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RNA polymerase II gene (*RPB2*) encoding the second largest protein subunit in *Phaeosphaeria nodorum* and *P. avenaria*

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ABSTRACT

A 5586 bp sequence (accession no. DQ278491), which includes the RNA polymerase II gene (*RPB2*) encoding the second largest protein subunit (RPB2), was obtained from the wheat biotype *Phaeosphaeria nodorum* (PN-w) by PCR amplification. The 3841 bp full length *RPB2* gene contains two exons and a 52 bp intron, and encodes a complete 1262 amino acid protein. Similar to the C-terminals of the β subunits of prokaryotes and yeast RNA polymerases, the deduced RPB2 protein contained many structural features needed for gene transcription. Based on the phylogenetic analysis with the deduced RPB2 polypeptide sequences, the PN-w was closely related to the maize pathogen *Cochliobolus heterostrophus*. Size differences were found in the full length *RPB2* gene of cereal *Phaeosphaeria* species, mainly due to differences in intron size. No nucleotide substitutions were found in homothallic *P. avenaria* f.sp. *triticea* (Pat1) and barley biotype *P. nodorum* (PN-b) isolates used in this study. The nucleotide and deduced amino acid sequences of the *RPB2* gene in Pat1 were closely related to that in PN-w.

Introduction

Phaeosphaeria nodorum (syn. *Leptosphaeria nodorum*; anamorph: *Stagonospora nodorum*, Syn. *Septoria nodorum*) and *Phaeosphaeria avenaria* (Syn. *Leptosphaeria avenaria*; anamorph: *Stagonospora avenae*, Syn. *Septoria avenae*), are two of the most important causal agents of cereal Stagonospora leaf blotch diseases (Couture 1989; Noble & Montgomerie 1954; Scharen & Sanderson 1985; Sprague 1950; Weber 1922a,b). The identification of these two pathogens is based largely on morphology of the anamorph (Cunfer 1997, 2000; Cunfer & Ueng 1999; Richardson & Noble 1970). Based on its pathogenicity to wheat (*Triticum aestivum*) or barley (*Hordeum vulgare*), *P. nodorum* was further recognized as having two special forms, a wheat biotype (PN-w) and a barley biotype (PN-b) (Holmes & Colhoun 1970; Smedegård-Petersen 1974). *P. avenaria* was also divided

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into two groups; *P. avenaria* f.sp. *avenaria* (Paa) which infects oats (*Avena* spp.), and *P. avenaria* f.sp. *triticea* (Pat) which infects wheat, barley, rye (*Secale cereale*) and several grasses (Johnson 1947; Shaw 1957).

In recent years, genetic relatedness and differentiation of cereal *Phaeosphaeria* species have been examined at the molecular level. Sequence data from rDNA ITS, mating type gene (*mat1*) conserved regions, and genes encoding glyceraldehyde-3-phosphate dehydrogenase (*gpd*), β-glucosidase (*bgl1*) and β-tubulin (*tubA*) have been used to define phylogenetic relationships among PN-w and PN-b, three genetically distinct groups of Pat (homothallic Pat1, heterothallic Pat2 and Pat3), and Paa (Malkus *et al.* 2005; Reszka *et al.* 2005; Ueng *et al.* 1998, 2003a,b). In 1995, based on sequence diversity, a new *Phaeosphaeria* sp. isolated from Polish winter rye was identified (Reszka *et al.* 2006). This new species has similar asexual pycnidiospore morphology and causes the same disease symptoms in cereals as *P. nodorum*. However, this new species has different intron sizes and sequences in the *bgl1* and *tubA* genes (Malkus *et al.* 2005; Reszka *et al.* 2005).

