Mycoscience (2006) 47:41–47
DOI 10.1007/s10267-005-0265-5

FULL PAPER

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Ciborinia gentianae sp. nov., the causal organism of sclerotial flower blight of cut-flower gentians

Received: April 25, 2005 / Accepted: November 8, 2005

Abstract A new *Ciborinia* causing sclerotial flower blight of cut-flower gentians (*Gentiana triflora* var. *japonica* and interspecific hybrids between related species or varieties) is described as *Ciborinia gentianae* on the morphological basis of sclerotia and apothecia. The characteristics of *Ciborinia gentianae* are (1) an abundant production of spermodochia in the hollow cavity of host stems; (2) flat and thin sclerotia produced beneath the epidermis and the inclusion of host vascular remnants within their medulla; (3) globose cells composed of ectal excipulum of apothecia; (4) elongated cells with a slight apical swelling in ectal excipulum at the apothecial margin; and (5) tetra nucleate ascospores. Asci and ascospores mounted in Melzer's reagent measured 156–208 × 8–12 μm and 11.8–15 × 5.5–7.1 μm, respectively.

Key words *Ciborinia gentianae* sp. nov. · Cut-flower gentian · *Gentiana triflora* var. *japonica* · Sclerotial flower blight · Taxonomy

Introduction

Cut-flower gentians consist of Japanese native species of the section *Pneumonante* in the genus *Gentiana* (Toyokuni 1963). The major species for cut flower production is *Gentiana triflora* Pall. var. *japonica* (Kuzen.) H. Hara f. *japonica*, but currently horticultural varieties that originated from interspecific hybrids between the species and *G. scabra* Bunge var. *buergeri* Maxim. or *G. triflora* Pall. var. *japonica* H. Hara f. *montana* Toyok. are extensively cultivated (Yoshiike 1992). Of the nine major fungal diseases, sclerotial flower blight is of economic significance because the disease occurs not only in gentian plants in fields but also in harvested cut flowers (Yoshiike 1992). The disease was first found in Nagano Prefecture (Imamura et al. 1975). Afterward, it occurred in Hokkaido (Mano 1980) and in Fukushima Prefecture (Kaji et al. 1990). The fungus responsible for the disease was first referred to *Sclerotinia* sp. (Imamura et al. 1975); however, it was suggested that the organism belonged to the genus *Ciborinia* Whet. because its sclerotial anamorph apparently had characteristics of that genus (Mano 1980). Later, Kaji et al. (1990) also considered that it should be a *Ciborinia* fungus. In this article, we consider the characteristics of the organism that agree with the known generic features of *Ciborinia* (Whetzel 1945; Batra 1960; Kohn 1979a) and describe it as a new species.

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Materials and methods

Isolation and culture

Monoascosporic isolation from a mature apothecium was carried out by methods described elsewhere (Batra and Korf 1959), and the isolates obtained were stored in potato dextrose agar slants (PDA-Difco) in a refrigerator (3°C) until utilization. To observe the cultural appearance, the organism was grown in PDA plates in 9-cm Petri dishes at 20°C in the dark. For observation of the relationship of temperature to mycelial growth, a monoascosporic isolate Ci -2 obtained in Fukushima Prefecture was used.

Production of apothecia

Sclerotia collected from infected stems were seeded on moistened polyurethane sponges in glass containers (Saito 1977). The sponges were previously cut with a razor blade

to make shallow slits in which sclerotia were inserted. They were incubated at a distance of 60 cm under continuous illumination by two daylight fluorescent lamps (20 W) at 20°C. Apothecia produced were freeze-dried in a small glass container by means of a freeze dryer (Tokyo Rikaki; type FD-80) and heated for 24 h at 60°C to prepare them as the specimens.

