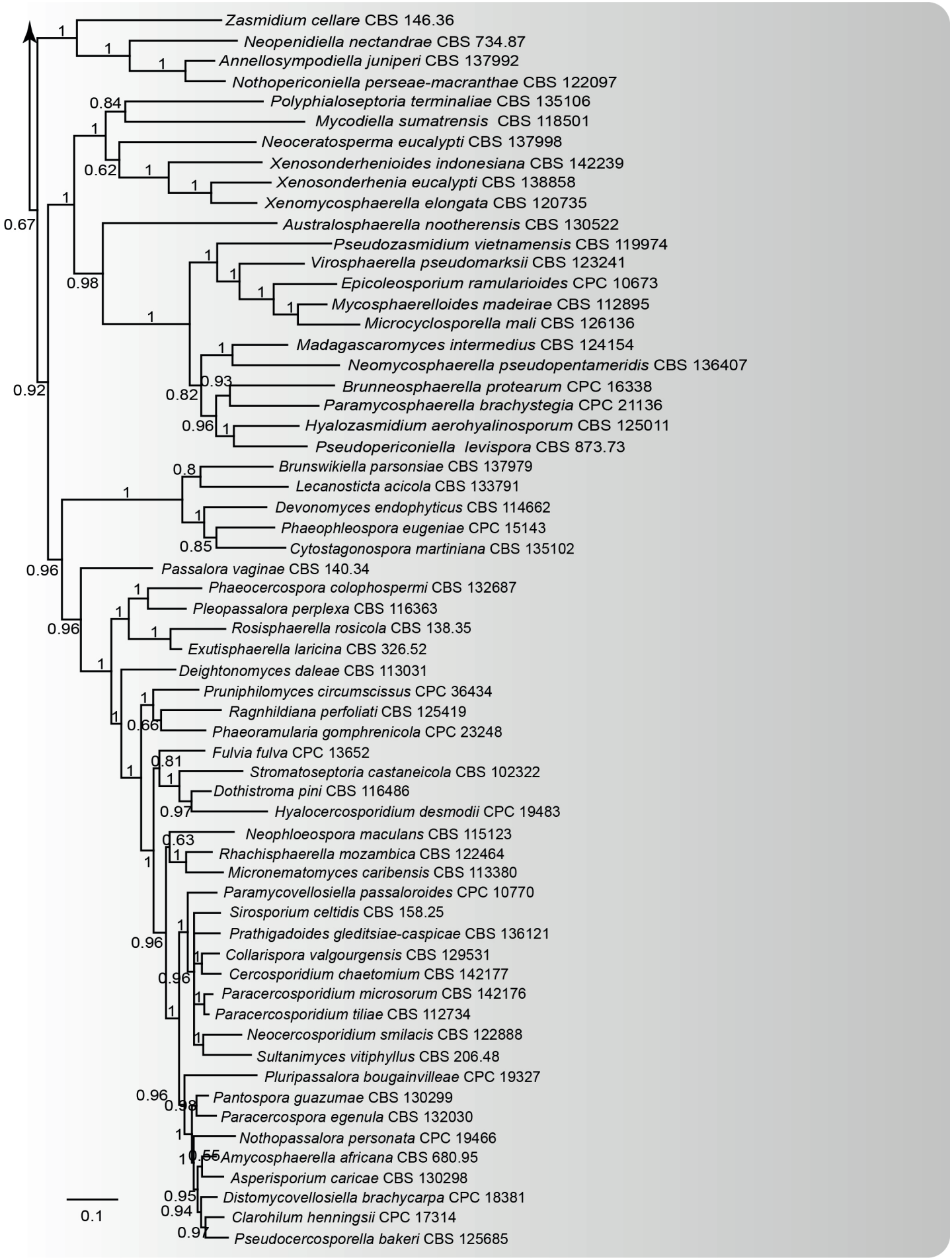

Fig. 1. (Continued).

in both directions using the PCR primers by Microsynth Company (Balgach, Switzerland).