In addition to ITS and LSU and SSU rDNA, sequence data from genes encoding structural and functional proteins have recently been used to broaden the base of molecular characters that play important roles in phylogenetic studies of eukaryotes. It was suggested that the single-copy genes, which encode polypeptides with sufficient length (~400 amino acids), and ≥65–70 % identities among all taxa are good candidates for resolving the deep phylogenetic relations in fungi (Baldauf *et al.* 2000; Sicheritz-Pontén & Andersson 2001). RNA polymerase II is one of the three RNA polymerases in eukaryotes which catalyse the transcription of messenger RNA. In yeasts, RNA polymerase II holoenzyme is composed of ten to twelve protein subunits (RPB1–RPB12), and the genes encoding these protein subunits are scattered throughout the genome (Archambault & Friesen 1993; Ishihama *et al.* 1998; Sakurai & Ishihama 1997; Young 1991). The RNA polymerase II gene (*RPB2*), which encodes the second largest protein subunit, is suggested to be a single-copy gene in fungi. The second largest protein subunit of RNA polymerase II (RPB2) is well conserved. Twelve highly conserved regions of the RPB2 protein subunit have >85 % amino acid identity among fungi, plants, and animals (James *et al.* 1991). Therefore, in combination with numerous morphological characteristics and other nucleotide and deduced polypeptide sequence data, the partial *RPB2* gene sequence has been used in parsimony analysis to detect phylogenetic links and to understand evolution in higher ascomycetes and lichen-forming lower ascomycetes (Chaverri & Samuels 2003; Chaverri *et al.* 2003; Hansen *et al.* 2005; Liu & Hall 2004; Liu *et al.* 1999; Lumbsch 2000; Lutzoni *et al.* 2004; Miller & Huhndorf 2005; Reeb *et al.* 2004; Staats *et al.* 2005; Zhang & Blackwell 2001, 2002; Zhong & Pfister 2004).

Based on the sequences of ITS, *mat1* conserved regions and the *bgl1*, *gpd* and *tubA* genes, the phylogenetic relationships of *Phaeosphaeria* species have been discussed (Malkus *et al.* 2005; Reszka *et al.* 2005; Ueng *et al.* 1998, 2003a,b). Whether the *RPB2* gene and its deduced amino acid sequences could be used to support the phylogenetic relationships of cereal *Phaeosphaeria* species remained to be studied. Developing a reliable molecular method for *Phaeosphaeria* identification will not only help to diagnose cereal Stagonospora leaf blotch diseases, but also help to develop potential disease control strategies. Hence, in this study, the sequence variation of the *RPB2* gene was used to develop a rapid assay for these two important cereal *Phaeosphaeria* species. In addition, the *RPB2* gene may assist in the detection and differentiation of other grass *Phaeosphaeria* species.

Materials and methods

The *RPB2* gene from wheat biotype *P. nodorum* was amplified from the isolate Sn37-1. Procedures for fungal culture in a liquid medium and for genomic DNA (gDNA) isolation were described previously (Ueng *et al.* 1992). The partial *RPB2* gene was first PCR-amplified with degenerate primer sets, *f*RPB2-5F/*f*RPB2-7cR and *f*RPB2-7cF/RPB2-11bR, which represent three separate amino acid-conserved motifs (5th, 7th and 11th) in the RPB2 polypeptide throughout eukaryotes (Figs 1–2, Table 1) (Liu *et al.* 1999). These two DNA sequences were joined by a fragment amplified with the primer set 2A/