Microscopy

The measurement of asci, ascospores, and paraphyses was carried out on freeze-dried specimens of apothecia using Melzer's reagent as a mounting fluid. The specimens were crushed in a drop of Melzer's reagent on a glass slide, given a cover slip and observed under a light microscope, Olympus BH2. To make Melzer's reagent, 7.5 g KI, 0.25 g iodine, and 10 g chloral hydrate were dissolved in sequence in 10 ml distilled water. For the purpose of staining nuclei in ascospores, a fresh apothecium was placed to make its upper face contact with a glass slide, allowing ascospores to be ejected onto the slide. The ascospores were fixed in Carnoi's fluid (Sass 1951) and stained with the HCl-Giemsa technique (Wilson 1992). Observations of the tissue structure of apothecia and sclerotia were carried out for the resin-embedded sections prepared by the following methods: tissues of apothecia and sclerotia were fixed and stored in 4% (v/v) glutaraldehyde solution in 1/15 M phosphate buffer, pH 7.0 at 3°C, then dehydrated with an acetone series, embedded in Spurr's low-viscosity resin, and sectioned in 1–2 µm thickness with glass knives on a Porter Blum MT 1 microtome. Sections of sclerotia and apothecia were stained according to the dichromatic staining methods of Bennell et al. (1978) using methylene blue/azure II and basic fuchsin. To detect remnants of the host plant contained in medullary tissue, sclerotia in stems of infected gentian were fixed with FAA (Sass 1951) for 24 h, dehydrated by an ethanol–butanol series, and embedded in paraffin blocks. As paraffin-embedded sclerotia were too hard to be sectioned on a rotary microtome, the paraffin blocks were cut to expose one end of the sclerotium and soaked in distilled water for 12 h to give appropriate softness to the sclerotial tissue. They were then sectioned 10 µm thick on a rotary microtome and stained with 0.05% (w/v) toluidine blue in 0.2 M acetate buffer (pH 4.0).

Results

Symptomatology

The first sign of infection is the development of water-soaked lesions on petals, which were elliptical or irregularly shaped, 3–4 mm in diameter (Fig. 1). The lesions then become tan to brown in color, enlarging over the whole flowers to give a typical flower-blight symptom (Fig. 3). The blight often extends to the stems through the base of the infected flowers, resulting in degeneration and dieback of the plant (Fig. 2). No visible development of mycelium is observed on the surface of the host plant. Sclerotia are produced in the basal part of the flowers, beneath the epidermis of stems, and in the hollow cavity of the stems (Fig. 4A–C).

