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SHORT COMMUNICATION

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Flowers for *Neotyphodium* endophytes detection: a new observation method using flowers of host grasses

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Abstract *Neotyphodium* endophytes are vertically transmitted fungal symbionts of grasses. Being pest-repelling and growth-promoting agents for their hosts, and also potential mycotoxin producers, their detection in plants is important. Observation of chemically cleared flowers of infected grasses (*Festuca arundinacea*, *F. pratensis*, *Lolium perenne*, and *L. multiflorum*) using differential interference contrast microscopy revealed the existence of endophytes within immature ovaries of host plants. This observation method provides an accurate and easy way to detect and distinguish *Neotyphodium* endophytes in flowering host grasses and to investigate the seed transmission process, which is critical to their life cycle, and the practical use of infected plants.

Key words Detection · Differential interference contrast microscopy · *Neotyphodium* endophyte · Seed transmission · Symbiosis

Neotyphodium Glenn, Bacon & Hanlin species are asexual fungal symbionts (endophytes) of cool-season grasses and are closely related to the choke pathogen *Epichloë* (Fries) L.R. & C. Tulasne species. They had been classified as *Acremonium* Link: Fries sect. *Albo-lanosa* Morgan-Jones & Gams, but were reclassified following phylogenetic studies based on ribosomal RNA gene (rDNA) sequences (Glenn et al. 1996). Detection of those Clavicipitaceous endophytes in infected plants is important because of their ecological and economical influences as biocontrol and growth-promoting agents for host plants (Clay 1989; Malinowski and Belesky 2000) and also as potential mycotoxin producers (Siegel and Bush 1997). Although immunological (Hill et al. 2002; Hiatt et al. 1999) and DNA-based methods (Groppe et al. 1995; Groppe and Boller 1997; Moon et al. 1999, 2000) are utilized to detect endophytes in host grasses, microscopic observation of leaf sheaths, stems, or seeds (Saha et al. 1988; Ohkubo et al. 2000) is still commonly used due to the simplicity of the procedures. When seeds are used for the detection of endophytes, there is the risk of other flower or seed pathogens, such as other Clavicipitaceous fungi, being misidentified as *Neotyphodium* spp. Considering that the sole confirmed transmission method of *Neotyphodium* endophytes is by infection of embryos in inflorescences of host plants (i.e., vertical transmission; Clay and Schardl 2002), an understanding of the growth of endophytes in inflorescences (Fig. 1) is important. To our surprise, there are only limited references concerning floret infection of these endophytes (Philipson and Christey 1986) and no reports of flowers being used for endophyte detection. In this article, we report how *Neotyphodium* endophytes infect ovaries in developing inflorescences and discuss the feasibility of using microscopic observations of flowers as a routine detection procedure and to investigate seed transmission of these fungi.

Endophyte-infected tall fescue (*Festuca arundinacea* Schreb. cv. Kentucky 31), meadow fescue (*F. pratensis* Huds. cv. Harusakae), perennial ryegrass (*Lolium perenne* L. cv. SR4000), and Italian ryegrass (*L. multiflorum* Lam. clone no. 42-6-3-3 and no. 54-10-3-4) were used as plant materials. As a noninfected control, *L. multiflorum* cv. Wase-yutaka, an endophyte-free cultivar for pasture use, was used. Commercial cultivars used were purchased from local seed suppliers. The origin of these cultivars and the presence of *Neotyphodium* endophytes were reported by Bacon et al. (1997), Fergus and Buckner (1972), Meyer et al. (1989), and Takai et al. (2001). The infected *L. multiflorum* clones used were collected from a naturalized population in Shizuoka Prefecture, Japan. These grasses were grown in a glass house and in a field at the National Institute of Livestock and Grassland Science, Nishinasuno, Tochigi, Japan. Infection status of the grasses was confirmed microscopically by observing the inner cortex of leaf sheaths stained with lactic acid containing 0.1% (w/v) acid

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fuchsin (Ohkubo et al. 2000). Inflorescences of those grasses were collected before flower opening and soaked in a solution made by mixing lactic acid, glycerol, and distilled water in the ratio of 1:2:1 (v/v/v) (lactic acid in glycerol; Kirk et al. 2001) for 4 h to overnight at room temperature. After soaking, florets were dissected under a dissecting microscope to isolate the ovaries. Isolated ovaries were mounted on glass slides using lactic acid in glycerol, the same solution used for soaking. Mounted samples were observed with differential interference contrast (DIC) microscopy (Olympus BX50 with interference contrast filters; Olympus, Tokyo, Japan). The mycelium in flowers was also observed alive by digesting infected ovaries of *L. multiflorum* clone no. 54-10-3-4, using cellulase and pectinase following the

