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FULL PAPER

Phylogenetic affinity of *Mycochaetophora gentianae*, the causal fungus of brown leaf spot on gentian (*Gentiana triflora*), to *Pseudocercosporella*-like hyphomycetes in Helotiales

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Abstract *Mycochaetophora gentianae*, the causal agent of brown leaf spot on gentian (*Gentiana scabra*), is characterized by its hyaline besom-like sporophore, although its conidiogenesis and phylogenetic position have so far remained unknown. We isolated the causal fungus from a new host, *G. triflora*, in Iwate, Japan. Both the *G. triflora* isolate and the ex-type *M. gentianae* isolate produced symptoms on *G. triflora* but not on *G. scabra*. Microscopic observations of the diseased leaves indicated that conidiogenesis was blastic from short conidiophores, and schizolytic secession of conidia left unthickened and inconspicuous conidial scars on the conidiogenous cells. Conidia were catenate, in branched acropetalous chains; secondary conidia were blastically produced from the first or second cell at the base of primary conidium. The *G. triflora* isolate was identified as *M. gentianae* because of its identity to the ex-type in characteristics of culture, pathogenicity, and conidia. Phylogenetic analyses using three ribosomal DNA (rDNA) sequences combined [small subunit (SSU) + large subunit (LSU) + 5.8S rDNA] indicated that both isolates clustered with *Rhexocercosporidium carotae*, and the cluster was placed within Helotiales–Rhytismatales. Additional analyses using internal transcribed spacers including 5.8S rDNA sequences revealed that both isolates were monophyletic and that they were closely related to three helotialean *Pseudocercosporella*-like hyphomycetous genera: *Helgardia*, *Rhexocercosporidium* and *Rhynchosporium*.

Keywords Conidiogenesis · *Helgardia* · rDNA sequence · *Rhexocercosporidium* · *Rhynchosporium*

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Introduction

In recent years, gentian (*Gentiana* L.) has become a major ornamental flower in Japan. The popular cultivars in Japan are mainly derived from two closely related species, *G. triflora* Pall. and *G. scabra* Bunge var. *buergeri* (Miq.) Maxim. Moreover, interspecific hybrids between these two species are bred (Yoshiike 1992). Gentian plants are mainly cultivated outdoors; hence, fungal diseases occur during the growing season.

Brown leaf spot disease (*Kappan-byo*, in Japanese) on gentian plants was first observed in Okayama, Japan (Kasuyama and Idei 1987) and then in Iwate (Nakatani and Takahashi 1991) and Fukushima (Horikoshi et al. 2003). Early symptoms are characterized by the formation of minute, grayish white, powdery spots on the upper surface of diseased leaves. The spots progressively enlarge up to approximately 5 mm in diameter, and the surrounding region turns yellowish. Finally, the lesions show brown rot symptom. The disease leads to economic losses in the production of gentian cut flowers because fungicide spray after disease occurrence is ineffective to prevent disease spread.

The taxonomic position of the causal fungus has not been determined since the disease was first reported (Kasuyama and Idei 1987). Recently, Kobayashi et al.

(2009) classified the causal fungus isolated from *G. scabra* in Okayama as a new species of the anamorphic genus *Mycochaetophora*, *M. gentianae* Tak. Kobay., Kasuyama & Nasu. The genus *Mycochaetophora* Hara & Ogawa is characterized by a hyaline besom-like sporophore and contains only two species. The species type *M. japonica* Hara & Ogawa infects Japanese zelkova (*Zelkova serrata* Makino) (Hara 1931). Kirk et al. (2008) stated that the genus *Mycochaetophora* is insufficiently described on conidiogenous events such as ontogeny and secession.

With regard to the taxonomic position of the genus *Mycochaetophora*, Hara (1931) speculated that the genus is related to *Cercosporella* Sacc. *sensu lato* on the basis of morphology. *Cercosporella* and its allied genera were later reclassified based on their morphological characteristics (Deighton 1973; Braun 1995, Braun 1998). Several *Cercosporella* species were classified under the genus *Pseudocercosporella* Deighton due to the presence of unthickened and inconspicuous conidial scars (Deighton 1973; Braun 1995), which includes anamorphs of *Mycosphaerella* Johanson. Later, Braun (1995) recognized a group of 16 genera comprising *Pseudocercosporella* and its allied genera (*Pseudocercosporella*-like hyphomycetes) on the basis of unthickened and inconspicuous conidial scars. Several genera have been accommodated in the Capnodiales, Helotiales, and Pleosporales (Goodwin et al. 2001; Goodwin 2002; Shoemaker et al. 2002; Crous et al. 2003, 2007; Harrington and Mcnew 2003). *M. gentianae* is phylogenetically related to a helotialean anamorph *Cadophora* Lagerb & Melin, although their conidial morphology is quite distinct from each other (Kobayashi et al. 2009).

In this study, we compared the fungal materials from *G. scabra* in Okayama and those from *G. triflora* in Iwate. The aims of this study were to: (1) compare *M. gentianae* isolates from *G. triflora* and from *G. scabra* in terms of cultural, pathogenic, and microscopic characteristics; (2) characterize conidiogenesis of *M. gentianae*; and (3) determine the phylogenetic position of *M. gentianae* using sequences of three ribosomal RNA genes (rDNA) and those of internal transcribed spacer (ITS) regions.

Materials and methods

Isolates

Isolates used in this study and their sources are listed in Table 1. The isolates of MAFF 239231 and MAFF 239284 were obtained from National Institute of Agrobiological Sciences (NIAS) Genebank, Tsukuba, Japan. Isolates J4 (=MAFF 241067) and K18 (=MAFF 241068) were established in September 2006 from lesions of gentian (*G. triflora*) leaves with typical symptoms in Karumai and Joboji, Iwate, respectively. The isolates were stored in a refrigerator (4°C) until use.

