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New host record of *Heterosphaeria linariae* (Heterosphaeriaceae, Helotiales) from *Peucedanum cervaria* in Italy

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Abstract

Heterosphaeria (Helotiales) comprises ten species characterized by apothecial fruiting bodies with thick-walled tissues of interwoven excipulum, cartilaginous hyphae covering the external part of the cortex and dark-coloured cells. A species of *Heterosphaeria* was collected from a dead stem of *Peucedanum cervaria* in Italy. Phylogenetic analysis based on combined gene regions of the internal transcribed spacer (ITS) and the large subunit of nuclear ribosomal RNA (LSU) sequence dataset along with the morphological characteristics confirmed that our isolate is *Heterosphaeria linariae*. Here, we report *H. linariae* as a new host record from *Peucedanum cervaria* in Italy. A detailed description and illustrations of the sexual and asexual morph of *H. linariae* and an updated phylogenetic tree for *Heterosphaeria* are provided.

Key words – Ascomata – asexual morph – Helotiales – phylogeny – taxonomy

Introduction

Heterosphaeria is the only genus in Heterosphaeriaceae (Helotiales, Leotiomycetes). It was introduced by Greville (1824) and is typified by *Heterosphaeria patella* (Wijayawardene et al. 2017, 2018). This species is mainly found on dried stems of Apiaceae and previously placed within *Sphaeria* (Tode 1791). However, *Heterosphaeria* is distinct from *Sphaeria* in having globose apothecia when immature, becoming patelliform and depressed at maturity with a coriaceous excipulum (Leuchtmann 1987). Leuchtmann (1987) accepted eight species in *Heterosphaeria*: *H. alpestris*, *H. intermedia*, *H. lojkae, H. ovispora*, *H. patella*, *H. pulsatillae, H. veratri* and *H. viriditingens*.

Asexual morph of *Heterosphaeria* that belonged to *Heteropatella* Fuckel. was discovered by Tulasne & Tulasne (1865) (Leuchtmann 1987). Then, Gremmen (1970) confirmed the connection between sexual and asexual morphs based on cultural studies. Grove (1937) linked *Heteropatella lacera* as the asexual morph of *Heterosphaeria linariae*, while Leuchtmann (1987) linked *Heterosphaeria patella* with *Heteropatella lacera*. Johnston et al. (2014) recommended the use of *Heterosphaeria* rather than *Heteropatella.* Through molecular data, Ekanayaka et al. (2019) confirmed the link between the sexual and asexual stages. Ekanayaka et al. (2019) and Li et al. (2020) confirmed *Heteropatella lacera* as the asexual morph of *Heterosphaeria linariae*.

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The host plant, *Peucedanum cervaria* (Apiaceae) belongs to a polyphyletic genus comprising more than 120 epithets. It is found widely in Africa, Asia, Europe and North America (Pimenov & Leonov 1993). However, only 69 species were accepted, and many names regarded as synonyms or unverified (Sarkhail 2014). External and anatomical features have been used to classify species in Apiaceae. *Peucedanum* spp. are identified using flattened orthospermus fruits with more and less developed lateral wings, a broad commissure and the lack of prominent dorsal ribs (Drude 1897). *Peucedanum* species produce many essential oils, flavonoids, phenolics and coumarins that are used in folk medicine for the treatment of coughs, cramps, pain, rheumatism, asthma and angina (Sarkhail 2014). *Peucedanum cervaria* is a herbaceous plant used as an expectorant, diaphoretic, diuretic, stomachic, sedative and antimicrobial agent (Skalicka-Wozniak et al. 2007).

In this study, we report a new host record of *Heterosphaeria linariae* collected from *Peucedanum cervaria* in Italy. Identification of the fungus is based on both morphological characteristics and molecular phylogenetic data.