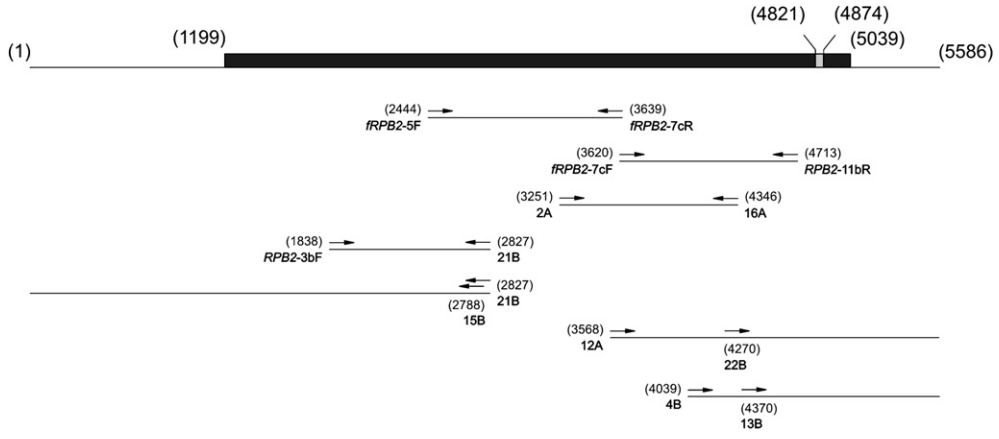

Fig 1 – PCR strategies for isolation of the full-length *RPB2* gene encoding the second largest subunit of RNA polymerase II in wheat biotype *Phaeosphaeria nodorum*. Two exons (nt1199–nt4821 and nt4874–nt5039) are shown in black boxes, and one intron (between nt4822 and nt4873) in grey box. The PCR primers with their amplifying directions are indicated in arrows, and nucleotide positions corresponding to the full-length sequence of the *RPB2* gene are shown in parentheses.

