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Connection of *Gnomonia intermedia* to *Discula betulina* and its relationship to other taxa in *Gnomoniaceae*☆

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ABSTRACT

Discula betulina is a foliar pathogen on birch (*Betula*) and *Gnomonia intermedia* is found on overwintered birch leaves. Perithecia of *G. intermedia* developed *in vitro* on colonies of *D. betulina* isolated from birch tissues in late summer, and single ascospores of *G. intermedia* consistently developed into colonies similar to *D. betulina*, producing typical *D. betulina* conidia. Isolates of *D. betulina* could be grouped into two mating types, which produced fertile perithecia of *G. intermedia* when mated with each other. Mycelia from single-ascospore and single-conidial isolates were inoculated onto shoots of downy birch, causing lesions and die-back from which *D. betulina* was consistently isolated. ITS region ribosomal DNA sequence analysis confirmed the results of the morphological and mating studies, and found that the closest known relatives of *G. intermedia*/*D. betulina* are *Gnomoniella nana* and *Sirococcus clavigignenti-juglandaceae-rum*. The conclusion from these studies is that *D. betulina* is the anamorph of *G. intermedia*.

Introduction

Discula betulina (syn. *Gloeosporium betulinum*, *Myxosporium devastans*) is a common foliar pathogen of birch (*Betula* spp.) in the UK, continental Europe and North America, causing leaf spots that can lead to premature defoliation (Phillips & Burdekin 1982; Sinclair *et al.* 1987). The fungus is frequently observed on young, planted birch in Scotland, together with the other leaf spot fungi, *Marssonina betulae* (Green 2004) and *Septoria betulae* (Green 2005a). *Discula betulina* forms brown lesions with dark margins on both sides of the leaves, but fruits predominantly on the undersides. Although generally regarded as a leaf disease, *D. betulina* has also been found on diseased and healthy shoots of birch in Scotland (Green 2004), and caused die-back of young shoots when inoculated onto birch saplings (Paetzholdt & Schneider 1966; Green 2004). It is thought that *D. betulina* may contribute to the widespread die-back of young birch recently reported in Scotland, hence the increased recent interest in the biology of this fungus (Green 2004, 2005b).

Currently, little is known about the life cycle of *D. betulina*, and a sexual state has not yet been reported (Sinclair *et al.* 1987). When isolations were made from shoots of silver birch (*Betula pendula*) in Scotland during late summer, perithecia belonging to a species of *Gnomonia* developed on culture plates containing colonies of *D. betulina* (Green 2004). Based on the morphology of the perithecia and ascospores, this fungus was identified as *G. intermedia* by the Centraalbureau voor Schimmelcultures

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(CBS), Utrecht, The Netherlands. *G. intermedia* has been described from overwintered leaves of birch in Europe and North America (Barr 1978) but an association with *D. betulina* has not been made.

This paper reports on studies undertaken to test the hypothesis that *D. betulina* is the anamorph of *G. intermedia*. These include cultural, morphological and life-cycle studies that link the two fungal states. Isolates of *D. betulina* and *G. intermedia* were mated to assess sexual compatibility, and inoculated onto birch to compare pathogenicity and to satisfy Koch's Postulates. Finally, DNA analysis was undertaken to confirm the connection between *D. betulina* and *G. intermedia*, and to determine relatedness to other known members of *Gnomoniaceae*. The molecular data are discussed in terms of their implications for the taxonomy of genera within *Gnomoniaceae*.

Materials and methods

Fungal material, morphology and life cycle

Healthy and diseased young shoots and leaves of silver birch and downy birch (*Betula pubescens*) were collected from various locations in Scotland during August and September 2002 and 2004. Leaf and shoot pieces, approximately 5 mm^2, were cut from the margin of lesions or from healthy tissue, surface sterilised in 70 % ethanol for 1 min, 5 min in 1.25 % sodium hypochlorite and 30 s in 70 % ethanol. They were then rinsed twice in sterile distilled water, blotted dry on sterile filter paper, plated onto 2 % malt agar and incubated in the dark at room temperature for three to four weeks. Single-ascospore cultures of *Gnomonia intermedia* were obtained by squashing perithecia which developed *in vitro*, spreading the contents onto 2 % malt agar, incubating overnight at room temperature in the dark, and transferring germinated individual ascospores to fresh agar plates using a stereomicroscope. Cultures of *Discula betulina* were obtained by transferring mycelium or individual conidia from isolated colonies also growing *in vitro*. All cultures were grown on 2 % malt agar and incubated at 20/15 °C day/night temperatures with a 12 h photoperiod consisting of cool white fluorescent and near-ultra violet light. The sizes of 10 asci, 20 ascospores, and 20 conidia from each of five isolates of *G. intermedia* or *D. betulina* were measured using a compound microscope containing an eyepiece micrometer, making a total of 50 asci, 100 ascospores and 100 conidia measured.