Taxonomy

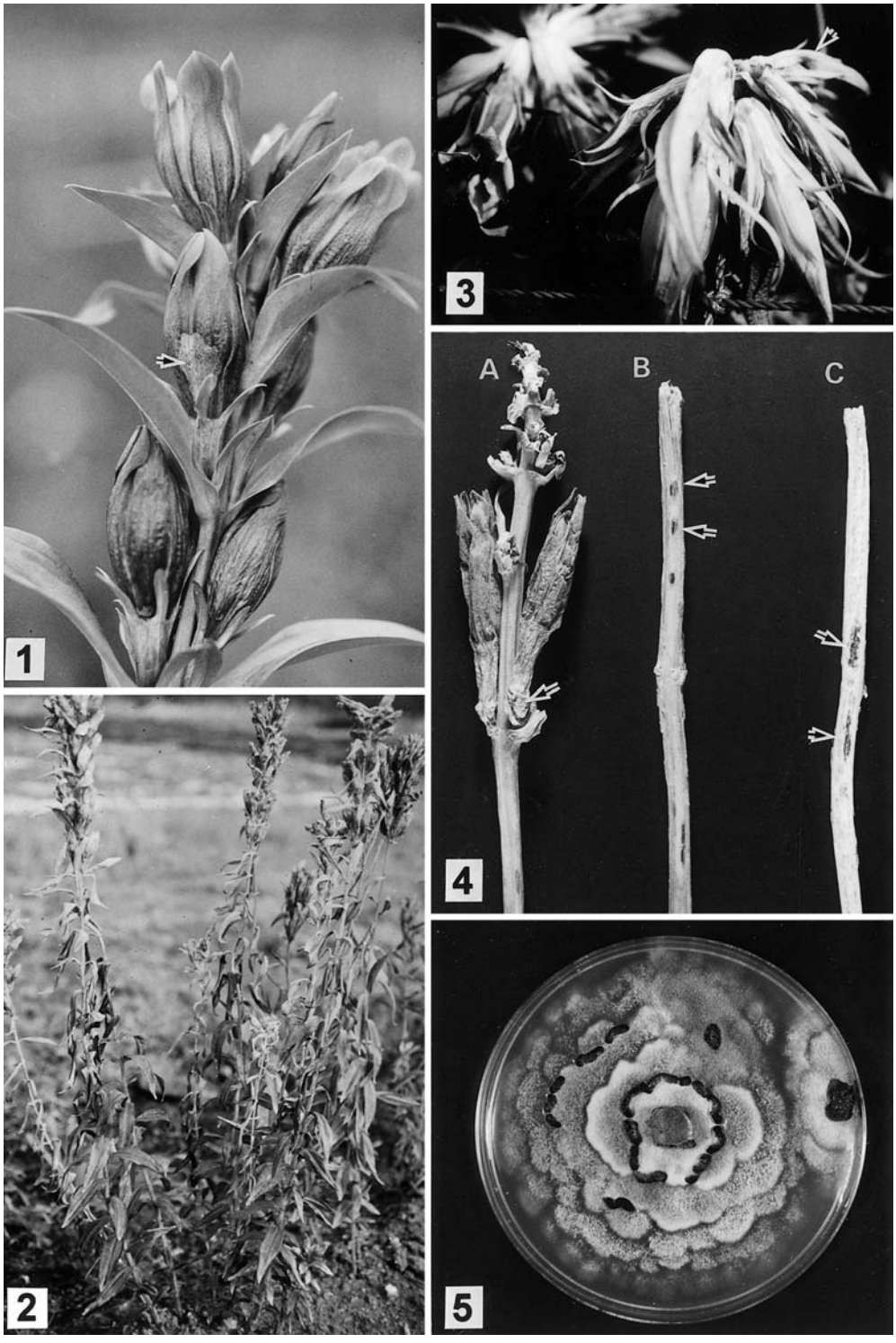
Ciborinia gentianae I. Saito et Kaji, sp. nov. Figs. 6-15
Sclerotia subcutanea, nigra, plana, tenuia; medulla sclerotiorum vestigia vascularia hospitis includens; microconidia globosa, 2–4 µm diametro, e phialide hyalina formantia; apothecia e sclerotio surgentia, nonnulla, stipitata; discus discoideus vel subdiscoideus, medilacunosus, posterius medilacunosi-convexus, carnosus, fulvus vel cinnamomeus, 2.4–5.3 mm diametro; excipulum medullare 131–245 µm crassum, "textura intricata," ex hyphis hyalinis 4–10 µm latis compositum; excipulum ectale 29–50 µm crassum, "textura prismatica" vel "textura globulosa," ex cellulis globosis hyalinis vel leptodermis 5–20 × 5–14 µm compositum; subhymenium pallide brunneum, "textura intricata," 61–90 µm crassum; stipites cylindracei, deorsum attenuati, disco paene concolores sed ad basim infuscati, 6.1–8.2 × 1–2 mm; asci cylindracei, deorsum paulo attenuati, inoperculati, poro apicali jodo non vel obscure cyanescenti praediti, octospori, 156–208 × 8–12 µm; ascosporae monostichae, continuae, ellipticae, hyalinae, tetranucleatae, 11.8–15 × 5.5–7.1 µm; paraphyses filiformes, ramosae, septatae, ad apicem latiusculae, 75–120 × 2–4 µm.

Habitat: Parasitic on *Gentiana triflora* Pall. var. *japonica* (Kuzen.) H. Hara f. *japonica* and the interspecific hybrids between *G. triflora* Pall. var. *japonica* H. Hara f. *montana* Toyok. or *G. scabra* Bunge var. *buergeri* (Miq.) Maxim.

Holotype: I. Saito ISNAD 23-6, with apothecia produced in culture on July 1, 1980, on sclerotia collected from infected *Gentiana triflora* var. *japonica* f. *japonica* in the field of the Hokkaido Prefectural Central Agricultural Experiment Station, Naganuma, Hokkaido, on October 25, 1979 by I. Saito; deposited in the Herbarium of the National Science Museum, Tokyo (TNS-F-11558).

Other specimens examined: (1) Dry specimens of infected plants including sclerotial and microconidial anamorph (I. Saito ISNAD23–7, deposited in the Herbarium of the National Science Museum, Tokyo (TNS–F–11559)) collected at the Hokkaido Prefectural Central Agricultural Experiment Station, Naganuma, Hokkaido. (2) Sclerotial anamorph (I. Saito ISNAD23–8) collected by K. Kaji in the experimental fields of gentian at the Fukushima Prefecture Agricultural Experiment Station, Yamaguchi, Nango–mura, Minamiaizu–gun, Fukushima Prefecture. (3) Teleomorph (I. Saito ISNAD23–9) produced in the Agroscience Research Laboratories, Hokkai Sankyo Co. Ltd., from sclerotia that were collected by K. Kaji in experimental fields of gentian at the Fukushima Prefecture Agricultural Experiment Station.