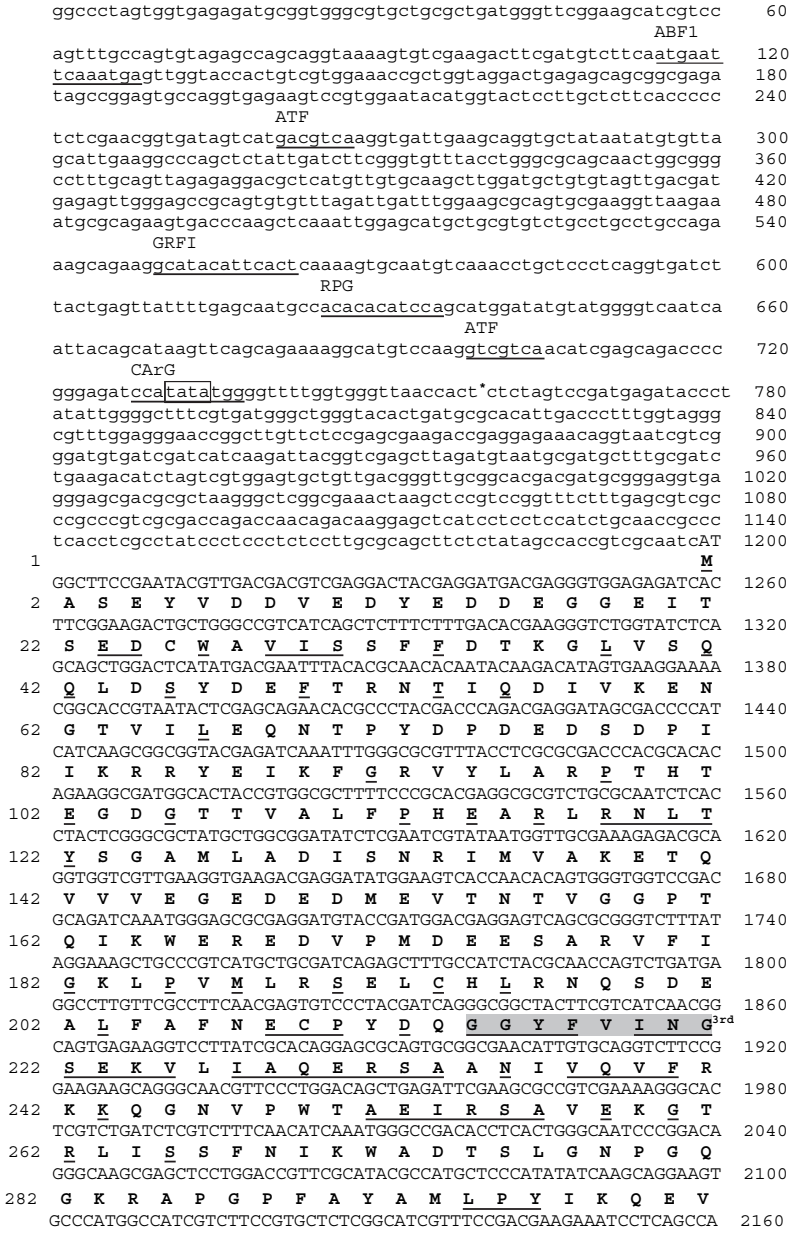

Fig 2 – Nucleotide and deduced peptide sequences of the *RPB2* gene encoding the second largest subunit of RNA polymerase II in wheat biotype *Phaeosphaeria nodorum* isolate Sn37-1. The numbers in the right column are for nucleotide numbering, and the ones in the left column for amino acids. The transcription factor binding sites ABF1, ATF, CArG, GRFI and RPG are underlined. The putative TATA box is boxed. The asterisk (*) indicates the potential transcription starting site (TSS). Lowercase letters indicate the non-translated regions of the *RPB2* gene including an intron. The double-underlined nucleotides denote the position of a putative polyadenylation signal. Letters in bold, uppercase and below the nucleotide coding sequence indicate the putative peptide sequence. Four amino acid motifs (3rd, 5th, 7th and 11th) that are conserved throughout eukaryotes and were used for the design of degenerate primers for PCR amplification are shaded. Underlined amino acids are conserved in full-length RPB2 subunit of RNA polymerase II found in 11 ascomycetes. Period (.) is the stop codon. The open circles above amino acids (GX_5GKT) (aa942-aa950) are the purine nucleotide-binding motif, and the solid circles ($CX_2CX_{15}CX_2C$) (aa1198–aa1220) indicate the zinc-binding motif. Two amino acid sequence clusters (aa997–aa1014 and aa1129–aa1137) involved in the formation of the active site of RNA polymerase are over-lined, and two potential lysine amino acids (K_{1014} and K_{1137}) for nucleotide analogues affinity labelling within the active site are indicated by open squares. Solid triangles indicate the bacterial RNase conserved amino acids residues.