The DNA sequences were analysed and consensus sequences were computed using MEGA v. X software (Kumar *et al.* 2018). Blast searches using consensus regions of LSU, ITS, *rpb2* and *tef1* sequences were performed for each isolate and the closest matches were retrieved from NCBI's GenBank and included in the phylogenetic analyses. Multiple sequence alignments for individual loci were performed by MAFFT v. 7 online interface (http://mafft.cbrc.jp/alignment/server/) (Katoh & Standley 2013). The alignments of individual loci were concatenated using Mesquite v. 3.61 (Maddison & Maddison 2018). Phylogenetic trees were constructed using Bayesian inference (BI) performed with MrBayes v. 3.2.6 (Ronquist *et al.* 2012) as explained in Bakhshi *et al*. (2019). The tree topologies were printed with Geneious v. 8.1.8 (Kearse *et al.* 2012), and the layout of the trees was done in Adobe ® Illustrator v. CC 2017. Sequence data were deposited in the GenBank database (Table 1) and the alignments and trees in TreeBASE (http://www.treebase.org).

Morphology

Slide preparations were mounted in Shear's mounting fluid from structures observed on fungarium material. Observations were made with a Zeiss Stemi 305 dissecting microscope and with an Olympus BX51 (Olympus, Tokyo, Japan) compound microscope using differential inference contrast (DIC) illumination, and images were recorded on an Olympus DP25 camera with associated software. Colony characters and growth rates were noted after 20 d on MEA incubated at 25 °C in the dark. Colony colours were defined using the mycological colour charts of Rayner (1970).

RESULTS

Phylogeny

Mycosphaerellaceae phylogeny: The final concatenated alignment comprised 119 isolates within the family *Mycosphaerellaceae* and *Schizothyrium pomi* (isolate CBS 486.50, *Schizothyriaceae*) which was used as outgroup. The final alignment contained a total of 2 181 characters divided in three partitions containing 753 (LSU), 808 (*rpb2*) and 620 (ITS) characters, including alignment gaps. Based on the results of MrModeltest v. 2.3 (Nylander 2004), dirichlet (1, 1, 1, 1) base frequencies and the GTR+I+G model was used for all loci (LSU, *rpb2* and ITS) for the Bayesian analysis. The Bayesian analysis generated 3 862 trees after 1 930 000 generations from which 2 898 trees were sampled after 25 % of them were discarded as burn-in and were used for calculating posterior probabilities (PP) in the majority rule consensus tree (Fig. 1). The alignment contained a total of 1 204 unique site patterns (227, 568 and 409 for LSU, *rpb2* and ITS, respectively). The results revealed that *Hirudinaria* is a member of *Mycosphaerellaceae* (*Mycosphaerellales*) and forms a highly stable clade, distinct from all other genera in this family.

Hirudinaria phylogeny: The final concatenated alignment comprised four isolates within the genus *Hirudinaria* and *Distocercosporaster dioscoreae* (isolate CPC 11513) which was used as outgroup. The final alignment contained a total of 2 480 characters divided in four partitions containing 733 (LSU), 482 (*rpb2*), 683 (ITS) and 582 (*tef1*) characters, including alignment gaps. Based on the results of MrModeltest, dirichlet base frequencies and the GTR+I+G model was used for LSU, *rpb2* and ITS loci and HKY+G model was used for *tef1*. The Bayesian analysis generated 12 trees after 5 000 generations from which 10 trees were sampled after 25 % of them were discarded as burn-in and were used for calculating posterior probabilities (PP) in the majority rule consensus tree (Fig. 2). The alignment contained a total of 109 unique site patterns (10, 19, 59 and 21 for LSU, *rpb2*, ITS and *tef1*, respectively).

Taxonomy

Hirudinaria Ces., *Hedwigia* **1**(15): 104, Tab. XIV, G, 1–3. 1856.
Synonym: *Hippocrepidium* Sacc., *Bull. Soc. Imp. Naturalistes Moscou*: no. 85, 1875, *fide* Hughes 1951.

Classification: *Ascomycota*, *Pezizomycotina*, *Dothideomycetes*, *Dothideomycetidae*, *Mycosphaerellales*, *Mycosphaerellaceae*.

Phytopathogenic, causing leaf spots. *Stromata* absent. *Mycelium* mostly external and superficial. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* arising from external hyphae, macronematous or semi-macronematous, solitary, not branched, slightly swollen, hyaline to pale brown, monoblastic or polyblastic with broad, dark brown loci. *Conidia* solitary, brown to dark brown, V-shaped or U-shaped, with two arms (rarely three arms), thick-walled, smooth, rugulose or verrucose, septate.

Table 1. List of fungal isolates included in this study and their corresponding GenBank accession numbers.