Ex-holotype culture: The strain CG–10 isolated by I. Saito on July 7, 1980 from an apothecium produced on sclerotia collected by I. Saito from infected *Gentiana triflora*

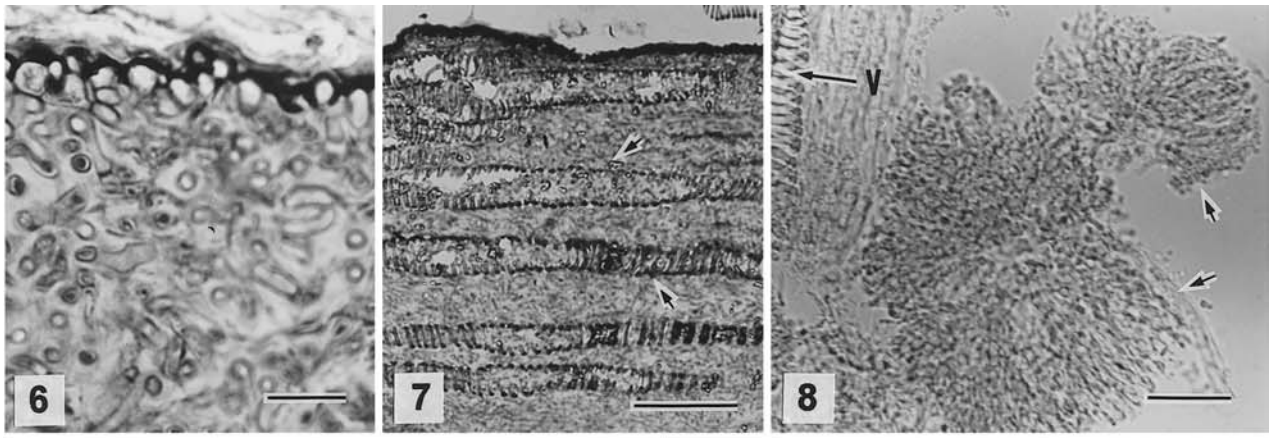


Figs. 1–5. Symptoms of sclerotial flower blight of *Gentiana triflora* var. *japonica*. **1** Infected flower with water-soaked lesions (*arrow*) in the early stage of the disease. **2** Appearance of infected gentian plants in the field. **3** Infected flower showing dead sepals and petals with sclerotia (*arrow*) in the late stage of the disease. **4 A** Top of infected plant showing tuberoid sclerotia (*arrow*) produced in flower base. **B** An infected stem with flat sclerotia (*arrows*) produced beneath the epidermis. **C** Section of an infected stem with sclerotia (*arrows*) produced inside. **5** Colony of *C. gentianae* on PDA showing a petaloid pattern of mycelial growth

var. *japonica* f. *japonica* in the field of the Hokkaido Prefectural Central Agricultural Experiment Station, Naganuma, Hokkaido on October 25, 1979, is deposited in the Japan Collection of Microorganisms (JCM), Wako as JCM 13253.

Etymology: Latin, *gentianae* = of gentian, referring the host of the fungus.