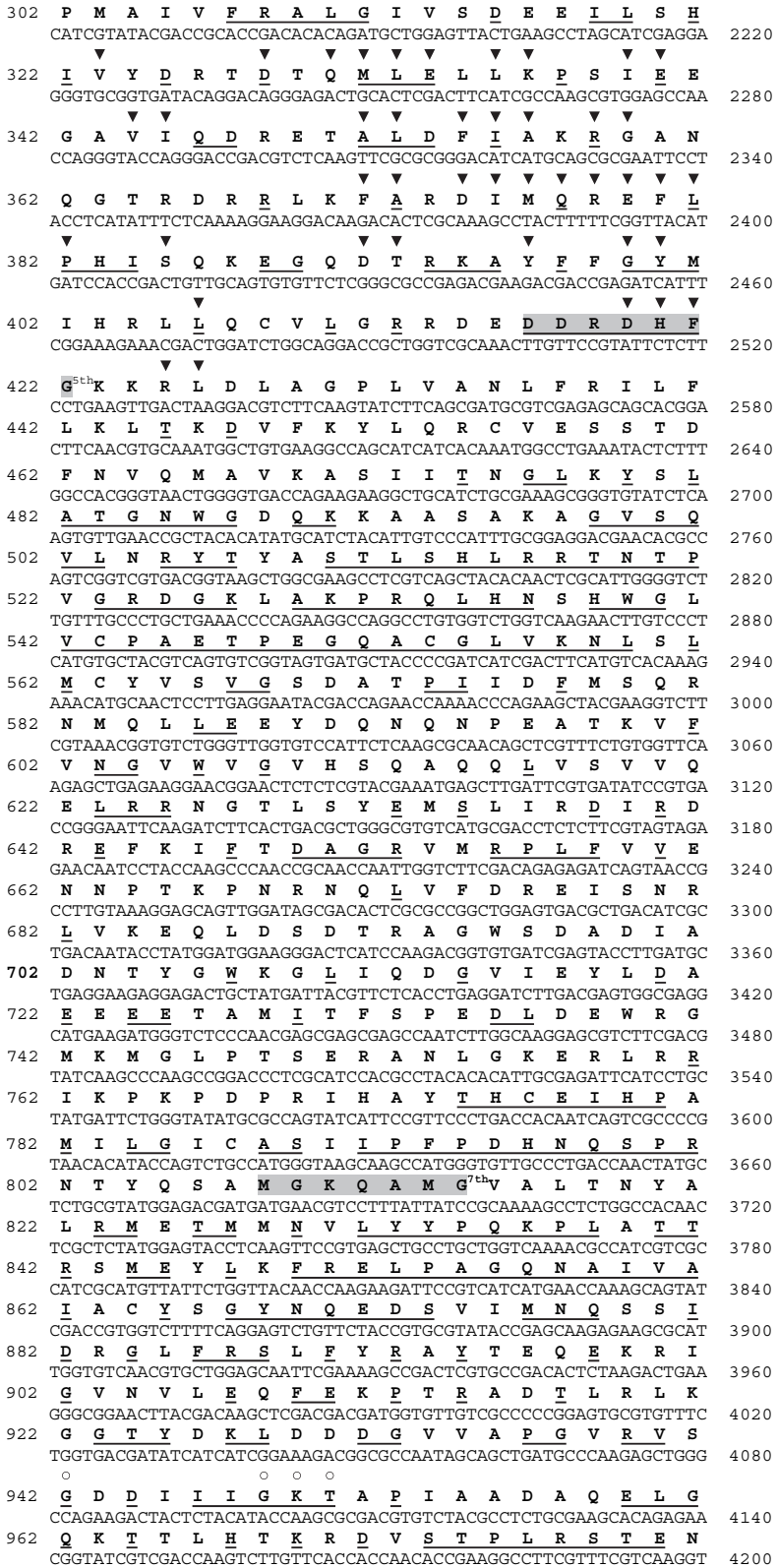

Fig 2 (*continued*)

16A, and extended to the 5′ end of the *RPB2* gene with the primer set *RPB2*-3bF/21B (Liu *et al.* 1999) (Figs 1–2, Table 1). PCR amplification was performed in 50 μl reaction mixtures containing 1× reaction buffer (50 mM KCl, 10 mM Tris–HCl, pH 9.0 at 25 °C, 0.1 % Triton X-100), 1.25 mM $MgCl_2$, 0.2 mM dNTPs, 2 μM of each primer, 80 ng gDNA, and 1.0 unit of *Taq* DNA polymerase (Promega, Madison, WI). Reaction parameters were: denaturation (94 °C, 3 min) followed by 40 cycles