Species	Culture accession number(s)[1]	Host	GenBank accession numbers[2]			
			LSU	ITS	*rpb2*	*tef1*
Hirudinaria macrocarpa	IRAN 4547C = CBS 150858 (T)	*Crataegus* sp.	OR785993	OR785997	_	OR790970
	IRAN 4548C	*Crataegus* sp.	OR785994	OR785998	_	OR790971
Hirudinaria mespili	IRAN 4550C = CBS 150859 (T)	*Mespilus germanica*	OR785996	OR786000	OR790975	OR790973
	IRAN 4549C	*Mespilus germanica*	OR785995	OR785999	OR790974	OR790972

[1] CBS: Culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; IRAN: Culture collection of the Iranian Research Institute of Plant Protection, Tehran, Iran.

[2] LSU: Large subunit of the nrDNA, ITS: internal transcribed spacers and intervening 5.8S nrDNA, *rpb2*: partial RNA polymerase II gene, *tef1*: translation elongation factor 1-alpha.

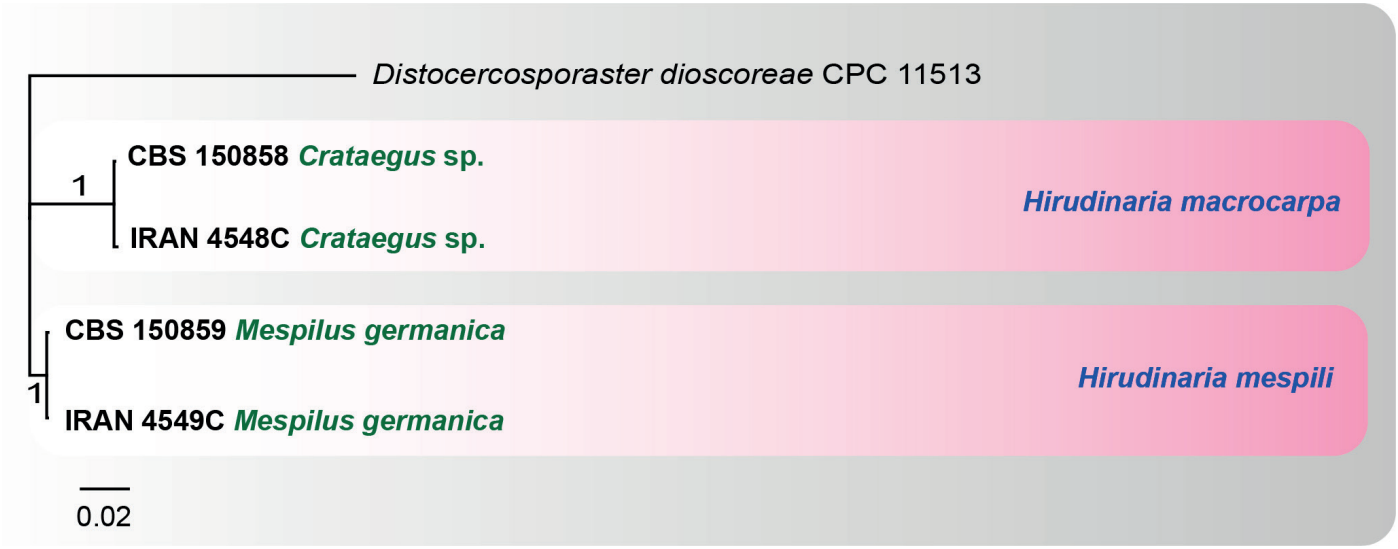

Fig. 2. Bayesian phylogram constructed from LSU, *rpb2*, ITS and *tef1* sequences of *Hirudinaria* spp. The phylogenetic tree was rooted to *Distocercosporaster dioscoreae* (CPC 11513). The scale bar indicates 0.02 expected changes per site.

Type species: *Hirudinaria mespili* Ces., lectotype *fide* Clements & Shear (1931)

Hirudinaria mespili Ces., *Hedwigia* **1**(15): 104, Tab. XIV, G, 3. 1856. Fig. 3.