Cultural and morphological characteristics

Five-mm plugs were taken from fresh colonies on potato dextrose agar [(PDA): 200 g potato, 20 g dextrose, 15 g agar, 1,000 ml distilled water], and each PDA plate received the plug in the center. Cultures were incubated in

Table 1 List of isolates and accession numbers of DNA sequences for data set of small subunit (SSU) + large subunit (LSU) + 5.8S

Isolates	Taxon	Host plant and locality	DDBJ/GenBank/EMBL accession no.[a]		
			SSU	LSU	ITS[b]
K18 (=MAFF 241068)	*Mycochaetophora* sp.	*Gentiana triflora* var. *japonica*, Karumai, Iwate, Japan[d]	**AB469687**	**AB469686**	**AB469685**
J4 (=MAFF 241067)	*Mycochaetophora* sp.	*Gentiana triflora* var. *japonica*, Joboji, Iwate, Japan[d]	–	–	**AB469684**
MAFF 239231[c]	*Mycochaetophora gentianae*	*Gentiana scabra* var. *buergeri*, Sanyo, Akaiwa, Okayama, Japan[e]	**AB496936**	**AB496937**	AB434661
MAFF 239284	*Mycochaetophora gentianae*	*Gentiana scabra* var. *buergeri*, Sanyo, Akaiwa, Okayama, Japan[e]	–	–	AB434662
CBS 418.65[c]	*Rhexocercosporidium carotae*	*Daucus carota*, Höyland, Norway[f]	AF487897	**AB469688**	AF487895

[a] The sequences determined in this study are in bold

[b] Internal transcribed spacer (ITS) regions include the 5.8S rDNA

[c] Ex-type culture

[d] This study

[e] Described by Kobayashi et al. (2009)

[f] Described by Årsvoll (1965)

the dark at 5–35°C. Two perpendicular diameters of each colony were measured 14 days after inoculation. The experiments were conducted twice with three replicates.

Inoculation of gentian

Two gentian species, *G. triflora* cv. Ashiro-no-aki and *G. scabra* cv. Ashiro-no-sawakaze, were used for inoculation. Plants were cut to about 40 cm long and placed in 300 ml Erlenmeyer flasks containing distilled water. Cultures were grown in potato–carrot liquid medium (20 g potato, 20 g of carrot, 1,000 ml distilled water) at 20°C for 5 days and shaken at 120 rpm on a rotary shaker. Cultures were filtered with gauze to remove mycelia, and conidia were adjusted to about 10^5 conidia/ml. The conidial suspension was sprayed to the plants, and the plants were covered with polyethylene bags for 3 days at 25°C. After removing bags, the plants were subsequently maintained at 25°C for 2 weeks.

Microscopy

To facilitate conidial formation on the lesion, diseased leaves were placed in a moist chamber (plastic box lined with paper towels moistened with sterile, distilled water) at 20°C for 2–7 days. Five-mm-long segments were cut off from the lesions, stained with lactophenol cotton blue for 1 min, and rinsed twice with lactophenol. Conidial morphology was observed under a light microscope (Olympus BX51; Olympus, Tokyo).

Molecular techniques

The isolates of K18, J4, MAFF 239231, and CBS 418.65 (Table 1) were cultured in darkness at 20°C on PDA plates, and mycelia were harvested 14 days after. DNA was extracted from approximately 0.1 g of mycelia using ISOPLANT Kit (Nippon Gene, Tokyo) according to the manufacturer's instructions. Sequence data were generated from three rDNA regions: partial nuclear small subunit (SSU) rDNA, large subunit (LSU) rDNA, and internal transcribed spacer (ITS) regions, including 5.8S rDNA. Primers used for polymerase chain reaction (PCR) were: NS1 and NS4 (White et al. 1990) for SSU rDNA, LR0R and LR7 (Rehner and Samuels 1994) for LSU rDNA, and ITS1 and ITS4 (White et al. 1990) for ITS regions including 5.8S rDNA. Amplification was conducted in 25 µl of PCR mixtures containing 1 µM each of primers, 0.125 U TaKaRa Ex Taq (TaKaRa Bio, Otsu), deoxyribonucleotide triphosphate (dNTP) mixture (2.5 mM each), and Ex Taq reaction buffer (containing 2 mM Mg^{2+}). PCR was carried out as follows: initial denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 1 min; annealing for 1 min at 48.8°C for SSU rDNA, 46.2°C for LSU rDNA, and 61.5°C for ITS; extension at 72°C for 1 min; and final extension at 72°C for 7 min. PCR products were gel-purified using QIAquick gel extraction kit (Qiagen) and sequenced using the ABI PRISM BigDye-terminator v1.1 cycle sequencing kit (Applied Biosystems) according to the manufacturers' protocols. Primers used for sequencing were NS1, NS4, SR3, SR7, SR7R, and SR8R for SSU rDNA; LR0R, LR7, LR3R, LR5, and LR16, for LSU rDNA; and ITS1 and ITS4 for ITS regions including 5.8S rDNA (these primer sequences are available online from the mycology lab of Duke University, http://www.biology.duke.edu/fungi/mycolab/primers.htm). Sequencing reactions were purified using Montage SEQ96 sequencing reaction cleanup kit (Millipore) and run on an ABI PRISM 3130xl genetic analyzer (Applied Biosystems). Sequences were edited with 4Peaks (Freeware; http://4peaks.en.softonic.com/), ATSQ and GENETYX (GENETYX corporation). Sequences were deposited in DNA Data Bank of Japan (DDBJ) (see Table 1).

Phylogenetic analysis

A data set comprising data from three genes, SSU rDNA (950 bp), LSU rDNA (914 bp), and 5.8S rDNA (156 bp), were prepared for higher-level analysis, according to Wang et al. (2006a). Alignments were downloaded from TreeBASE (accession number M2780; Wang et al. 2006b). Sequences of three rDNA segments for the higher-level analysis were used with a data set of 58 taxa, consisting of 55 taxa selected from the downloaded alignments and two *Mycochaetophora* isolate (K18 and MAFF 239231) and an isolate of *Rhexocercosporidium carotae* U. Braun (CBS 418.65), which were sequenced in this study. The outgroup taxa consisted of *Candida albicans* (C.P. Robin) Berkhout, *Neolecta irregularis* (Peck) Korf & J.K. Rogers, and *Saccharomyces cerevisiae* Meyen ex E.C. Hansen.