Sclerotia formed beneath the epidermis of the host or sometimes in stem cavity, black, flat, thin, elliptical or elongated or irregularly shaped, 1.2–9.8 mm in length, 0.5–3.5 mm in width, 0.1–0.3 mm in thickness; sclerotia formed in sepals irregularly shaped, sometimes tuberous; vertical section of a sclerotium shows a rind layer composed of thick-walled, darkly pigmented cells and a white medulla of densely interwoven hyphae, 3–6.1 µm in width, embedded in a gelatinous matrix (Fig. 6). Sclerotia produced in the stem cavity always contain vascular remnants of the host (Fig. 7). Spermatia (microconidia), produced in phialides developing from the mycelium in PDA culture, globose,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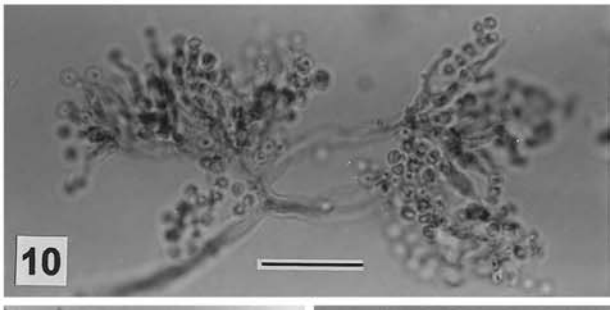
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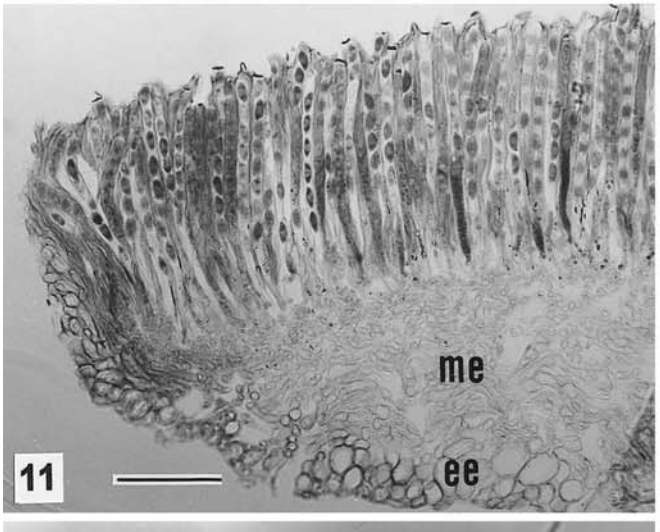
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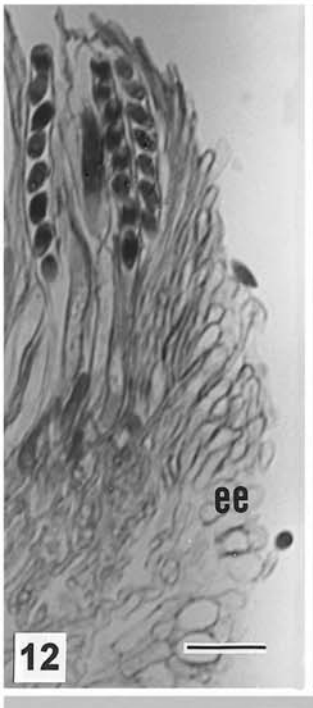
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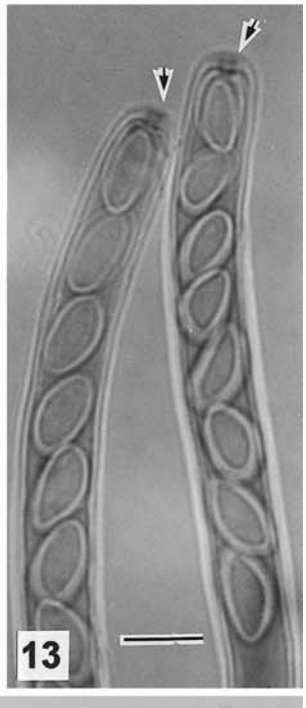
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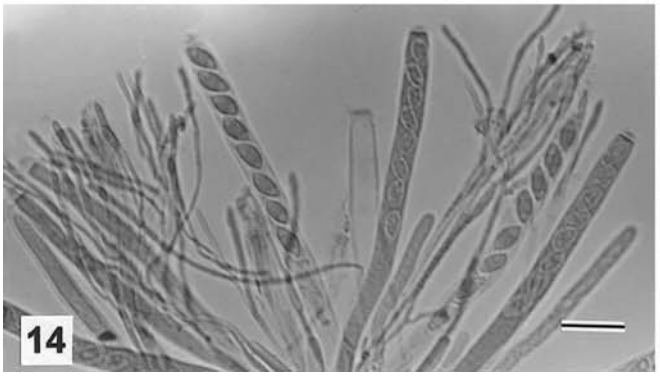
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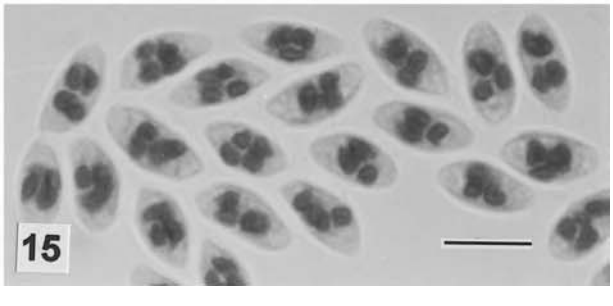
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hyaline, 2–4 μm in diameter (Fig. 10). In nature, spermatia are abundantly produced in the hollow cavity of stems of host forming tufts of spermodochia (Fig. 8). No macroconidia were observed in culture or in infected gentian plants. Apothecia stipitate, arising singly or multiply from a sclerotium (Fig. 9). Disks first saucer-shaped with central depression, involute by the margin at maturity, becoming convex with central depression, carnose, tawny, or cinnamon (Ridgway 1912), 2.4–5.3 mm diameter. Stipes cylindrical, 4.6–8.2 × 1–2 mm, tapering toward the base, almost concolor with the outer surface of the disc but usually darkening downward. Vertical section of apothecium (Fig. 11) shows hymenium; subhymenium, 61–90 μm thick; medullary excipulum 131–245 μm thick, textura intricata of loosely interwoven hyaline hyphae, 4–10 μm wide; ectal excipulum 29–50 μm thick, textura prismatica or textura globulosa, composed of hyaline to light brown, thin-walled globose cells, 5–20 × 5–14 μm; component cells of ectal excipulum at apothecial margin elongated, sometimes septate, slightly swollen at the tip (Fig. 12); asci cylindrical, slightly attenuate toward the base, eight-spored, 156–208 × 8–12 μm, apex rounded, without pretreatment in 2% (w/v) KOH apical pore apparatus weakly J+ or J–, with KOH apical pore apparatus strongly J+; ascospores one-celled, hyaline, ellipsoid, tetranucleate (Figs. 13, 14, 15), 11.8–15 × 5.5–7.1 μm; paraphyses hyaline, filiform, septate, 75–120 × 2–4 μm, often swollen at the tip.