Leaf spots hypophyllous, subcircular to circular, dark blackish brown to black, velvety, 2–10 mm diam., without forming chlorosis or necrosis. *Caespituli* hypophyllous, velvety. *Stromata* lacking. *Mycelium* mostly external and superficial; consisting of branched, septate, hyaline to pale brown hyphae, 1.5–2.5(–3) µm wide, smooth or somewhat verrucose. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* arising from external hyphae, intercalary, lateral or terminal, macronematous or semi-macronematous, solitary, not branched, slightly swollen, hyaline to pale brown, usually monoblastic or polyblastic, 6–7(–9) × (3–)4.5–5 µm, loci broad, dark brown, 2–3 µm diam. *Conidia* solitary, brown to dark brown, V-shaped or U-shaped, with two arms (rarely three arms), straight or curved, thick-walled, smooth, rugulose or verrucose, (4–)8–12(–18)-septate, tapering gradually to the paler, rounded apex, (20–)55–80(–110) × (2–)4–7(–8) µm.

Culture characteristics: Colonies on MEA, surface raised, folded, dirty white, with fluffy aerial mycelium and entire margin, reverse dark grey, reaching 11 mm after 20 d at 25 °C.

Typus: **Switzerland**, Tessin, Lugano ['Ad Ceresium lacum (L. di Lugano) in Helvetia insubrica'] on *Mespilus germanica* (*Rosaceae*), Oct. 1848 [Rabenh., Klotzschii Herb. Viv. Mycol., Ed. Nova, Cent. 3: no. 269] (HAL, s.n.) – **lectotype**, designated here, MBT 10016603. **Iran**, Mazandaran Province, Amol, N36°33'30", E52°18'30", on *Mespilus germanica*, 13 Oct. 2017, *M. Bakhshi* (IRAN 18303F **– epitype** designated here**,** MBT 10016604, IRAN 4550C = CBS 150859 – ex-epitype culture).

Additional material examined: **Iran**, Mazandaran Province, Chamestan, Lavig forest, N36°25'27", E52°03'43", on the lower surfaces of the leaves of *M. germanica*, 12 Oct. 2017, *M. Bakhshi* (IRAN 18304F, culture IRAN 4549C).

Notes: The genus *Hirudinaria* was introduced by Cesati (1856), and *H. mespili* was designated as type species by Clements & Shear (1931). Based on a megablast search of NCBI's GenBank nucleotide database, the closest hits using the ITS sequence had highest similarity to *Pseudocercosporella magnusiana* [isolate CBS 114735, GenBank KF251327; identities = 463/471 (98 %), 1 gap (0 %)], *Cercospora eucommiae* [isolate CBS 131932, GenBank MH866054; identities = 466/477 (98 %), 2 gaps (0 %)], and *Sphaerulina azaleae* [isolate KACC44865, GenBank MK578200; identities = 466/478 (97 %), 4 gaps (0 %)]. Closest hits using the LSU sequence are *Septoria hibiscicola* [isolate CBS 128615, GenBank MH876482; identities = 725/728 (99 %), no gaps], *Septoria abei* [isolate CBS 128598, GenBank KF251837; identities = 725/728 (99 %), no gaps], and *Acericercospora hyrcanica* [as *Mycosphaerellaceae* sp.; isolate IRAN 4553C, GenBank ON212671; identities = 723/728 (99 %), no gaps]. Closest hits using the *rpb2* sequence are *Neopseudocercosporella capsellae* [isolate MAFF 237605, GenBank MF951550; identities = 557/697 (80 %), 6 gaps (0 %)], *Apseudocercosporella trigonotidis* [isolate CPC 10865, GenBank KX288413; identities = 534/666 (80 %), 2 gaps (0 %)], and *Sphaerulina tirolensis* [isolate CBS 109018, GenBank MF951680; identities = 575/731 (79 %), 12 gaps (1 %)]. Closest hits using the *tef1* sequence are *Lecanosticta brevispora* [isolate CMW50526, GenBank MK015434; identities = 126/135 (93 %), no gaps], *Nowamyces globulus* [isolate CBS 144601, GenBank MN162343; identities = 114/118 (97 %), no gaps], and *Septoria linicola* [isolate SE15195, GenBank CP099418; identities = 126/136 (93 %), no gaps].

Cesati (1856) introduced the name *Hirudinaria mespili*, and described it almost simultaneously in Rabenhorst, Klotzschii Herb. Viv. Mycol., Ed. Nova, Cent. 3: no. 269, 1856, in Bot. Zeitung **14**: 445. 1856, and in Flora **39**: 377. 1856 (see Braun 2018: 9). The specimens distributed in Rabenh., Herb. Viv. Mycol. 269 represents syntypes that must be used for a lectotypification.

