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Phylogenetic relationships between members of the crucifer pathogenic *Leptosphaeria maculans* species complex as shown by mating type (*MAT1-2*), actin, and β-tubulin sequences

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Abstract

The dothideomycetous fungus *Leptosphaeria maculans* comprises a complex of species differing in specificity and pathogenicity on *Brassica napus*. Twenty-eight isolates were investigated and compared to 20 other species of the Pleosporales order. Sequences of the mating type *MAT1-2* (23), fragments of actin (48) and β-tubulin (45) genes were determined and used for phylogenetic analyses inferred by maximum parsimony, distance, maximum likelihood, and Bayesian approaches. These different approaches using single genes essentially confirmed findings using concatanated sequences. *L. maculans* formed a monophyletic group separate from *Leptosphaeria biglobosa*. The *L. biglobosa* clade encompasses five sub-clades; this finding is consistent with classification made previously on the basis of internal-transcribed sequences of the ribosomal DNA repeat. The propensity for purifying and neutral evolution of the three genes was determined using sliding window analysis, a technique not previously applied to genes of filamentous fungi. For members of the *L. maculans* species complex, this approach showed that in comparison to actin and β-tubulin, exonic sequences of *MAT1-2* were more diverse and appeared to evolve at a faster rate. However, different regions of *MAT1-2* displayed different degrees of sequence conservation. The more conserved upstream region (including the High Mobility Group domain) may be better suited for interspecies differentiation, while the more diverse downstream region is more appropriate for intraspecies comparisons.
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Keywords: Mating type; *Leptosphaeria maculans*; Phylogeny; Pleosporales; uORFs; Sliding window analyses

1. Introduction

Leptosphaeria maculans (Desm.) Ces. & de Not. (anamorph: *Phoma lingam* (Tode ex Fr.) Desm.) is a dothideomycetous fungus (order: Pleosporales). It comprises a species complex of plant pathogens, some of which cause blackleg (phoma stem canker), a disease responsible for significant yield losses of the *Brassica napus* (canola/oilseed rape) worldwide. Blackleg epidemics occurred in Europe in the 1960s and when the crop was first introduced to Australia in the early 1970s. Since then yield losses in Australia have been about 5% annually, but in 2003 losses of up to 90% occurred in some regions when blackleg resistance conferred by a single gene was overcome. In Europe and Canada, losses are significant but not as high as in Australia (Howlett, 2004).

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The members of this species complex look similar both macroscopically and microscopically. Accordingly the taxonomy and nomenclature associated with this fungus is confusing. Historically, isolates have been divided into two pathotypes (A and B groups or A and NA groups) on the basis of a range of characters including RFLP analysis and secretion of a phytotoxin, sirodesmin PL, into culture. Other isolates classified as *L. maculans* have been cultured from cruciferous weeds such as *Thlaspi arvense*, *Sisymbrium* spp., *Descurainia* spp., *Lepidium* spp., and *Erysimum* spp. (for reviews, see Howlett et al., 2001; Williams and Fitt, 1999). Isolates of the 'A' group are distributed worldwide and cause blackleg. They have been subdivided into several pathogenicity groups (e.g., PG2, PG3, and PG4) based on differential disease reactions on cotyledons of *B. napus* cultivars (Koch et al., 1991). Restriction fragment length polymorphism (RFLP) analysis has shown that these isolates have a high degree of genetic similarity and are most likely a single species, whilst 'B' group isolates belong to three genetically distinct sub-groups (NA1, NA2, and NA3) (Koch et al., 1991). Isolates of the NA1 sub-class cause less severe symptoms than those of the 'A' group on *B. napus*. They have a more restricted distribution and are not found in Australia or central Canada. Isolates of the NA2 and NA3 sub-groups are not found in Europe and do not cause symptoms on *B. napus*. Isolates cultured from cruciferous weeds are genetically dissimilar to isolates of 'A' and 'B' groups and do not cause symptoms on *B. napus* (Purwantara et al., 2000; Voigt et al., 2001).