To elucidate relationships between *Mycochaetophora* isolates and anamorphic fungi such as *Pseudocercosporella*-like hyphomycetes and *Cadophora* (Helotiales), ITS sequences were used for lower-level analysis. The data set contained four *Mycochaetophora* isolates and 26 other taxa selected on the basis of previous phylogenetic trees, including *M. gentianae* and *R. carotae* (Harrington and Mcnew 2003; Kageyama et al. 2008; Kobayashi et al. 2009). ITS sequences were obtained from the DDBJ database. Outgroup taxa included *Sclerotinia sclerotiorum* (Lib.) de Bary and *Scleromitrula shiraiana* (Henn.) S. Imai.

Sequence data for all matrices were aligned with ClustalW (Thompson et al. 1994) included in the MEGA4 (Tamura et al. 2007). Introns were deleted from the alignments, and ambiguously aligned positions were excluded from the data sets before performing analyses.

Alignment gaps were treated as missing data. The alignments were deposited in TreeBASE (http://www.treebase.org/treebase/index.html) under the study number S2485. The phylogenetic trees were constructed by maximum parsimony using the heuristic search (CNI level = 1) option in MEGA4. Bootstrap values were subsequently calculated with 1,000 replications for reliability of the tree topologies.

Results

Cultural characteristics

Both colonies of *G. triflora* isolate K18 and *M. gentianae* MAFF 239231 on PDA were at first white to gray and then turned greenish dark gray. Colonies were later covered with aerial mycelium and had yellowish pigmentation. All cultures produced few conidia on submerged hyphae. No sporulation was observed on aerial hyphae. The isolates K18 and MAFF 239231 were capable of growth at temperatures from 5 to 30°C but did not grow at temperatures above 32.5°C; the optimal temperature for mycelial growth was at about 20°C for both isolates (Fig. 1).

Pathogenicity

In inoculation tests, both *G. triflora* isolate K18 and *M. gentianae* MAFF 239231 failed to produce brown leaf spot symptoms on *G. scabra* cv. Ashiro-no-sawakaze. On the other hand, both isolates developed severe symptoms on *G. triflora* cv. Ashiro-no-aki, with initial small grayish white and powdery spots forming 10 days after inoculation, and typical lesions, with a diameter of approximately 1 mm developing after 20 days (Fig. 2a).

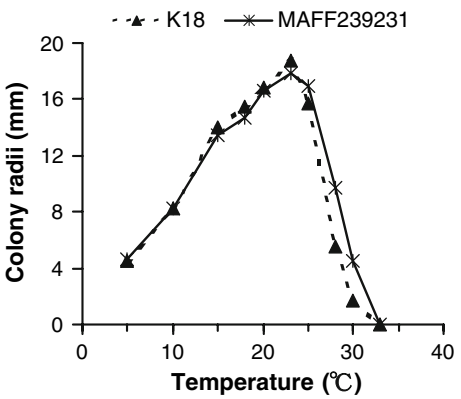

Fig. 1 Effect of temperature on colony growth of *Mycochaetophora gentianae* isolates MAFF 239231 and K18. Colony diameter was determined 14 days after incubation on potato dextrose agar (PDA)

Morphological characteristics

Observations were made on inoculated leaves of *G. triflora* with typical symptoms. *G. triflora* isolate K18 produced besom-like sporophores (Fig. 2b), comprising numerous conidia (Fig. 2c). Sporophores of isolate K18 ranged 40–93 × 20–113 μm in size (Fig. 2h). Conidiophores hyaline, 7–20 × 4–5 μm, unicellular or multicellular, frequently branched in the upper part; conidiogenous cells integrated, terminal or intercalary; conidial scars unthickened and inconspicuous; conidial secession schizolytic (Fig. 2i). Conidia hyaline, 20–60 × 3–5 μm, clavate to cylindrical or subcylindrical, straight, when matured, constricted at septum, 3- to 6-cellular with septa at 6- to 7-μm intervals, apex often rounded, base rounded to truncate, occasionally with remnant tissue of subtending cell, hilum unthickened and colorless (Fig. 2k), often catenate, in branched acropetalous chains (Fig. 2j).

Conidial size was almost identical between *G. triflora* isolate K18 (20–60 × 3–5 μm) and *M. gentianae* MAFF 239231 (19–39 × 3.5–5 μm). Conidial apex of *G. triflora* isolate K18 varied from that of *M. gentianae* MAFF 239231; the uppermost cells of the latter were slightly sharper than that of the former. Based on the morphology of the sporophore, the *G. triflora* isolate was identified as the genus *Mycochaetophora*.

Sporophores developed as follows in isolate K18: conidiophores emerged from submerged mycelia in the leaves (Fig. 2d) and then extended and became 1–2 septate (Fig. 2e); the apical cell was swollen at maturity, seceded by schizolytic secession and then turned into conidia, whereas remaining cells blastically formed conidiogenous cells or conidia at the apical part (Fig. 2f); the conidiogenous cells were turned into conidia except for one or two cells at the base (Fig. 2g); and finally, conidiophores were frequently branched in the upper part (Fig. 2j). Conidia were usually form secondary spores. These primary conidia often blastically formed secondary conidia from the first or second cells at the base (Fig. 2i, j). Formation of secondary conidia resulted in besom-like sporophore.