Hirudinaria macrocarpa Ces., *Hedwigia* **1**(15): 104, Tab. XIV, G, 1–2. 1856. Fig. 4.
Synonym: *Hirudinaria macrospora* Ces., In Rabenhorst, *Klotzschii Herb. Viv. Mycol.*, Edn Nov, Ser. Sec., Cent. **3**: no. 269. 1856; *Bot. Zeitung* **14**: 445. 1856; and *Flora* **39**: 377 1856.

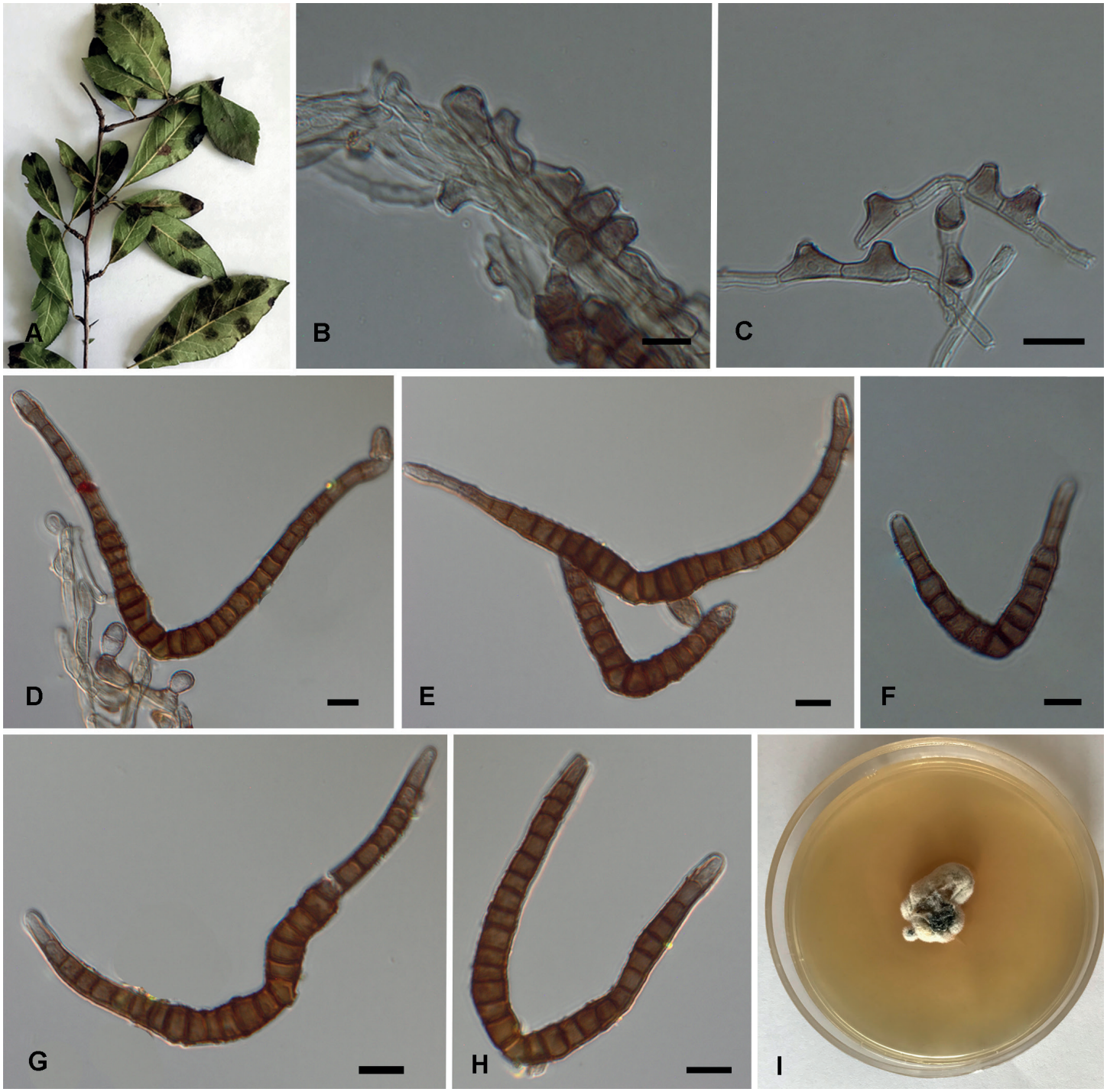

Fig. 3. *Hirudinaria mespili* CBS 150859.. **A**. Leaf spot symptoms on *Mespilus germanica*. **B–H**. Hypha, conidiophores and conidia. **I**. Culture on MEA. Scale bars = 10 µm.