Materials & Methods

Fungal isolation and morphology

A specimen belonging to *Helotiales* on *Peucedanum cervaria* was collected in Italy in 2017. The specimen was examined using a Motic SMZ-168 stereomicroscope. Sections of the fruiting bodies were mounted in water and preserved in lacto-glycerol for the examination of morphological characters. The photographs were taken with a Canon EOS 600D digital camera attached to a Nikon Ni compound microscope. Measurements were taken using Tarosoft (R) Image Frame Work program v. 0.9.7. The specimen was deposited in the herbarium of Mae Fah Luang University (MFLU), Chiang Rai, Thailand. The photo-plate was made using Adobe Photoshop CS6 Extended version 13.0 × 64 (Adobe Systems, USA).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted directly from the ascomata using the Forensic DNA Kit-D3591-01 (OMEGA bio-tek). The internal transcribed spacer (ITS), the large subunit of nuclear ribosomal RNA (LSU), translation elongation factor 1-alpha (TEF1-α) and RNA polymerase II (RPB2) gene regions were amplified using primer pairs ITS5/ITS4 (White et al. 1990), LROR/LR5 (Vilgalys & Hester 1990), TEF1-728F/TEF1-986R and fRPB2-5F/fRPB2-7cR (Carbone & Kohn 1999, Liu et al. 1999), respectively. The total volume of PCR mixtures was 25 µl, containing 8.5 µl ddH_2O, 12.5 µl 2× Easy Taq PCR SuperMix (a mixture of Easy Taq TM DNA Polymerase, dNTPs, and optimized buffer, Beijing Trans Gen Biotech Co., Beijing, PR China), 2 µl of DNA template, and 1 µl of each forward and reverse primers (10 pM). PCR amplification conditions for all regions consisted of an initial denaturation step of 5 min at 94°C, 35 cycles of denaturation at 94°C for 1 minute, annealing at 53°C for 50 seconds and elongation at 72°C for 3 minutes and a final extension step of 10 minutes at 72°C. The quality of PCR products was checked on 1% agarose gels stained with ethidium bromide. The PCR products were sequenced at the Sangon Biotech, Shanghai, China.

Phylogenetic analyses

The consensus sequences were assembled in Geneious Prime v2019.1.1. Sequences of available *Heterosphaeria* species were analyzed (Table 1) using the nucleotide BLASTn search in GenBank and data obtained from Li et al. (2020). *Mycochaetophora gentianae* (MAFF 239231) and *Oculimacula acuformis* (CBS 495.80) (Ploettnerulaceae, Helotiales) were used as outgroup taxa. The datasets were compiled with BioEdit v.7.2.5 (Hall 2004), manually trimmed and aligned with MAFFT v. 7 using default settings (http://mafft.cbrc.jp/alignment/server; Katoh et al. 2017). The individual gene alignments were analyzed separately by comparing any conflicts of the tree. The LSU and ITS datasets were combined to construct the phylogenetic tree. The phylogeny

website tool “ALTER” (Glez-Peña et al. 2010) was used to convert the alignment file for maximum likelihood (ML), maximum parsimony (MP) and Bayesian analyses formats.

Reconstruction of the ML analysis was performed via RAxML v.8 on the CIPRES web portal (Stamatakis et al. 2014) as part of the “RAxML-HPC2 on XSEDE 8.2.10 on TG tool” (http://www.phylo.org/potal2/; Miller et al. 2010). RAxML rapid bootstrapping and subsequent ML search used distinct model/data partitions with joint branch length optimization executing 1,000 rapid bootstrap inferences and thereafter a thorough ML search. All tree model parameters were estimated by RAxML and ML estimate of 25 per site rate categories. Likelihood of the final tree was evaluated and optimized under GTR+GAMMA. GAMMA model parameters were estimated to an accuracy of 0.1000000000 log-likelihood units. Every 100th tree was saved. Phylogenetic trees were illustrated using FigTree v1.4.0 program (Rambaut 2014) and Adobe Illustrator CS3 (Adobe Systems, USA).

The Bayesian command was generated using Fabox 1.41 (Villesen 2007). The best-fit model has resulted as TrNef+I for the LSU gene and K80+I for the ITS gene using MrModeltest v.2.3 (Nylander et al. 2008). The Bayesian posterior probability analysis was performed using MrBayes 3.2.6 run on XSEDE at the CIPRES web portal (Ronquist & Huelsenbeck 2003), using the parameter settings of two parallel runs, four chains, run for 1,000,000 generations, and sample frequency at every 100th generations. The first 1,000 trees, representing the burn-in phase of the analyses, were discarded and the remaining 9,000 trees (post burn-in) were used for calculating posterior probabilities (PP) in the majority rule consensus tree (critical value for the topological convergence diagnostic set to 0.01). Bayesian posterior probabilities (BYPP) values greater than 0.95 were accepted.