Phylogenetic analyses

Phylogeny based on SSU rDNA + LSU rDNA + 5.8S rDNA: the positions of *G. triflora* isolate K18 and *M. gentianae* MAFF 239231 were estimated using three combined rDNA sequences (SSU + LSU + 5.8S; Fig. 3). The data matrix comprised 58 taxa and 2,020 aligned characters with 745 variable positions (36.9%) and 503 parsimony-informative positions (24.9%). Fourteen ambiguous characters as identified by Wang et al. (2006a) were excluded from the analysis. Maximum parsimony analysis yielded three equally parsimonious trees (MPTs) of 2,483 steps, with a

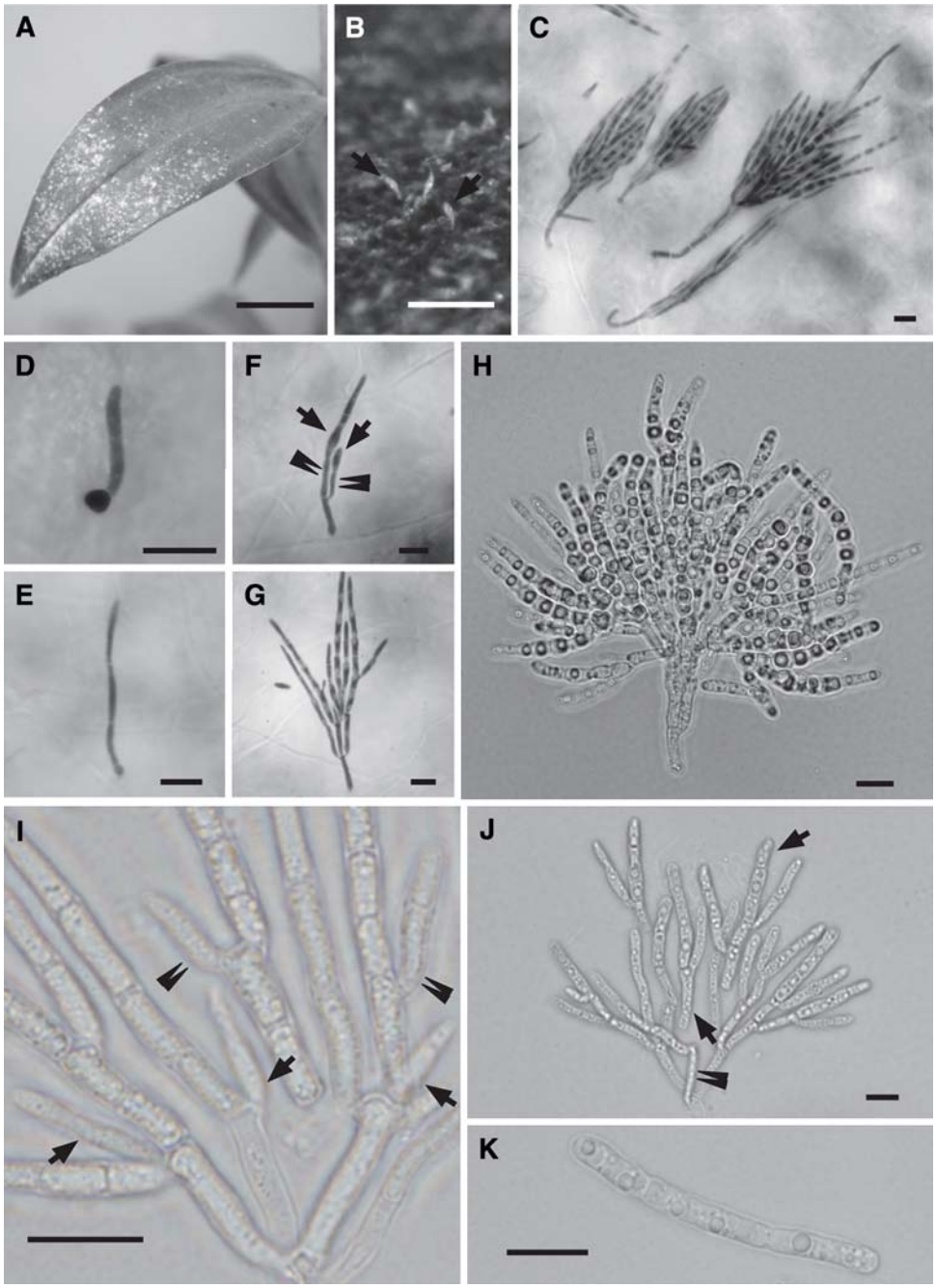

Fig. 2 *Mycochaetophora gentianae* isolated from *Gentiana triflora*. **a** Plants showing early symptoms of infection (minute grayish white, powdery spots). **b** Sporophores (*arrows*) on diseased leaf. **c** Besom-like sporophores on diseased leaf. Sporophore development. **d** Conidiophore erumpent on cuticle. **e** Multicellular conidiophore with septa. **f** Conidia (*arrows*) produced from branched conidiophores (*double arrows*). **g** Secondary conidia produced. **h** Mature sporophore. **i** Primary conidia (*arrows*) and secondary conidia (*double arrows*) blastically produced. **j** Conidia in branched acropetalous chains (*arrows*) left from conidiophores (*double arrows*). **k** Conidia. *Bars* **a** 10 mm; **b** 1 mm; **c–k** 10 μm

consistency index (CI) and retention index (RI) of 0.443 and 0.580, respectively; a consensus tree was constructed on the basis of these MPTs.

Phylogenetic analyses revealed that both *G. triflora* isolate K18 and *M. gentianae* MAFF 239231 were included in Helotiales–Rhytismatales (Leotiomycetes) with 86% bootstrap support (BP). These two isolates were strongly supported as monophyletic (BP = 97%). The two *Mycochaetophora* isolates and *R. carotae* constituted a clade with strong support (BP = 100%); the clade was placed as a sister group of *Vibrissea* (Vibrisseaceae), *Mollisia* (Dermateaceae), *Loramyces* (Loramycetaceae), and an anamorphic genus *Phialocephala* with low bootstrap support. The clade was also positioned in proximity to several helotialean families, such as Hyaloscyphaceae, Geoglossaceae, Helotiaceae, Sclerotiniaceae, and Leotiaceae. These families were not monophyletic, consisting of two clades.