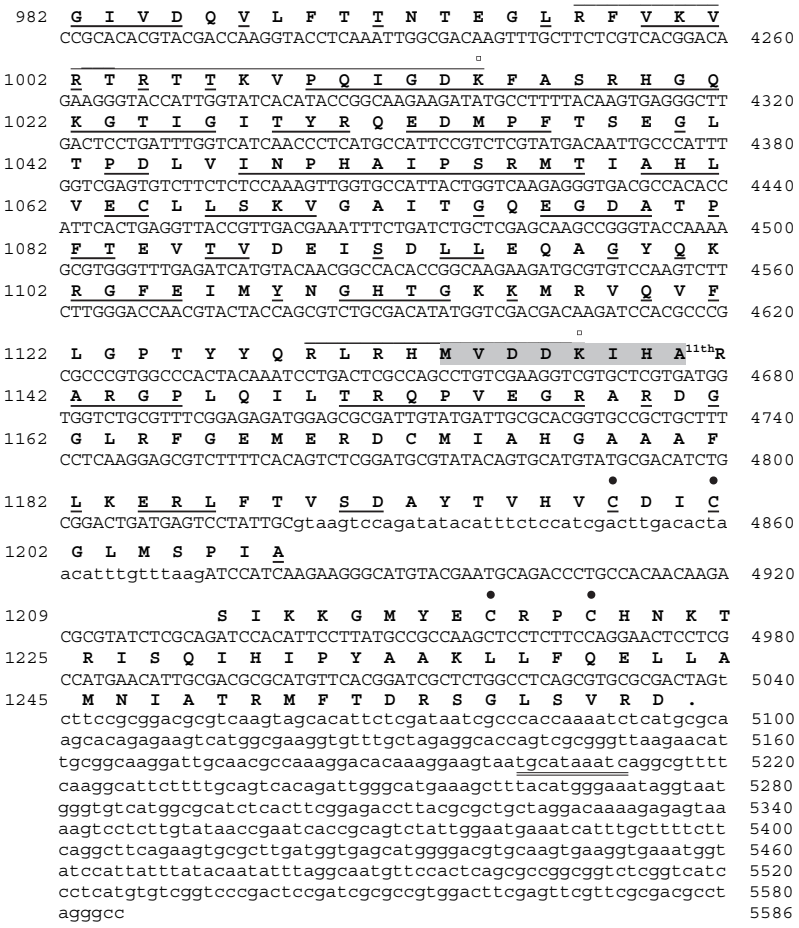

Fig 2 (*continued*)

of 94 °C (20 s), 55 °C (30 s), and 72 °C (1 min), and a final incubation at 72 °C (10 min). In this way, a 2876 bp partial *RPB2* gene sequence was obtained.

The 5′ and 3′ flanking genomic *RPB2* gene sequences were obtained with a 'step-down' PCR amplification technique (Zhang & Gurr 2002). Endonuclease restrictions and adaptor ligations of gDNA followed the protocols as described (Zhang & Gurr 2002). PCR amplification was performed in 20 µl reaction mixtures containing 1× reaction buffer (15 mM potassium acetate, 40 mM tricine–potassium hydroxide, pH 9.2 at 25 °C, 3.5 mM Mg acetate and bovine serum albumin 3.75 µg ml^{-1}), 0.2 mM dNTPs, 10 µM of each primer, 10 ng ligated gDNA, and 1× Advantage cDNA polymerase mix (BD Biosciences-Clontech, Mountain View, CA). Reaction parameters were: three cycles of 94 °C for 2 s and 72 °C for 3 min, three cycles of 94 °C for 2 s and 70 °C for 3 min, and three cycles of 94 °C for 2 s and 68 °C for 3 min, followed by 26 cycles of 94 °C (2 s), 66 °C (20 s), and 68 °C (3 min), and a final incubation at 68 °C (8 min). Three DNA fragments representing the 5′ flanking region of the partial *RPB2* gene were first amplified from *Spe*I, *Xma*I and *Bsp*EI enzyme restricted/adaptor ligated gDNA fragments with the primer set 21B/PP1, and subsequently with the nested primer set 15B/PP2 (Fig 1, Table 1). For the 3′ flanking region, three *Spe*I, *Nhe*I and *Xba*I enzyme restricted/adaptor ligated gDNA fragments were amplified with the primer set 12A/PP1 and then with the nested primer set 22B/PP2. The fourth PCR product was amplified from *Bsp*EI restricted/adaptor ligated gDNA fragments with the primer set 4B/PP1 and the nested one, 13B/PP2 (Fig 1, Table 1). Both primers PP1 and PP2 recognized sequences on the adaptors 1 and 2, and served as the upstream primers for PCR (Zhang & Gurr 2002). Primers 21B, 15B, 12A, 22B, 4B and 13B were designed from the obtained 2876 bp partial *RPB2* gene sequence as described above. Isolation and direct sequencing of PCR products were conducted as described previously (Ueng *et al.* 2003b).