Maximum parsimony (MP) was performed in PAUP v. 4.0b10 (Swofford 2002) using 1,000 replicates of heuristic search with random addition of sequences and subsequent TBR branch swapping. Ambiguous regions in the alignment were excluded and gaps were treated as missing data. The stability of the trees evaluated by 1,000 bootstrap replications. Descriptive tree statistics such as tree length (TL), consistency index (CI), retention index (RI), relative consistency index (RC), and homoplasy index (HI) were calculated.

Results

Phylogenetic analyses

Newly generated sequences were searched using blast tool in GenBank to find closely related taxa, and the results indicated that our isolate belongs to Heterosphaeriaceae. Fifteen strains were included in the combined multi-locus tree. The final alignment comprised a total of 1322 characters (LSU: 842; ITS: 480).

The RAxML analysis of the combined dataset yielded the best-scoring tree (Fig. 1) with a final optimization likelihood value of -2685.257528. The matrix had 116 distinct alignment patterns, with 6.44% of undetermined characters or gaps. Parameters for the GAMMA+P model of the combined LSU and ITS were estimated base frequencies: A = 0.243709, C = 0.221122, G = 0.286559, T = 0.248610; substitution rates AC = 1.122036, AG = 1.486483, AT = 0.737894, CG = 0.213630, CT = 4.317528, GT = 1.000000; and gamma distribution shape parameter α = 0.835562.

The maximum parsimonious dataset consisted of 1,203 constant, 78 parsimony-informative and 41 parsimony-uninformative characters. The parsimony analysis of the data matrix resulted in one most parsimonious tree with the length of 150 steps (CI = 0.927, RI = 0.908, RC = 0.841, HI = 0.073) in the first tree. Bayesian posterior probabilities (BY) were evaluated with the final average standard deviation of split frequencies = 0.004574. Tree topologies (generated under MP and Bayesian criteria) generated from single-gene datasets were compared, and the overall tree topology was congruent to those obtained from the ML tree of the combined dataset (Fig. 1). The phylogeny showed that our isolate (MFLU 17-1693) grouped with other *H. linariae* strains with moderate statistical support (50% ML) (Fig. 1).