Leaf spots hypophyllous, subcircular to circular, dark blackish brown, velvety, 2–7 mm diam., without forming chlorosis or necrosis, which might be due to the semibiotrophic nature of the fungus. *Caespituli* hypophyllous, velvety. *Stromata* lacking. *Mycelium* mostly external and superficial; consisting of branched, septate, hyaline to pale brown hyphae, 1.5–2.5(–3) µm wide, smooth or somewhat verrucose. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* arising from external hyphae, intercalary, macronematous or semi-macronematous, solitary, not branched, slightly swollen, hyaline to pale brown, usually monoblastic but rarely polyblastic, 6–10(–15) × (4–)5–8 µm, loci broad, dark brown, 2–3 µm diam. *Conidia* solitary, brown to dark brown, V-shaped or U-shaped, with two arms, straight or curved, thick-walled, smooth, rugulose or verrucose, (1–)6–10(–13)-septate, tapering gradually to the paler, rounded apex, (10–)45–60(–80) × (3–)7–9(–10) µm.

Culture characteristics: Colonies on MEA, surface raised, folded, dirty white to grey olivaceous, feathery, with sparce aerial mycelium and lobate margin, reverse iron grey, reaching 7 mm after 20 d at 25 °C.

Typus: **Italy**, Piemont, Prov. Asti, Villafranca de'Asti [prope Villafranca de'Asti (Montisferr., Pedem. provinc.], on *Crataegus* sp. (*Rosaceae*), Oct. 1855 [Rabenh., Klotzschii Herb. Viv. Mycol., Ed. Nova, Cent. 3: no. 269] (HAL s.n.) – **lectotype**, designated here, MBT 10016605. **Iran**,