Cultural characteristics

Colonies of *C. gentianae* on PDA were at first white, then ivory, and changed to light brown. Mycelium with a few aerial growths developed irregularly to give characteristic broad, rounded, petaloid sectors in the colony, and the colony margin was lobed (see Fig. 5). Sclerotia on the colony were flat and partially embedded in the agar surface (Fig. 5). A monoascosporic isolate of *C. gentianae*, Ci-2, grew in PDA in the temperature range 10°–25°C but did not grow at 4° or 30°C. The optimal temperature for mycelial growth was 18°C (Fig. 16).

Discussion

Since the emendation of the generic diagnosis of *Ciborinia* by Batra and Korf (1959), Batra (1960) provided a key to 13 species. Thereafter, no monograph on the genus has been published, but several species were added through new combinations (Kohn 1979a,b; Schumacher and Kohn 1985; Palmer 1992) and findings of new taxa (Kohn 1982; Zhuang and Wang 1997). Thus, the genus *Ciborinia* currently circumscribed includes 19 species (Table 1). Further, a *Ciborinia* fungus on *Salix planifolia* was suspected to be a new taxon, although its epithet was not given (Huhtinen 1985) (Table 1). The traditional characters for identification of *Ciborinia* species are host, morphology of apothecia (size, presence or absence of rhizoidal tuft in stipe base), measurements of asci and ascospores (Seaver 1951; Groves and Bowerman 1955; Batra 1960; Huhtinen 1985; Palmer 1992), and number of ascospores in asci (Zhuang and Wang 1997). In addition, the nuclear number of ascospores appears to be a valid character to identify a species in the Sclerotiniaceae (Kohn 1979a; Saito 1997), and we revealed tetranucleate ascospores of *C. gentianae* in this study; however, the published descriptions on ciborinias lack comparable nuclear data except for *C. camelliae* (Kohn and Nagasawa 1984) and *C. ciborium* (Schumacher and Kohn 1985). With apothecial size, *C. gentianae* differs from species having minute apothecia (1–2 mm diameter) (*C. seaveri* Groves & Bowerman; *C. davidsoniana* Groves & Bowerman; *C. candolleana* (Lev.) Whet.; *C. allii* (Sawada) L.M. Kohn; *C. hirsuta* L.M. Kohn & Korf) and large apothecia (up to 14 or 18 mm diameter) (*C. whetzelii*