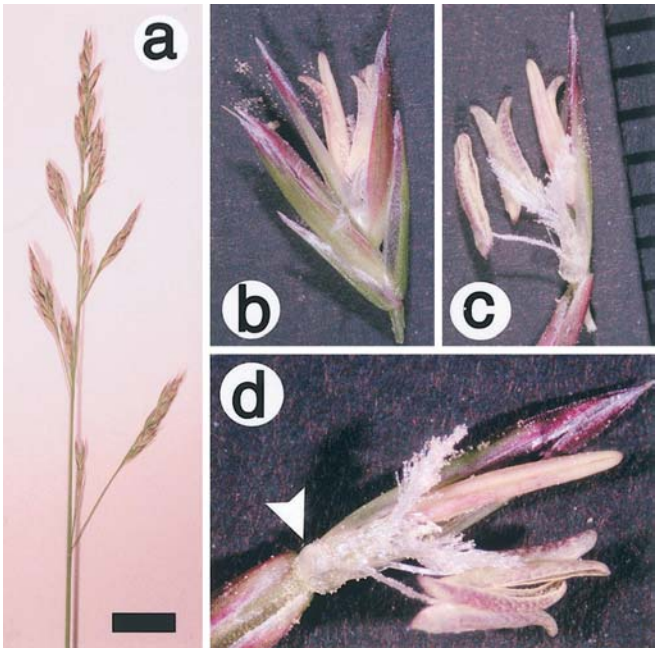

Fig. 1. Inflorescence/flowers of grass plant. **a** Inflorescence of tall fescue (*Festuca arundinacea*). *Bar* 1 cm. **b–d** Dissection process of a flower. *Scale* in **c** (right side) is an interval of 1 mm. *Arrowhead* in **d** indicates the ovary

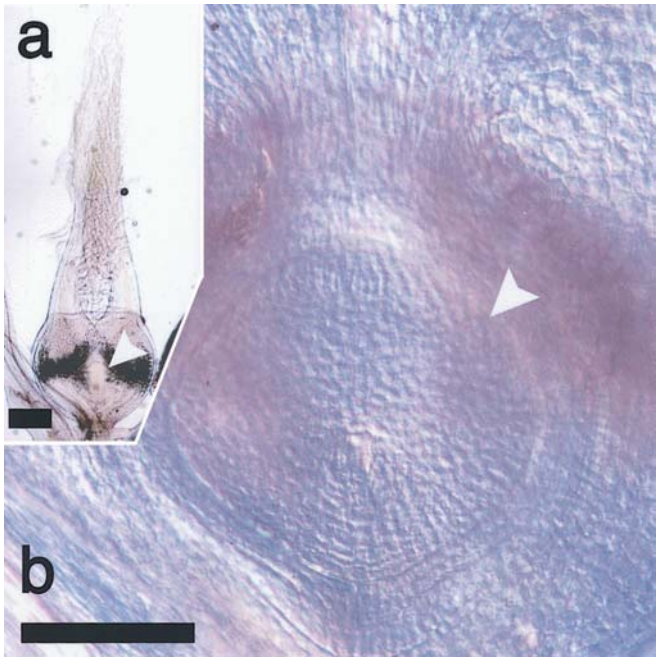

Fig. 2. Ovary isolated from *Lolium multiflorum* not infected with *Neotyphodium* endophyte. **a** Micrograph from bright-field microscopy. **b** Micrograph from differential interference contrast microscopy. *Arrowheads* indicate the ovule. *Bars* **a** 200 μm; **b** 100 μm