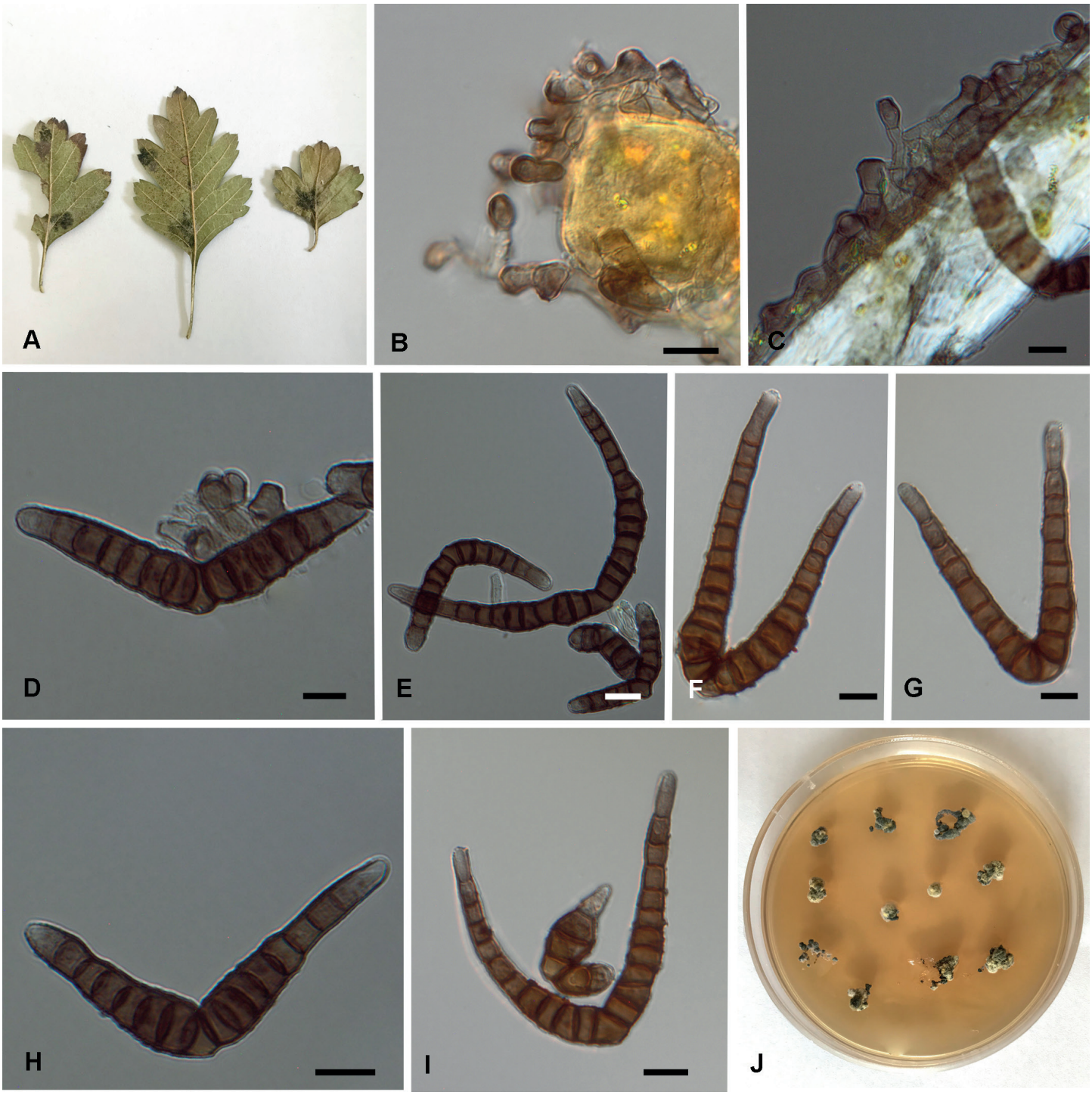

Fig. 4. *Hirudinaria macrocarpa* CBS 150858. **A**. Leaf spot symptoms on *Crataegus sp.* **B–I.** Hypha, conidiophores and conidia. **J.** Culture on MEA. Scale bars = 10 µm.

Mazandaran Province, Amol, Sorkhkola, on *Crataegus* sp., 10 Oct. 2017, *M. Bakhshi* (IRAN 18305F – **epitype** designated here, MBT 10016606, IRAN 4547C = CBS 150858 – ex-epitype culture).

Additional material examined: **Iran**, Mazandaran Province, Amol, Sorkhkola, on the lower surfaces of the leaves of *Crataegus* sp., 10 Oct. 2017, *M. Bakhshi* (IRAN 18306F, culture IRAN 4548C).

Notes: Based on a megablast search of NCBI's GenBank nucleotide database, the closest hits using the ITS sequence had highest similarity to *Pseudocercosporella magnusiana* [isolate CBS 114735, GenBank KF251327; identities = 458/471 (97 %), 1 gap (0 %)], *Sphaerulina azaleae* [isolate KACC44865, GenBank MK578200; identities = 461/477 (97 %), 2 gaps (0 %)] and *Cercospora eucommiae* [isolate CBS 131932, GenBank MH866054; identities = 461/477 (97 %), 2 gaps (0 %)]. Closest hits using the LSU sequence are *Septoria hibiscicola* [isolate CBS 128615, GenBank MH876482; identities = 724/728 (99 %), no gaps], *Septoria abei* [isolate CBS 128598, GenBank KF251837; identities = 724/728 (99 %), no gaps], and *Septoria anthurii* [isolate CBS 148.41, GenBank NG_069641; identities = 722/728 (99 %), no gaps]. Closest hits using the *tef1* sequence are *Lecanosticta brevispora* [isolate CMW50526, GenBank MK015434; Identities = 126/135 (93 %), no gaps], *Nowamyces globulus* [isolate CBS 144601, GenBank MN162343; identities = 114/118 (97 %), no gaps], and *Periconia cyperacearum* [isolate CPC 32138, GenBank MH327882; identities = 136/152 (89 %), 1 gap (0 %)].

Cesati (1856) introduced the name *Hirudinaria macrocarpa* and described this species almost simultaneously under the alternative name *H. macrospora* in Rabenhorst, Klotzschii Herb. Viv. Mycol., Edn Nov, Ser. Sec., Cent. **3**: no. 269. 1856, in Bot. Zeitung **14**: 445. 1856, and in Flora **39**: 377 1856 (see Braun 2018: 9). The specimens distributed in Rabenhorst, Klotzschii Herb. Viv. Mycol. 269 are syntypes for both names that have to be used for a lectotypification.

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Conflict of interest: The authors declare that there is no conflict of interest.

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