Phylogeny based on ITS regions: the relationship among *G. triflora* isolates, *M. gentianae*, and *Pseudocercosporella*-like hyphomycetes in Helotiales was estimated using ITS

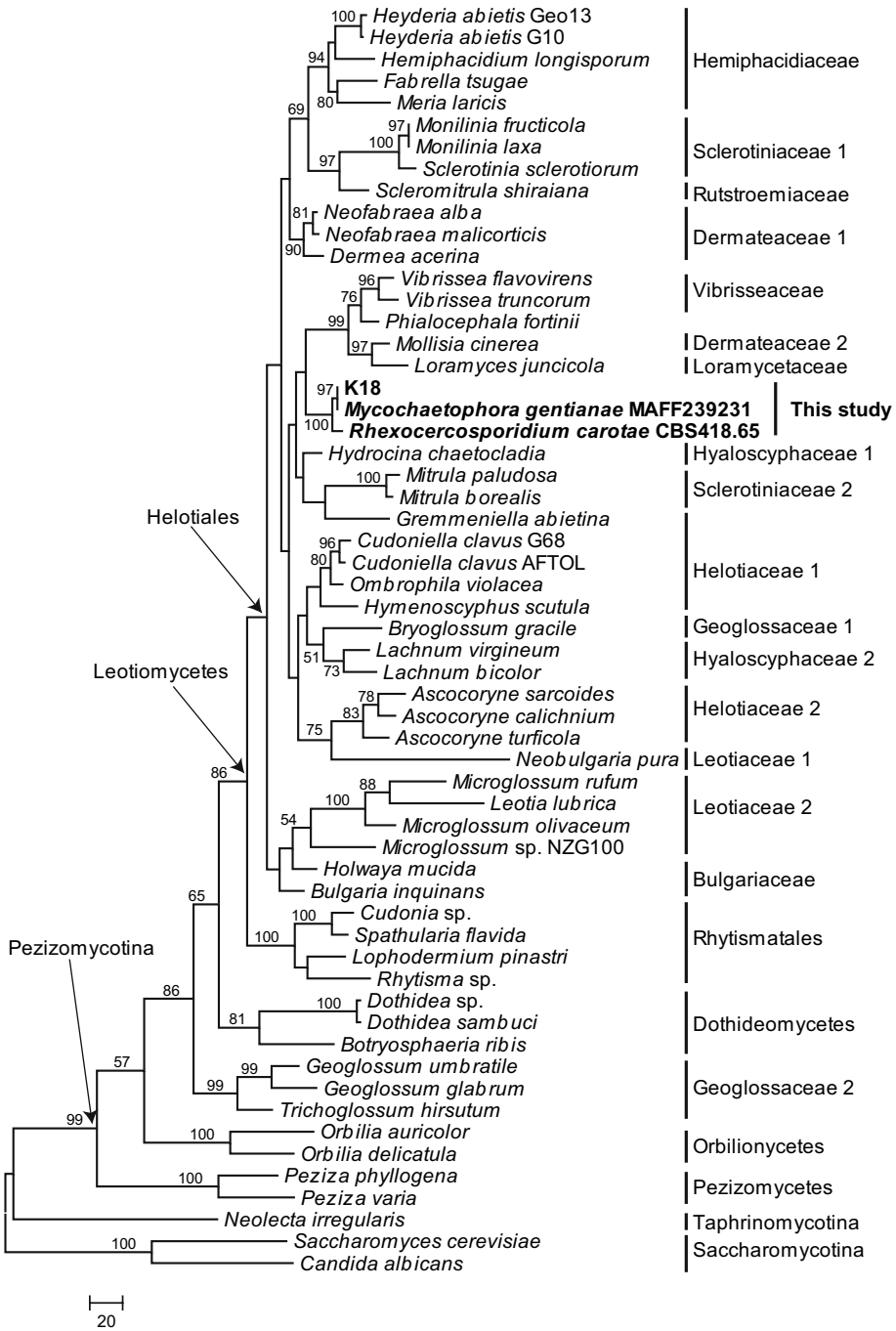

Fig. 3 Higher-level phylogenic tree of gentian isolates based on the data set of small subunit (SSU) + large subunit (LSU) + 5.8S by Wang et al. (2006a, b). Consensus trees of maximum parsimony analysis [length = 2,483, consistency index (CI) = 0.443, retention index (RI) = 0.580]. The *bold line* emphasizes the clade comprising *Mycochaetophora* isolates and *Rhexocercosporidium carotae*. Family-level designations of the clades are based on Lumbsch and Huhndorf (2007a). Confidence values >50% obtained from a 1,000-replicate bootstrap analysis are indicated at the *branch nodes*. The *scale bar* indicates the number of steps

regions that included 5.8S rDNA (Fig. 4). The data matrix comprised 30 taxa and 525 aligned characters, with 256 variable positions (48.8%) and 166 parsimony-informative positions (31.6%). Five ambiguous characters were excluded from the analysis. Maximum parsimony analysis yielded in 24 MPTs of 542 steps with CI and RI of 0.742 and 0.787, respectively; a consensus tree was constructed on the basis of these MPTs.

G. triflora isolates and *M. gentianae* were strongly supported as monophyletic (BP = 99%). The ITS sequences of *G. triflora* isolate K18 was different in two nucleotides from those of *M. gentianae* MAFF 239231 and MAFF 239284, which were identical. The clade was divided into two subclades, one including *G. triflora* isolate K18 and J4 (BP < 50%) and the other consisting of *M. gentianae* MAFF 239231 and MAFF 239284 (BP = 90%), each from Iwate and Okayama, respectively.