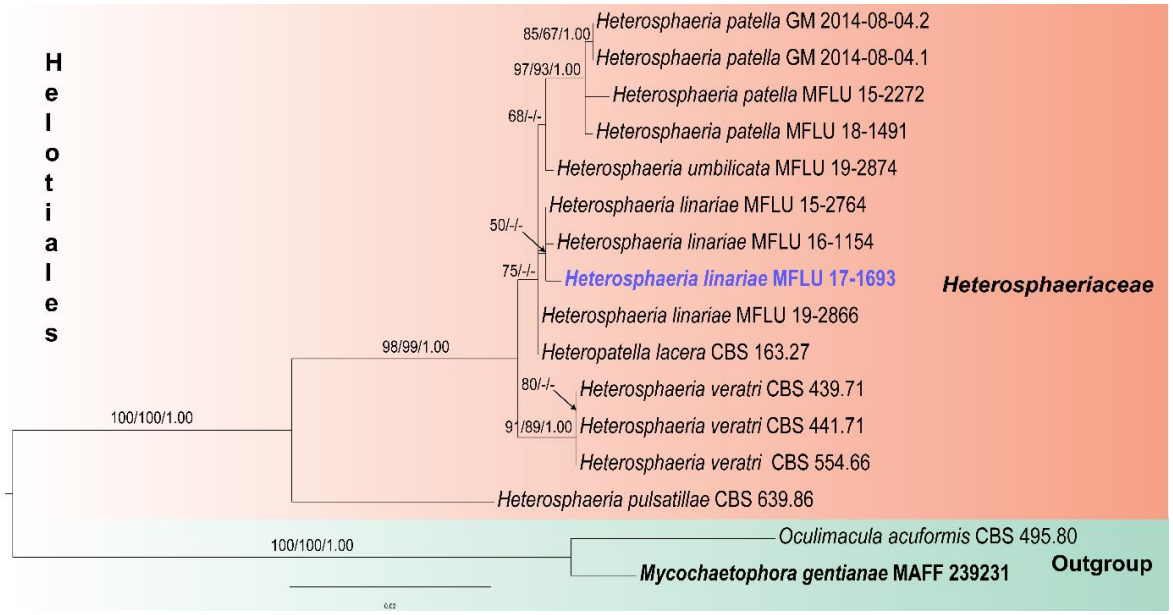

Fig. 1 – Phylogram generated from maximum likelihood analysis based on combined LSU and ITS sequence data for *Heterosphaeria* species. Bootstrap values for maximum likelihood (ML) greater than 50% and Bayesian posterior probabilities (BYPP) equal or greater than 0.95 are given at the nodes. The newly generated sequences are indicated in blue bold.

Heterosphaeria linariae (Rabenh.) Rehm, Rabenh. Krypt.-Fl., Edn 2 1.3(life. 30): 203 (1888) [1896] Fig. 2

Index Fungorum number: IF186485; Facesoffungi number: FoF05874

Saprobic on dead stem of *Peucedanum cervaria*. Sexual morph: *Apothecia* 255–500 × 200–400 µm ($\bar{x}$ = 388 × 258 µm, n = 10), black-brown, solitary or gregarious, scattered on wood, superficial, sessile, unilocular, glabrous, rugose. *Receptacle* rough surface, deep cupulate. *Disc* concave, black. *Margin* enclosed the hymenium at the beginning, become ruptured at maturity, slightly curved, black-brown. *Ectal excipulum* 25–60 µm ($\bar{x}$ = 45 µm, n = 10) in the vertical section, thick-walled, brown to black cells of *textura angularis. Medullary excipulum* 30–70 µm ($\bar{x}$ = 45 µm, n = 10) composed of thick-walled, cartilaginous, hyaline cells of *textura intricata* to *textura epidermoidea. Hymenium* hyaline, inner mixed with asci and paraphyses. *Paraphyses* 2–3 µm wide ($\bar{x}$ = 2.5 µm, n = 20), hyaline, filiform, slightly swollen at the apex, septate, unbranched, thin and smooth-walled, exceeding the asci. *Asci* 60–85 × 9–15 µm ($\bar{x}$ = 75 × 12 µm, n = 20), hyaline, 8-spored, unitunicate, cylindrical-clavate, trapezoidal at apex, sessile, thick-walled, amyloid ring with Lugol's reagent. *Ascospores* 10–15 × 4–6 µm ($\bar{x}$ = 12 × 5 µm, n = 20), hyaline, biseriate, ellipsoidal to inequilateral, 0–1-septate, straight or slightly curved, thick-walled, several small guttules in immature spores, a large guttule at each end in mature spores. Asexual morph: *Conidiomata* 255–500 × 200–400 µm ($\bar{x}$ = 388 × 258 µm, n = 10), black-brown, pycnidial, solitary or gregarious, superficial, globose to subglobose, unilocular, sessile, glabrous. *Conidiomatal wall* composed of the outer layer 25–60 µm ($\bar{x}$ = 45 µm, n = 10), thick-walled, brown to black cells of *textura angularis*, and an inner layer 30–70 µm ($\bar{x}$ = 45 µm, n = 10) thick-walled, hyaline cells of *textura intricata* to *textura epidermoidea*. *Conidiophores* hyaline, cylindrical, septate, branched, arising from the inner layer of pycnidium. *Conidiogenous cells* hyaline, holoblastic, sympodial, indeterminate, cylindrical, slightly wider at apex, with several apical conidiogenous loci, smooth-walled. *Conidia* 35–50 × 3–5 µm ($\bar{x}$ = 42 × 3.5 µm, n = 20), hyaline, long-fusiform to filiform or lunate, 2–3-septate, slightly constricted at the septa, guttulate, bearing a filiform, unbranched, attenuated appendage at each end.

Material examined – ITALY. Forlì-Cesena Province: Balze di Verghereto, on dead stem of *Peucedanum cervaria* (*Apiaceae*), 19 September 2017, Erio Camporesi, IT3491 (MFLU 17-1693, new host record).