To determine whether the teleomorph state is formed over winter, leaves of silver birch and downy birch, which were naturally infected with *D. betulina*, were placed on either wet or dry filter paper in plastic Petri dishes and maintained inside a cooled incubator at 4 °C with continuous darkness. Leaves maintained on the wet filter paper were misted lightly with distilled water at weekly intervals. Diseased leaves were also placed in 15 cm^2 nylon mesh bags and maintained outside on the ground. The study was set up in September 2004, and the leaves examined for formation of ascocarps in April 2005. Single ascospore cultures were obtained from fertile perithecia that developed on overwintered leaves.

Isolate crosses

This study was set up in October 2004 to test the ability of 20 isolates of *Discula betulina* and one isolate of *Gnomonia intermedia* (Table 1) to form fertile perithecia when mated. Pair wise crosses using all isolate combinations were performed on Petri plates containing 1 % water agar, with an autoclaved flat birch toothpick placed across the centre of each plate to provide a substrate for the formation of perithecia. Mycelial plugs of the two parental strains were placed on the agar surface, one either side of the toothpick. Inoculated Petri plates were incubated at room temperature in direct, natural light, and examined for the development of perithecia at two, four and six months after inoculation. When perithecia were observed in any pair-wise cross, they were crushed and the contents examined under a compound microscope to confirm the presence of mature ascospores. Single-ascospore isolates were obtained from fertile perithecia which developed during this study.

Pathogenicity tests

Pathogenicity tests were carried out on one-year-old seedlings of Scottish provenance downy birch, which were grown from seed in a greenhouse. Two isolates of *Discula betulina* and two isolates of *Gnomonia intermedia* were inoculated onto four replicate seedlings per isolate using 3 mm diameter mycelial plugs cut from the growing margin of single-conidial and single-ascospore cultures on 2 % malt agar. Four seedlings were inoculated with plugs of sterile 2 % malt agar as controls. The plugs were placed, mycelial side down, onto the expanding leading shoot of each seedling, 2–3 cm from the base of the current year's shoot extension. Shoots were wounded before inoculation by gently scraping back a 10×2 mm section of epidermis at the inoculation site using a sterile scalpel. After inoculation, a droplet of sterile distilled water was placed on each plug, and the plugs sealed in place with parafilm. Four mycelial plugs of each isolate were also plated onto 2 % malt agar, incubated in the dark at room temperature for 7 d, and examined for colony development to confirm inoculum viability. Inoculated seedlings were placed outside for 14 d, at which time the parafilm was removed and lesion lengths on the leading shoots measured. Back-isolations were carried out from a lesion on one seedling per isolate, and from the inoculation site on one control seedling, using methodology described above. Four tissue pieces per isolate or control treatment were plated onto 2 % malt agar, incubated in the dark at room temperature for three to four weeks, and colonies identified by morphological characteristics.

DNA analysis

All isolates sequenced were isolated from silver birch or were the result of crosses of isolates from silver birch (Table 1). Genomic DNA was extracted from approximately 50 mg of mycelia scraped from the surface of a 3–5 d old culture growing on Difco potato dextrose agar (PDA). The ITS regions 1 and 2 of the nu-rDNA including the 5.8 S nu-rDNA were amplified using primers ITS 5 and ITS 4 (White *et al.* 1990). Five isolates of *Discula betulina* (CBS 119202 = 2006a, CBS 119193 = 2041a, CBS 119518 = 2043a, CBS 119188 = 2044a, CBS 119352 = 2081) and four isolates of *Gnomonia intermedia* (CBS 119194 = 2048a, CBS 119196 = 2070a, CBS 119192 = 2083b, CBS 119197 = 2087a; Table 1) were sequenced. In addition, the ITS gene region was sequenced for representatives of taxa within the