Mycochaetophora isolates constituted a heterogeneous clade with *Rhexocercosporidium*, *Helgardia* Crous & W. Gams (teleomorph: *Oculimacula* Crous & W. Gams),

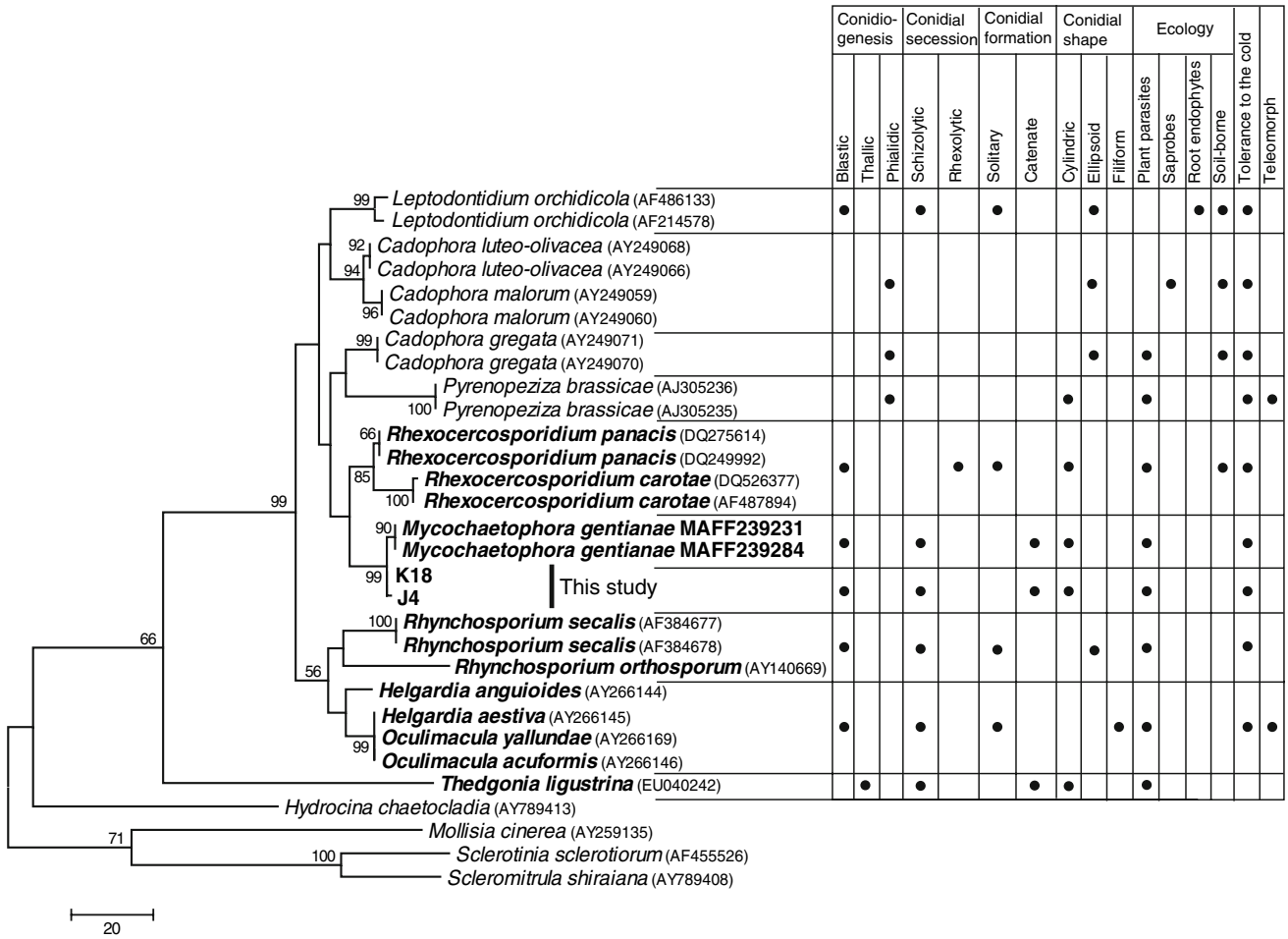

Fig. 4 Lower-level phylogenic tree of *Gentiana triflora* isolates, *Mycochaetophora gentianae*, and helotialean *Pseudocercosporella*-like hyphomycetes based on the data set of internal transcribed spacer (ITS) regions. Consensus trees of maximum parsimony analysis [length = 542, consistency index (CI) = 0.742, retention index (RI) = 0.787]. *Bold* emphasize gentian isolates and helotialean *Pseudocercosporella*-like hyphomycetes. Confidence values >50% obtained from a 1,000-replicate bootstrap analysis are indicated at the *branch nodes*. The *scale bar* indicates the number of steps

Rhynchosporium Heinsen ex A.B. Frank, *Cadophora*, *Leptodontidium orchidicola* Sigler & Currah, and *Pyrenopeziza brassicae* B. Sutton & Rawl (BP = 99%) and was placed as a sister group of *Rhexocercosporidium* (BP < 50%). In addition, the clade was divided into two subclades, one comprising *Helgardia* and *Rhynchosporium* (BP = 56%) and the other comprising *Cadophora*, *L. orchidicola*, *Mycochaetophora*, *P. brassicae*, and *Rhexocercosporidium* (BP < 50%). The most closely related teleomorph to the *Mycochaetophora* isolates was *Oculimacula* in the family Dermateaceae; the ITS sequences of *O. yallundae* and *O. acuformis*, which were identical, differed from that of *G. triflora* isolate K18 in 30 nucleotides. The second teleomorph closely related was *P. brassicae* in the family Dermateaceae; the ITS sequence of isolate K18 differed from those of the two isolates of *P. brassicae* in 33 nucleotides.

Discussion

Identity of the isolate from *G. triflora*

Cultural, pathogenic, and morphological characteristics of the *G. triflora* isolate (isolate K18) almost agreed with those of *M. gentianae* MAFF 239231 (ex-type from *G. scabra*) except for shape of conidial apex. Although immature conidia of *M. gentianae* MAFF 239231 were characteristically acute, the mature conidia liberated from sporophores were sharp or round at the apex. Thus, its conidia were indistinguishable from those of isolate K18. The conidial width of *M. gentianae* MAFF 239231 was slightly different from that of the original description (3.5–5 μm vs. 2–3.5 μm; Kobayashi et al. 2009). The description appears to be based on immature conidia because they were first filiform in both isolates. Consequently, *G. triflora*

isolates should be included in the species *M. gentianae* despite a difference of two nucleotides between the ITS sequences of isolate K18 from *G. triflora* and the ex-type isolate of *M. gentianae* (MAFF 239231).