GenBank numbers – ITS: MK875979, LSU: MK875978, RPB2: MN557394, EF1α: MN557395.

Notes – In the phylogenetic analyses, our strain grouped sister to *H. linariae* (MFLU 16-1154) with moderate statistical support (50% ML; Fig.1). However, the morphology of our strain is very similar to *H. linariae* in the heteromorph. Our strain MFLU 17-1693 differs from the type species in having larger, ellipsoidal ascospores (10–15 × 4–6 vs 9–14 × 3–4), that are often 1-septate with large guttules at each end. The type of *Heterosphaeria linariae* has oblong, aseptate ascospores (Rehm 1896). The ectal excipulum of our strain is of *textura angularis* and thicker than that of *H. linariae* (MFLU 15-2764) (25–60 vs 17–22 µm). The medullary excipulum of our strain is composed of *textura intricata* to *epidermoidea*, but *H. linariae* (MFLU 15-2764) has *textura porrecta* and is thinner than our strain (40–47 vs 30–70 µm) (Ekanayaka et al. 2019). Our strain also produces both conidia and ascospores in the same ascomata. We compared the asexual morph characters of *H. linariae* (MFLU 17-1693) with *Heteropatella lacera* (= *H. linariae*). The morphology of conidia is similar to the other strains by being hyaline fusiform, lunate, 3–4-septate, and guttulate with both apical and basal appendages. However, the conidia of our strain are larger than those of *Heteropatella lacera* in Leuchtmann (1987) (35–50 × 3–5 vs 16–24 × 2–3 µm).

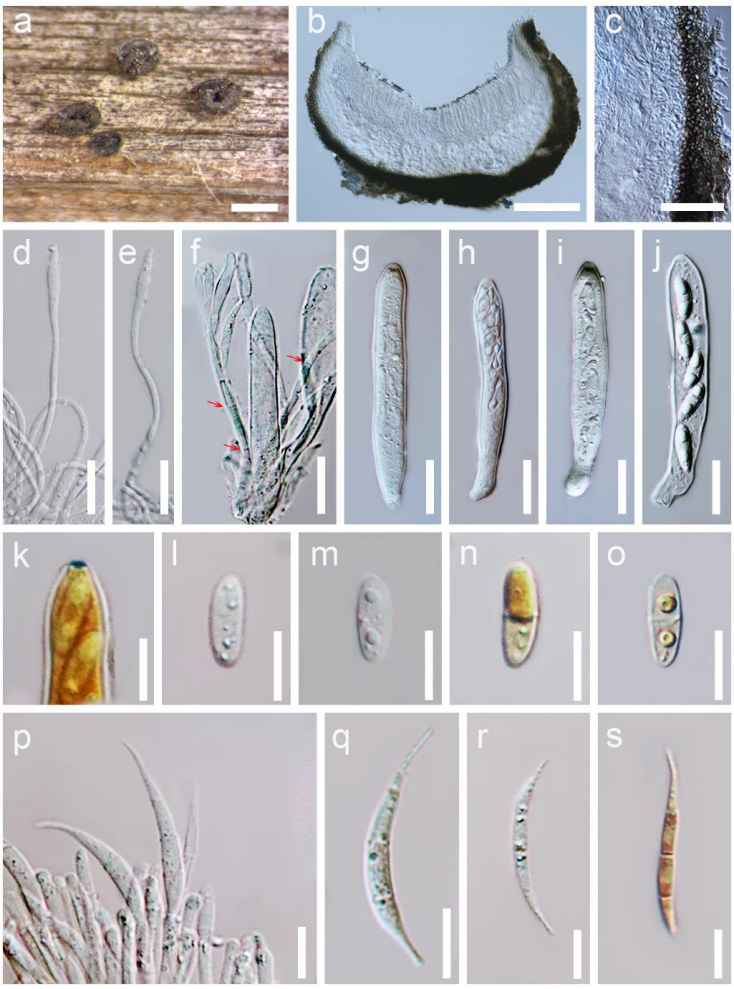

Fig. 2 – *Heterosphaeria linariae* (MFLU 17-1693, new host record). a Apothecia on *Peucedanum*. b Cross section of apothecium. c Apothecium wall. d, e Filiform paraphyses swollen at the apex. f Septate paraphyses (arrows). g–j Cylindric-clavate asci. k Apical part of ascus in Lugol's reagent. l–o Ellipsoid ascospores (n, o in Lugol's reagent). p Conidiogenous cells and developing conidia. q–s Lunate fusiform conidia (s in Lugol's reagent). Scale bars: a = 400 µm, b = 50 µm, c = 20 µm, d–j = 20 µm, k–s = 10 µm.