To determine the expressed *RPB2* gene sequence, total RNA was isolated from the wheat biotype *P. nodorum* isolate Sn37-1 culture grown in a liquid medium containing 0.5 % malt extract, 0.5 % yeast extract and 1 % cellulose with shaking at 125 rev min^{-1} for 14 d at 27 °C. The mycelia were harvested, washed with 2 % sodium chloride solution and flash-frozen in liquid nitrogen. The total RNA was extracted from mortar and pestle pulverized mycelia using the RNeasy Plant Mini Kit and RNase-Free DNase I enzyme (Qiagen Inc., Valencia, CA). Using the First Strand cDNA Synthesis Kit with the Oligo-p(dT)$_{15}$ primer (Roche Diagnostics Corporation, Indianapolis, IN) and the thermocycler settings (25 °C for 10 min, 42 °C for 60 min, 99 °C for 5 min, and 4 °C for 5 min), the first

strand cDNA synthesis was performed. To determine the *RPB2* gene structure, seven primer sets, m1A1/m1B, m1A3/m2B, m2A/m3B, m3A/m4B, m4A/m5B, m5A/m6B and m6A/m8B, were used to amplify the cDNA with the Advantage cDNA PCR Kit (BD Biosciences-Clontech, Mountain View, CA; Table 1). These primer sets provide the PCR fragments that represent the transcribed *RPB2* gene-coding region. Reaction parameters were: 94 °C for 1 min, 34 cycles of 94 °C for 30 s, 55 °C for 3 min and 68 °C for 3 min. The fragments were isolated, directly sequenced and compared with the *RPB2* genomic sequence (Ueng *et al.* 2003b).

Seven primer sets designed from the *RPB2* gene sequence of PN-w isolate Sn37-1 were used for PCR amplification of the same gene from ten PN-b, ten Paa, fourteen Pat1, two Pat2 from wild barley (foxtail barley, *Hordeum jubatum;*), one Pat3 from state of Washington, two *Phaeosphaeria* spp. from Polish ryes, one from dallis grass (*Paspalum dilatatum*) and sixteen other PN-w (Tables 1–2). Two oligonucleotide primers, m52A and m52B, were designed from the known sequences of PN-b, Paa and Pat1 for amplifying the corresponding PCR fragments in these *Phaeosphaeria* species (Table 1). These primer sets produced overlapping fragments that together represented the full-length *RPB2* gene coding region. Protocols for PCR amplification were as those for the partial *RPB2* gene. Isolation and direct sequencing of PCR products were conducted (Ueng *et al.* 2003b).

Based on the nucleotide and deduced polypeptide sequences of the *RPB2* gene, phylogenetic relationships within two cereal *Phaeosphaeria* species were analysed using the Phylogeny Inference Package (PHYLIP) Version 3.6 (alpha2) (http://evolution.genetics.washington.edu/phylip.html) (Felsenstein 1989). The nucleotide and polypeptide sequences were aligned with CLUSTAL_X (1.83) in a multiple sequence alignment mode (Thompson *et al.* 1997). From the aligned sequences, 1K data sets were generated by BS re-sampling in the 'seqboot' program. The bootstrapped data sets were evaluated by the ML method with 'dnaml' and 'proml' programs. Finally, the 'consense' program was used to construct a 'tree'.

Results

Using PCR amplification with degenerate primer sets and the 'step-down' technique, a 5586 bp sequence (accession no.

Table 1 – Oligonucleotide primers used to amplify PCR products from the gene encoding the second largest protein subunit of RNA polymerase II (*RPB2*)