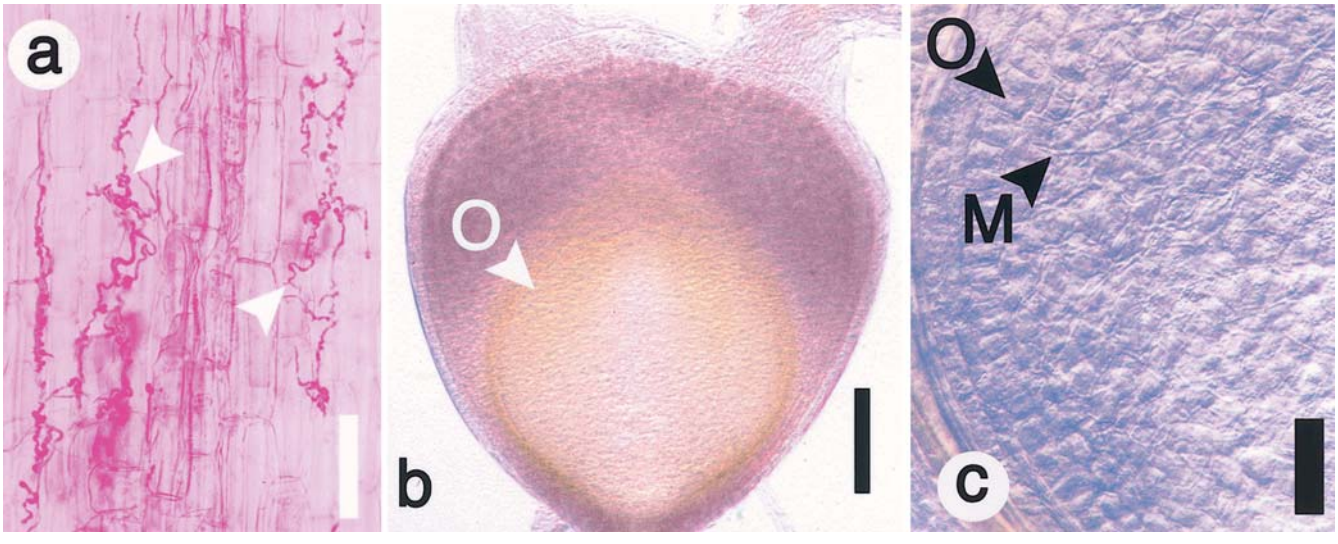

Fig. 3. *Neotyphodium* endophtye in tall fescue. **a** Mycelium of endophyte (*arrowheads*) observed in inner cortex of leaf sheath stained with lactic acid containing 0.1% (w/v) acid fuchsin (bright-field microscopy). **b** Ovary isolated from the same plant. **c** Close-up view of the area around the *arrowhead* in **b** (differential interference contrast microscopy). *O*, ovule; *M*, mycelium of endophyte. *Bars* **a** 100 μm; **b** 200 μm; **c** 50 μm

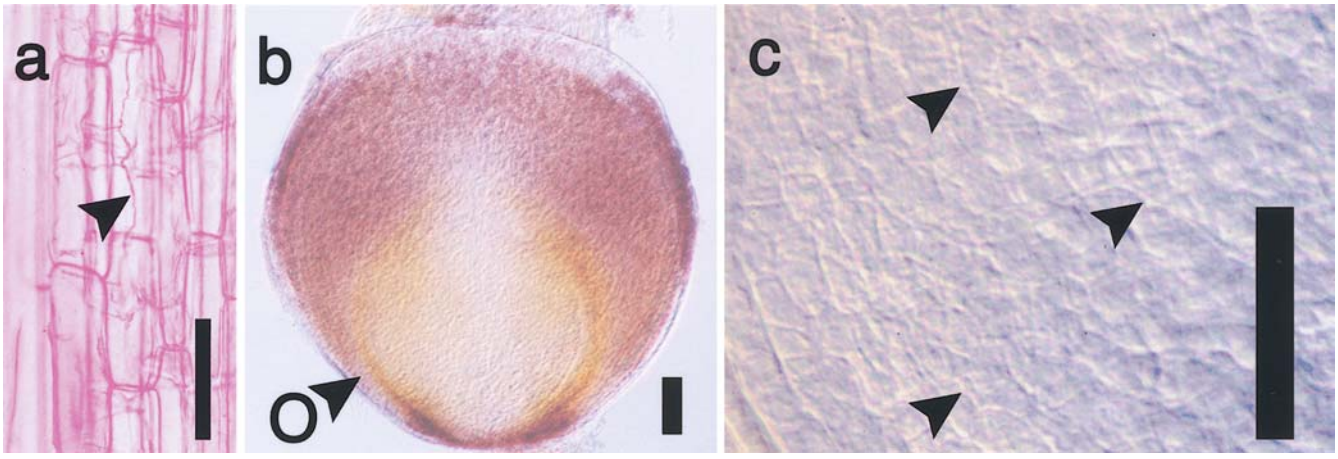

Fig. 4. *Neotyphodium* endophtye in meadow fescue (*Festuca pratensis*). **a** Mycelium of endophyte (*arrowheaded*) in inner cortex of leaf sheath stained with lactic acid containing 0.1% (w/v) acid fuchsin (bright-field microscopy). **b** Ovary isolated from the same plant. *O*, ovule. **c** Close-up view of the area around the *arrowhead* in **b** (differential interference contrast microscopy). *Arrowheads* indicate mycelium of endophyte. *Bars* **a,b** 100 μm; **c** 50 μm