Two recent studies have addressed taxonomic relationships and nomenclature of members of this species complex. First, two B isolates were classified as *Leptosphaeria biglobosa* based on morphological characters and the inability of isolates to sexually cross with the 'A' group isolates (Shoemaker and Brun, 2001). This new species differs from 'A' group isolates by having a conspicuous beak on pseudothecia that is enlarged at the apex. This study did not describe the B sub-group to which the *L. biglobosa* type isolate belonged. The second study proposed relationships of *L. biglobosa* and *L. maculans* based on the sequence of the internal-transcribed spacer (ITS) region of the ribosomal DNA (rDNA) repeat and also identified the *L. biglobosa* reference isolates as belonging to the NA1 sub-group (Mendes-Pereira et al., 2003). The *L. maculans* species complex fell into two clades corresponding to *L. maculans sensu stricto* and *L. biglobosa*. Within these clades, sub-clades were given names that corresponded to host or geographic origin. These were *L. maculans* 'brassicae' and *L. ma culans* 'lepidii' (one isolate only cultured from *Lepidium* spp.) and *L. biglobosa* 'brassicae,' *L. biglobosa* 'thlaspii' (two isolates cultured from *Thlaspi* spp.), *L. biglobosa* 'erysimii' (one isolate cultured from *Erysimum* spp.), *L. biglobosa* 'canadensis' and *L. biglobosa* 'australensis.' As these authors point out, in the absence of formal descriptions, these sub-clades cannot be assigned to defined, distinct species (Mendes-Pereira et al., 2003). To avoid confusion this nomenclature is used in this paper.

Since the taxonomic revision of this economically important group of plant pathogens is so recent, it is desirable to test its validity using additional characters to rDNA. Ribosomal DNA has some limitations as an indicator of phylogenetic relationships. Mutations occur in rDNA without immediate effects at the functional, and thus the selective level. Also there is generally a low degree of interspecific polymorphism between closely related organisms, due to concerted evolution. Furthermore, arrangement of rDNA in isolates of *L. maculans* 'brassicae' appears to differ to that in other haploid fungi. Copy number is highly variable; in a previous study, four isolates examined had rDNA copy numbers ranging from 56 to 225 and the non-transcribed intergenic spacer regions were of different sizes, even within individual isolates (Howlett et al., 1997). Mating type (*MAT*) genes are useful determinants of phylogenetic relationships of closely related species (Pöggeler, 1999; Turgeon, 1998). Dothideomycetes have a single *MAT* locus with two alternate forms (idiomorphs) that must be different for two isolates to mate. These idiomorphs encode single proteins with DNA-binding domains, such as α boxes for *MAT1-1* isolates and high mobility group (HMG) DNA-binding domain for *MAT1-2* isolates (Turgeon, 1998). Nucleotide comparisons among *MAT* genes from ascomycetes *Cochliobolus*, *Neurospora*, and *Sordaria* revealed that these genes appear to evolve at a faster rate than other genome sequences. Also *MAT* sequences appear to be conserved within species, in contrast to high variability between species (Pöggeler, 1999; Turgeon, 1998). The *MAT* locus has been characterised in *L. maculans* 'brassicae,' but not in any other *Leptosphaeria* species. This locus includes a *MAT1-2* gene which encodes a predicted protein of 399 amino acids, with a 55 bp intron and a small open reading frame directly upstream of the gene (Cozijnsen and Howlett, 2003).

For phylogenetic analyses, as well as comparing genes that evolve quickly, it is important to examine housekeeping genes where sequence divergence would be expected to be minimal. Such genes include glyceraldehyde-3-phosphate dehydrogenase, the translation elongation factor 1α and the cytoskeletal genes, actin and β-tubulin (Einax and Voigt, 2003; Kang et al., 2001; Pöggeler, 1999; Voigt and Wöstemeyer, 2000). In this paper, we compare phylogenetic relationships between 28 isolates belonging to the *L. maculans* species complex and 20 other dothideomycetes, using sequences of *MAT1-2* loci, actin, and β-tubulin to relationships inferred by Mendes-Pereira et al. (2003) on the basis of ITS

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