The original description with symptomatic drawings was based on diseased plants of *G. scabra* (Kobayashi et al. 2009), although the diseased plants did not have typical features of *G. scabra,* which has liner to lanceolate calyx lobes (Yonezawa and Kawano 1989) and outwardly spread corolla (Yoshiike 1992), and thus seem to be *G. triflora* rather than *G. scabra*. Our pathogenicity tests also showed that both *M. gentianae* isolates (MAFF 239231 from *G. scabra* and isolate K18 from *G. triflora*) were pathogenic to *G. triflora* but not to *G. scabra.* Consequently, we suspect that the plants used in the previous studies (Kobayashi et al. 2009) were probably *G. triflora* but not *G. scabra* and that the latter is highly resistant to infection with *M. gentianae*, although further study is required for testing the susceptibility in additional cultivars of *G. scabra*.

Conidiogenesis of *M. gentianae*

Microscopic observations indicate that conidia of *M. gentianae* were blastically formed from conidiogenous cells that integrated into short conidiophores. The presence of short conidiophores was described in the first report by Kasuyama and Idei (1987), although Kobayashi et al. (2009) did not refer to this in their original description of *M. gentiane*. Blastic conidiogenesis of *M. gentianae* was similar to that of the type species, *M. japonica*, whose conidiophores are blastically transformed into conidia excluding one or two cells at the base (Hara 1931). On the other hand, the position of conidial cells to form secondary conidia was different between *M. gentianae* and *M. japonica;* the former usually produce secondary conidium from the first or second cell at the base of primary conidium, whereas the latter do so from almost all cells (Hara 1931). Conidial secession was schizolytic and left unthickened and inconspicuous scars on the conidiophore. These morphological features, such as short conidiophore and blastic conidiogenesis, unthickened and inconspicuous conidial scar, and catenate and colorless conidia in *M. gentianae*, imply its affinity with *Pseudocercosporella*-like hyphomycetes.

Phylogenetic position of *Mycochaetophora gentianae* within Helotiales

A teleomorph of *M. gentianae* has not yet been discovered. To determine the phylogenetic position of *M. gentianae*, higher-level data set phylogenetic analysis of rDNA (SSU + LSU + 5.8S) was conducted (Fig. 3). *M. gentianae* was grouped with *R. carotae*, which is a helotialean *Pseudocercosporella*-like hyphomycete, with strong support (BP = 100%). The two taxa were members of the Helotiales–Rhytismatales clade with strong support (BP = 86%) and were placed in proximity to the helotialean families. This result strongly suggests that *M. gentianae* belongs to the order Helotiales.

The higher-level analysis did not reveal a family to which *M. gentianae* belonged because these families were not monophyletic, and whereas the clade consisting of *M. gentianae* and *R. carotae* are grouped within Helotiales, its position relative to other members of the Helotiales was unresolved and unsupported. Wang et al. (2006a) suggested that although analysis of data from three rDNA regions with a wide taxonomic sampling improves resolution of the Helotiales phylogeny, the usefulness of rDNA in resolving the deep relationships within the Leotiomycetes is limited.

On the other hand, a lower-level analysis using ITS regions indicate the clade involving *M. gentianae* contain two teleomorphic fungi, *Pyrenopeziza* and *Oculimacula*, in the family Dermateaceae (Fig. 4). Goodwin (2002) speculated that if teleomorphs of *Rhynchosporium* exists, they would belong to *Tapesia* (=*Oculimacula*). Thus, we predict that if teleomorphs exist for *Pseudocercosporella*-like hyphomycetes including *M. gentianae*, they would be nearly classified as *Oculimacula* or *Pyrenopeziza*.

Relationships between *Mycochaetophora gentianae* and helotialean *Pseudocercosporella*-like hyphomycetes

Analysis using the ITS region revealed the close phylogenetic relationship between *M. gentianae* and three *Pseudocercosporella*-like hyphomycetes, namely, *Rhexocercosporidium*, *Helgardia,* and *Rhynchosporium* (Fig. 4), all of which are characterized by unthickened and inconspicuous conidial scars and blastic conidiogenesis, as is the case with *M. gentianae*. Whereas *Thedgonia ligustrina* (Boerema) B. Sutton is also considered as a *Pseudocercosporella*-like hyphomycete, it was separated from the clade including *M. gentianae*. The phylogenetic separation may be due to conidiogenous difference between *Thedgonia* and other *Pseudocercosporella*-hyphomycetes, including *M. gentianae* (thallic vs. blastic). Moreover, the phylogenetic affinity of *M. gentianae* to a group of morphologically similar helotialean *Pseudocercosporella*-like hyphomycetes supports the speculation of Hara (1931) that *Mycochaetophora* is placed in proximity to *Cercosporella* sensu lato (=*Pseudocercosporella*).

Within this clade, four *M. gentianae* isolates were monophyletic and separated from the three helotialean *Pseudocercosporella*-like hyphomycetes, and are strongly supported (BP = 99%). It has been suggested that the three genera *Helgardia*, *Rhexocercosporidium* and *Rhynchosporium* are positioned in close proximity to each other in the