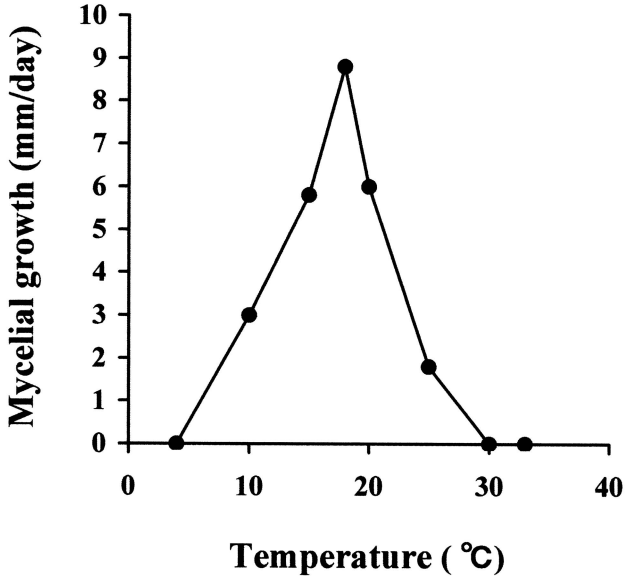

Fig. 16. Effect of temperature on the mycelial growth of *Ciborinia gentianae*

Figs. 6–15. Anamorph and teleomorph of *Ciborinia gentianae*. **6** Vertical section of a sclerotium produced beneath the host epidermis. **7** Section of a sclerotium produced in the hollow cavity of the host stem showing remnants of vascular tissues of the host in medullary tissue (*arrows*). **8** Tufts of spermodochia (*arrows*) produced in hollow cavity of an infected gentian plant. *V*, remnants of host vascular tissue. **9** Apothecia produced on sclerotia. **10** Spermodochia produced in potato dextrose agar (PDA) culture. **11** Vertical section of apothecium. *me*, medullary excipulum; *ee*, ectal excipulum. **12** Structure of apothecium margin showing elongated cells of ectal excipulum with a slight apical swelling. *ee*, ectal excipulum. **13** Apical pore rings (*arrows*) in asci. **14** Asci and paraphyses. **15** Ascospores containing four nuclei. *Bars* **6**, **10**, **12**, **14**,**15** 20 μm; **7** 100 μm; **8** 120 μm; **11** 50 μm; **13** 10 μm

Table 1. Comparison of *Ciborinia gentianae* with the *Ciborinia* species so far reported in the world

Species	Hosts	Apothecia diameter (mm)	Asci (μm)	Ascospores (μm)	Rhizoidal tuft in stipes	References[c]
Species on woody angiosperms:						
C. whetzelii[a]	*Populus tremuloides*	2–10	(100–)140–180 (–200) × 9–13	(9–)11–15(–17) × (5–) 6–8	–	A, B, D
C. seaveri	*P. tremuloides*	0.5–1.5	46–65 × 6–7.5	7–10 × 2.5–3.5	–	B, D
C. davidsoniana	*P. tremuloides*	0.5–1	153 × 7–9	9–13 × (3–)5–6	+	B, D
C. pseudobifrons	*Populus* sp.	1–3	110–150 (–180) × 7–9	(7.5–) 9–13 (–16.5) × 3.5–5	–	B, D
C. foliicola	*Salix humilis*	2–6	125–150 × 7–9	9–12 (–16) × 6–7 (–8)	+	D
C. wisconsinensis	*S. petiolaris*	2–6	(120–)165–180 (–210) × (7–) 8–10	(7–) 9–13 (–15) × (4–)5–6	–	D
Ciborinia sp.	*S. planifolia*	5 (dried)	150 × 10	9–11 × 4–5	–	G
C. candolleana	*Quercus rubra*	1–2	72–115 × 5–10	(6–) 7–9 (–10) × 3–4	+	A, D
C. bresadolae[b]	*Q. robur*	3	63–82 × 4.3–7.9	7.0–9.5 × 2.8–4.1	Not described	I
C. gracilipes	*Magnolia glauca*	2–3	50–65 × 4.5	4.5–6.2 × 3–4	+	A, D
C. hirsuta	*Vaccinium* sp.	1–2	56–75 × 5–5.8	Dimorphic in size (4:4, 6:2)	+	E
C. camelliae	*Camellia japonica*	3–18	(100–) 120–140.5 × (5–) 6–10.5	7.5–12.5 × 4.0–5.0 (–6.0)	Not described	F
C. hemisphaerica	Unidentified	3–6	97–110 × 7.2–7.6	8–10 × (2.5–)3–3.5	+	L
Species on herbaceous angiosperms:						
C. ciborium	*Eriophorum* spp.	Up to 14	140–190 × 7–12	9.5–17.8 × 4.5–7.5	–	I
C. allii	*Allium fistulosum*	1–2	190–210 × 10–12	Dimorphic in size (4:4)	–	H, K
C. gracilis	*Erythronium albidum*	2.5–3.5	182–250 × 9–14	20–30 × 6.5–7.5	–	C, D
C. erythronii	*Erythronium* spp.	2–5	170–275 × 8–17	(14–) 18–30 × 7–9	+	C, D
C. trillii	*Trillium* spp.	1–2.5	(50–) 65–90 (–103) × 3–4	(6–) 7–10 (–12) × 2.5–4	+	C, D
C. violae	*Viola* spp.	2–3	90–130 × 7–9	(7–) 8–10 (–11) × 2.5–4.5	–	C, D
C. jinggangensis	Unidentified	0.5–1	53–60 × 5.0–5.5	7–11 × 2.8–3.5	–	L
C. gentianae	*Gentiana triflora* var. *japonica*	2.4–5.3	156–208 × 8–12	11.8–15 × 5.5–7.1	–	This paper