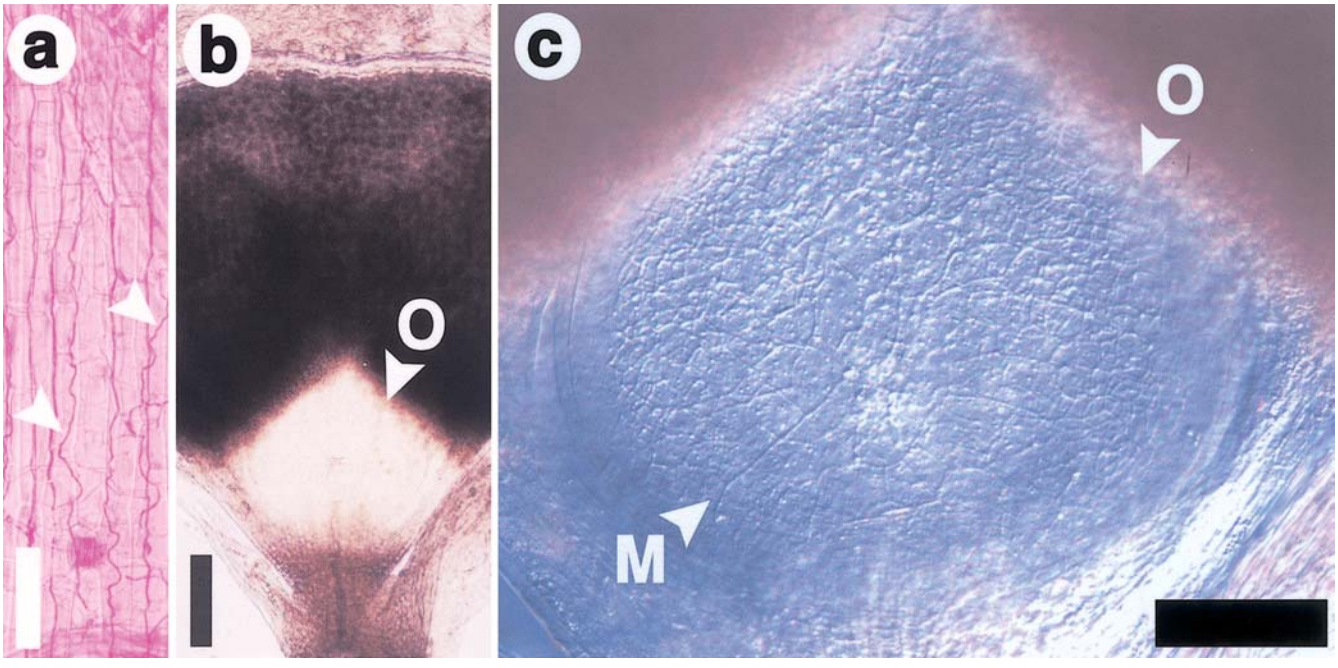

Fig. 5. *Neotyphodium* endophyte in perennial ryegrass (*Lolium perenne*). **a** Mycelium of endophyte (*arrowheads*) in inner cortex of leaf sheath stained with lactic acid containing 0.1% (w/v) acid fuchsin (bright-field microscopy). **b** Ovary isolated from the same plant. **c** Close-up view of the area around the *arrowhead* in **b** (differential interference contrast microscopy). *O*, ovule; *M*, mycelium of endophyte. *Bars* **a,c** 100 μm; **b** 200 μm

method described by Saito (1995). The mycelium visualized by the enzymic digestion was also used as a DNA source for sequencing rDNA to confirm the identity of the fungi. Total DNA was isolated from digested ovaries using a DNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) following the supplier's instruction. The nuclear rDNA region was amplified by polymerase chain reaction (PCR) using the primers ITS4 and ITS5 and sequenced directly with the same primers as described by White et al. (1990).