The sequences of our collection were obtained by direct DNA extraction. The LSU sequence of our strain (MFLU 17-1693) is 99–100% similar to the LSU sequence of other *H. linariae* isolates (MFLU 15-2764, MFLU 16-1154, MFLU 19-2866, CBS 163.27). The ITS sequence of our strain showed 99% similarity to the sequences of other isolates (MFLU 15-2764, MFLU 16-1154 and CBS 163.27). This is the first report of *H. linariae* on *Peucedanum cervaria*.

Table 1 Taxa used in the phylogenetic analyses and their GenBank accession numbers. The new host record of *Heterosphaeria linariae*, GenBank accession numbers are indicated in bold. Ex-type strain is indicated in ^{T}

Taxa	Isolate no.	Country	GenBank Accession no.	
			ITS	LSU
Heterosphaeria linariae	**MFLU 17-1693**	**Italy**	**MK875979**	**MK875978**
H. linariae	MFLU 19-2866	Italy	-	MT183474
H. linariae	MFLU 16-1154	Italy	MT185512	MT183475
H. linariae	MFLU 15-2764	Russia	MK585000	MK591955
H. patella	GM 2014-08-04-1	Luxembourg	MF196187	MF196187
H. patella	GM 2014-08-04-1	Luxembourg	KY462821	KY462821
H. patella	MFLU 18-1491	Italy	MN860230	MN860231
H. patella	MFLU 15-2272	Italy	MT185513	MT183476
H. pulsatillae	CBS 639.86	Switzerland	MH862006	–
H. umbilicata	MFLU 19-2874	Italy	MT185514	MT183477
H. veratri	CBS 439.71	Czechoslovakia	MH860207	MH871975
H. veratri	CBS 554.66	France	–	MH870540
H. veratri	CBS 441.71	Slovakia	MH860209	MH871976
Heteropatella lacera	CBS 163.27	United Kingdom	MH854915	MH866409
Mycochaetophora gentianae ^{T}	MAFF 239231^{T}	Japan	NR_121201 ^{T}	AB496937 ^{T}
Oculimacula acuformis	CBS 495.80	Germany	MH861289	MH873054

Discussion

Heterosphaeria can be distinguished from other helotialean genera by the apothecial characters and host plant (Leuchtmann 1987). The species in this genus are all very similar, and it is difficult to distinguish them based on morphology alone. The asexual morph of *Heterosphaeria* is *Heteropatella.* Many asexual morphs have been renamed in *Heterosphaeria.*

When the LSU sequence of *Heterosphaeria pulsatillae* was searched against the blastn tool in GenBank, it did not show any close matches to any of the available *Heterosphaeria* species. Therefore, it is unlikely to belong to this genus. Thus, there is taxonomic confusion in *Heterosphaeria* due to varied gene data available in the GenBank. We compared our isolate with the isolates from a recent study (Li et al. 2020). In both studies, the tree topologies were similar. Our strain of *Heterosphaeria linairae* clustered with the other strains of *H. linariae* with a moderate bootstrap support (50% ML). *Heterosphaeria* is recorded mostly from Europe, and our strain was found in Italy. With the recent introductions, ten species were accepted for *Heterosphaeria* including *H. linairae* and *H. umbilicata*, but sequence data are available for only five species. Fresh collections of *Heterosphaeria* from different geographical regions and sequence data from different genetic markers are needed to understand the species limits of the genus.

Our finding together with previous research findings show that *H. linariae* has a wide host range. Collecting more samples from potential plant hosts of *H. linariae* from a wider geographical range is recommended for future research studies. This is the first record of *H. linariae* from *Peucedanum cervaria.*

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