previous phylogenetic trees (Goodwin 2002; Crous et al. 2003; Harrington and Mcnew 2003; Kageyama et al. 2008), but the association between these fungi has not been discussed. *R. carotae*, which was placed nearest to *M. gentianae* both in higher- and lower-level analyses, is a soil-borne pathogen and causes black rot in carrot (*Daucus carota* L.) (Braun 1994; 1995). *Rhexocercosporidium* is characterized by rhexolytic conidial secession and semi-macronematous conidiophores (Braun 1995) and is similar to *M. gentianae* in conidial shape. However, rhexolytic conidial secession of *Rhexocercosporidium* was clearly distinct from the schizolytic one of *M. gentianae* (Fig. 4). Likewise, *Helgardia*, which was placed in a subclade separated from *M. gentianae* and *Rhexocercosporidium*, causes eyespot on cereals and forms conidial masses in slimy packets (Robbertse et al. 1995; Crous et al. 2003). The conidial masses in *Helgardia* resemble besom-like sporophores in *Mycochaetophora*. However, *Helgardia* and *M. gentianae* differ in patterns of conidial formation (solitary vs. catenate) and shape (filiform vs. cylindrical) (Fig. 4). Also, the shape of conidiophores in *Helgardia*, which is short and subcylindrical to geniculate sinuous (Robbertse et al. 1995; Crous et al. 2003), is quite different from that of *M. gentianae*. *Rhynchosporium*, which is the same subclade with *Helgardia*, causes scald on barley, rye, and grasses and is characterized by bicellular conidia bearing a beak on one end (Caldwell 1937). Conidia of *Rhynchosporium* are directly produced on the mycelia without conidiophore formation (Caldwell 1937). The conidial formation and shape of *Rhynchosporium* are clearly distinct from those of *M. gentianae* (Fig. 4). As a whole, the molecular and morphological data clearly provide evidence that *Mycochaetophora* is a distinct genus and not congeneric with other helotialean *Pseudocercosporella*-like hyphomycetous genus, although both fungi share ecological (plant parasite) and superficial morphological similarities to each other.

Relationship of *Mycochaetophora gentianae* with *Cadophora*, *Leptodontidium*, and *Pyrenopeziza brassicae*

Morphological relationships

ITS phylogeny indicates that *M. gentianae* is also monophyletic, with two anamorphic genera of *Leptodontidium* and *Cadophora* and a teleomorphic fungus *P. brassicae* (Fig. 4). They are a root endophyte, saprobe, and plant parasite, respectively. Previous studies suggest that these fungi have an affinity to helotialean *Pseudocercosporella*-like hyphomycetes (Goodwin 2002; Crous et al. 2003; Harrington and Mcnew 2003; Kageyama et al. 2008; Wu and Guo 2008). Blastic mode of conidiogenesis in *Leptodontidium* (Fernando and Currah 1995) is shared with that of *M. gentianae* and helotialean *Pseudocercosporella*-like hyphomycetes; *Cadophora* and anamorph of *P. brassicae* (*Cylindrosporium concentricum* Greville) produce conidia on phialides. Genetic association of *M. gentianae* with these fungi can be hardly deduced from morphological features alone.

Ecological relationships

Wang et al. (2006a) suggest that life style and ecological factors play a critical role in shaping the evolutionary history of helotialean fungi. Mycelial growth of *M. gentianae* on PDA occurred at low temperature conditions (Fig. 1), which the host plant, gentian, also prefers (Yoshiike 1992). *R. carotae* can infect the host and grow at low temperatures (Shoemaker et al. 2002; Kastelein et al. 2007; Reelder 2007). Goodwin (2002) pointed out that the ecological features of *Rhynchosporium* and *Ramulispora* (=*Helgardia*) overlap; both pathogens cause eyespot on winter wheat and scald on barley, respectively, autumn through winter (Caldwell 1937; Fitt et al. 1988), and their conidia are produced and germinate under low-temperature conditions (Caldwell 1937; Fitt et al. 1988). Likewise, *P. brassicae* can infect its host and then produce new conidia at low temperatures (Gilles et al. 2000). *L. orchidicola* has been isolated from plants growing in alpine and subalpine habitats in western Canada (Fernando and Currah 1995). *C. gregata* is a soil-borne fungus and causes brown stem rot of soybean; the disease development is favored at low temperatures (Gray and Grau 1999). Also, *Cadophora* spp. are soil inhabits in Antarctica, which seem to be native saprophytes (Held et al. 2005; Arenz et al. 2006). As a whole, phylogenetic affinity of these fungi implies their common feature, i.e., tolerance of cold environments, and they might constitute a cold-tolerant clade.

Furthermore, ITS phylogeny indicates the presence of a subclade comprising *M. gentianae* and soil-borne fungi, including *Cadophora*, *Leptodontidium*, and *Rhexocercosporidium*, with low bootstrap support. Given that the disease tends to occur in the same spot where it occurred the previous year, the result suggests a possibility that *M. gentianae* inhabits in the soil. We should further confirm the possibility in terms of epidemiologic context.

Phylogenetic position of cold-tolerant clade within Helotiales

Wang et al. (2006a) divided Helotiales into nine distinct clades based on three rDNA sequences. Lumbsch and Huhndorf (2007b) noted that these clades may form the basis for future family classification within this order. Wang et al. (2006a) predicted that *Rhexocercosporidium*

and phytopathogenic *Tapesia* species (=*Helgardia*) belong to the *Vibrissea-Loramyces* clade, which comprises aquatic saprobes, wood decomposer, and root endophytes (Fig. 4 in Wang et al. 2006a). Our results indicate that the clade including *R. carotae* and *M. gentianae* was not included in the *Vibrissea-Loramyces* clade but was placed as a sister group with low bootstrap support (Fig. 3). We speculate that a range of the *Vibrissea-Loramyces* clade may be larger than their prediction (Wang et al. 2006a). It is possible that the cold-tolerant clade, which was found in the ITS phylogeny and also includes plant-parasitic, saprobic, and root-endophytic fungi encompassing *M. gentianae*, will become a new clade within Helotiales.

Conclusions

This study reveals that the causal fungus of brown leaf spot on *G. triflora* in Iwate, Japan is identical to *M. gentianae* and that its conidiogenesis is blastic from short conidiophores, with schizolytic secession of conidia leaves unthickened and inconspicuous conidial scars on the conidiogenous cells. Molecular and morphological data clearly show that whereas *M. gentianae* have an affinity to three helotialean *Pseudocercosporella*-like hyphomycetes, they are a distinct genus and not congeneric with these fungi. In addition, ITS phylogeny indicates that *M. gentianae* is related to *Cadophora*, *Leptodontidium*, and *P. brassicae*, as well as helotialean *Pseudocercosporella*-hyphomycetes, which is reflected in ecological linkages such as tolerance to cold environment, although further ecological and epidemiologic studies on *M. gentianae* are required.

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