No hyphae were observed in florets from noninfected plants (Fig. 2). The presence or absence of endophytes in the plants included in this study was confirmed by examining leaf sheaths (Figs. 3a, 4a, 5a, 6a). Mycelium of the endophytes was clearly recognizable through the entire process of ovary development, from the very early stage (Fig. 6b,c) to just before opening of flowers (Fig. 5b,c). Examination of the florets revealed that in all the plants the *Neotyphodium* endophyte infected ovaries well before flower opening,

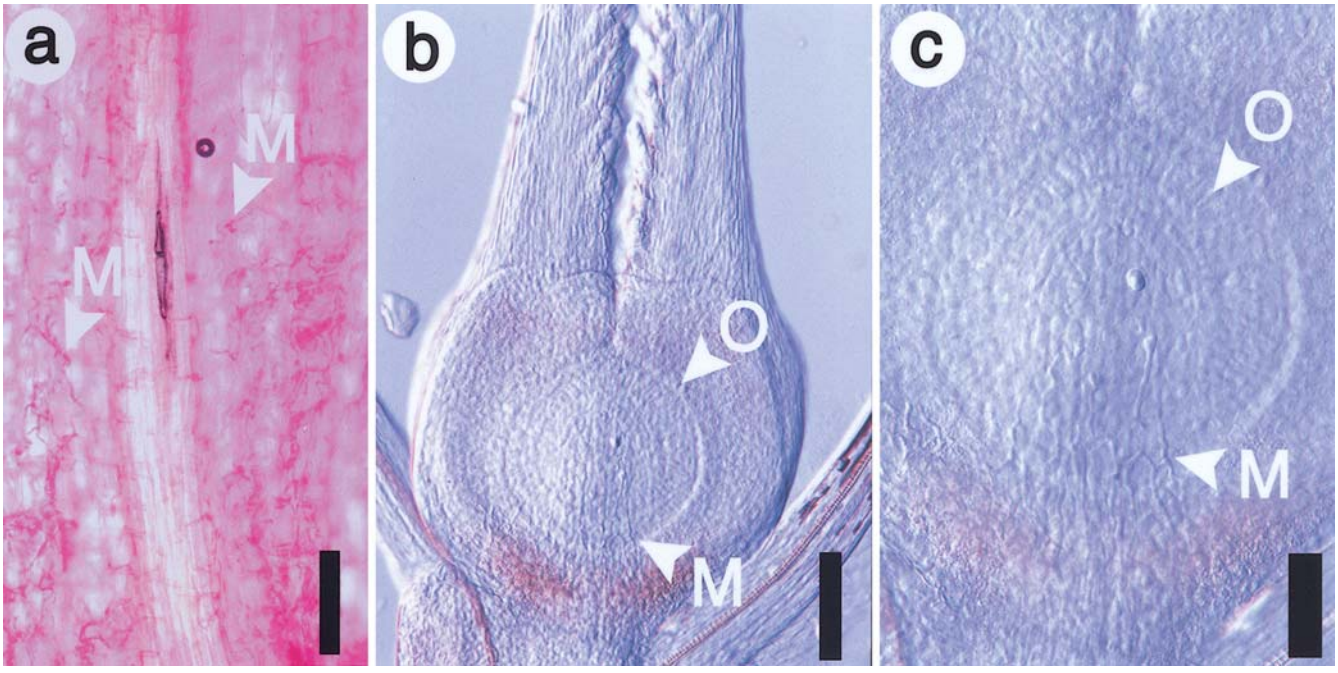

Fig. 6. *Neotyphodium* endophtye in Italian ryegrass (*Lolium multiflorum*, clone no. 42-6-3-3). **a** Mycelium of endophyte (*arrowheads*) in inner cortex of basal end of leaf sheath stained with lactic acid containing 0.1% (w/v) acid fuchsin (bright-field microscopy). **b,c** Ovary isolated from the same plant observed by differential interference contrast microscopy. *O*, ovule; *M*, mycelium of endophyte. *Bars* **a,b** 100 μm; **c** 50 μm