[a] *C. whetzelii* (≡*C. bifrons* (Whetzel) Whetzel) (≡*C. confundens* (Whetzel) Whetzel) [Batra and Korf (1959)]
[b] *C. bresadolae* (=*C. hirtella* (Boud.) Batra and Korf)) [Palmer (1992)]
[c] References: A, Seaver (1951); B, Groves and Bowerman (1955); C, Batra and Korf (1959); D, Batra (1960); E, Kohn (1982); F, Kohn and Nagasawa (1984); G, Huhtinen (1985); H, Lew and Wu (1985); I, Schumacher and Kohn (1985); J, Palmer (1992); K, Tamura et al. (1996); L, Zhuang and Wang (1997)

(Seaver) Seaver; *C. camelliae* (Hara) L.M. Kohn; *C. ciborium* (Vahl: Fr.) Schumacher & L.M. Kohn). In the rest of the species listed, *C. wisconsinensis* Batra has a comparable teleomorph with *C. gentianae* considering its published description on sizes of apothecia, asci, ascospores, and lack of rhizoidal tufts. However, *C. wisconsinensis* parasitizes only *Salix petiolaris* in artificial inoculation with ascospores to several *Salix* species (Batra 1960). The members of the genus appear to be host specific, and several species attack deciduous trees, even being biotrophic with great affinity to their hosts (Groves and Bowerman 1955; Batra 1960).

The genus *Gentiana* is the largest taxonomic group of plants in the family Gentianaceae and consists of 15 sections including 360 species widely distributed in the world (Chen and Wang 1999). However, there are no records with specific identification of sclerotiniaceous fungi causing diseases of cultivated *Gentiana* plants except *Botrytis cinerea* Pers. ex Fr. (macroconidial and sclerotial anamorph of *Botryotinia fuckeliana* Whet.) (Anonymous 1960; Morita 1988). Based on its specific host and teleomorphic characters, we conclude that *C. gentianae* is a different taxon from the *Ciborinia* species so far reported in the world. In Hokkaido, sclerotial flower blight first occurred in gentian fields of the Hokkaido Prefectural Central Agricultural Experiment Station where plants of *G. triflora* var. *japonica* collected from their natural habitats were cultivated (E. Miki, former Head of Horticultural Department of Hokkaido Prefecture Central Agricultural Experimental Station; personal communication). *Ciborinia gentianae* is, therefore, likely to be an endemic parasite surviving in native gentian plants in Japan.

Acknowledgments We gratefully acknowledge Dr. K. Katumoto, Ex-Professor of Yamaguchi University, for his critical reading and valuable suggestions for preparation of the manuscript. One of us (I.S.) thanks Dr. K. Takaoka, former president of Hokkai Sankyo Co. Ltd., for his continuous encouragement and the staff of Agroscience Research Laboratories, Hokkai Sankyo Co. Ltd. for their support during this study.

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