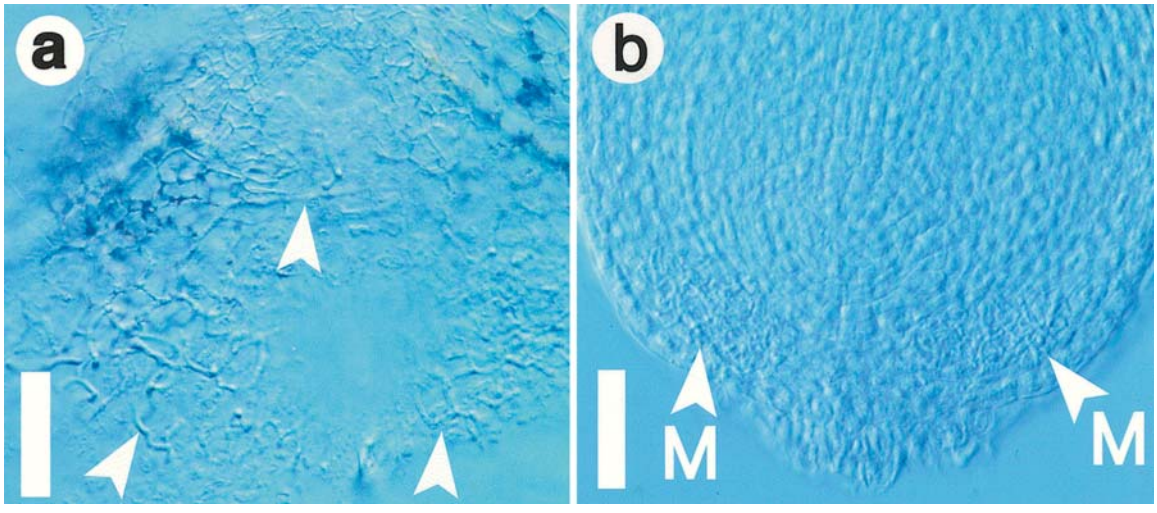

Fig. 7. *Neotyphodium* hyphae isolated by enzymic digestion of infected ovary from Italian ryegrass (*Lolium multiflorum*, clone no. 54-10-3-4), compared with its morphology in cleared infected ovary. **a** Mycelium in infected ovary visualized by digestion using cellulase and pectinase (*arrowheads*, differential interference contrast microscopy). **b** Ovary isolated from the same plant in **a**, cleared by lactic acid in glycerol (differential interference contrast microscopy). *M*, mycelium of endophyte. *Bars* 50 μm

passing through the placenta (Figs. 3b,c, 4b,c, 5b,c, 6b,c). Enzymic digestion of ovaries of *L. multiflorum* (clone no. 54-10-3-4) allowed us to observe the living mycelium in the plant tissue, and the morphology of the hyphae corresponded well with the results of observations using lactic acid in glycerol (Fig. 7). The PCR using total DNA extracted from the digested ovaries generated amplicons of two sizes, and the sequence alignment using BLASTN 2.2.6 in GenBank (www.ncbi.nlm.nih.gov/Genbank/index.html) revealed that the smaller one (approx. 600 bp; DDBJ accession number AB126352) had 99%–100% identity with that of five registered strains of *Neotyphodium* endophytes from *L. multiflorum* (Lrr1:AF176262, Lro1:AF176263, Lm1:AF176264, Lm2:AF176265, and Lrr2:AF176261;

Moon et al. 2000) and the larger one (approx. 700 bp; DDBJ accession number AB126353) had an identity greater than 90% with many *Lolium* and *Festuca* species.

Our observations on the endophyte infection process corresponded with descriptions by Philipson and Christey (1986) from infected flowers of *L. perenne* using electron microscopy, and our DNA data strongly support that the hyphae observed in those flowers were *Neotyphodium* species, although their morphology was not the identical to what observed in other parts of the host plants. Philipson and Christey (1986) mainly described the interaction between host cells and endophyte at high magnifications, whereas our method enabled us to acquire entire views of the infection process and the proliferation of the mycelium in ovaries. Chemical clearing of plant tissue has been widely used for microscopic observations (Herr 1971; Yadegari et al. 1994), but it has not been well applied for plant tissues infected with the endophyte. Although the method we reported here requires the use of DIC microscopy, it involves only simple, nontoxic chemicals and is very easy to perform. In addition, the method can be universally applied for *Neotyphodium* species considering their mode of infection, vertical transmission. Because flower morphology is a key to identify grass plants, collection and observation of inflorescences is essential for taxonomical and ecological studies of the grass family. Endophyte detection in flowers can add some value for these studies and has applications not only for mycologists but also for botanists, plant breeders, and agronomists. The method may be advanced further for histochemical and cytological analysis of the symbionts.

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