Name	Nucleotide position (no.)[a]	Sequence 5′→3′[c]
Genomic DNA fragments amplified from wheat biotype *Phaeosphaeria nodorum* isolate Sn37-1		
*f*RPB2-5F/*f*RPB2-7cR	2444–2463/3639–3620	GAYGAYMGWGATCAYTTYGG/CCCATRGCTTGYTTRCCCAT
*f*RPB2-7cF/*RPB2*-11bR	3620–3639/4713–4694	ATGGGYAARCAAGCYATGGG/CAATCWCGYTCCATYTCWCC
2A/16A	3251–3273/4346–4323	GAGCAGTTGGATAGCGACACTCG/GAGGGTTGATGACCAAATCAGGAG
RPB2-3bF/21B	1838–1860/2827–2806	GGWGGWTAYTTYATYATYAATGG/GCAAACAAGACCCCAATGCGAG
21B/PP1[b]	2827–2806/5′ end	GCAAACAAGACCCCAATGCGAG/GTAATACGACTCACTATAGGGC
15B/PP2[b]	2788–2769/5′ end	CTTCGCCAGCTTACCGTCAC/ACTATAGGGCACGCGTGGT
12A/PP1	3568–3586/3′ end	CATTCCGTTCCCTGACCAC/GTAATACGACTCACTATAGGGC
22B/PP2	4270–4292/3′ end	CATTGGTATCACATACCGGCAAG/ACTATAGGGCACGCGTGGT
4B/PP1	4039–4061/3′ end	CGGAAAGACGGCGCCAATAGCAG/GTAATACGACTCACTATAGGGC
13B/PP2	4370–4393/3′ end	ATTGCCCATTTGGTCGAGTGTCTT/GTAATACGACTCACTATAGGGC
Fragments amplified from total RNA of wheat biotype *Phaeosphaeria nodorum* isolate Sn37-1		
m1A1/m1B	1145–1167/1703–1682	CTCGCCTATCCCTCCCTCTCCTT/CCTCGCGCTCCCATTTGATCTG
m1A3/m2B	1595–1617/2005–1983	CGTATAATGGTTGCGAAAGAGAC/GATGTTGAAAGACGAGATCAGAC
m2A/m3B	1983–2005/2629–2608	GTCTGATCTCGTCTTTCAACATC/CAGGCCATTTGTGATGATGCTG
m3A/m4B	2608–2629/3193–3172	CAGCATCATCACAAATGGCCTG/GGTAGGATTGTTCTCTACTACG
m4A/m5B	3172–3193/3962–3943	CGTAGTAGAGAACAATCCTACC/CCTTCAGTCTTAGAGTGTCG
m5A/m6B	3943–3962/4727–4708	CGACACTCTAAGACTGAAGG/CGTGCGCAATCATACAATCG
m6A/m8B	4708–4727/5078–5056	CGATTGTATGATTGCGCACG/GATTATCGAGAATGTGCTACTTG
Genomic DNA fragments amplified from cereal *Phaeosphaeria* species		
M12-2/1Bm	1103–1125/1709–1687	CAGACAAGGAGCTCATCCTCCTC/GTACATCCTCGCGCTCCCATTTG
SM2/5B1	1588–1610/2467–2444	CTCGAATCGTATAATGGTTGCGA/CTTTCCGAAATGATCTCGGTCGTC
m2A/m3B	1983–2005/2629–2608	GTCTGATCTCGTCTTTCAACATC/CAGGCCATTTGTGATGATGCTG
5A1/7B1	2444–2467/3641–3619	GACGACCGAGATCATTTCGGAAAG/CACCCATGGCTTGCTTACCCATG
m31A/m5B (m31A/m52B)[d]	2610–2629/3962–3943 (2610–2629/3962–3943)	GCATCATCACAAATGGCCTG/CCTTCAGTCTTAGAGTGTCG (GCATCATCACAAATGGCCTG/CCTTCAATCGTAGAGTGTCG)
7A1/51	3619–3641/4617–4592	CATGGGTAAGCAAGCCATGGGTG/GCGTGGATCTTGTCGTCGACCATATG
m5A/m8B (m52A/m10B)[d]	3943–3962/5078–5056 (3943–3962/5039–5017)	CGACACTCTAAGACTGAAGG/GATTATCGAGAATGTGCTACTTG (CGACACTCTACGATTGAAGG/CTAGTCGCGCACGCTGAGGCCAG)

a Nucleotide positions are relative to the RNA polymerase II (*RPB2*) gene sequence of wheat biotype *Phaeosphaeria nodorum* isolate Sn37-1 (accession no. DQ278491).

b PP1 and PP2 are used to amplify the ligated adaptors 1 and 2 sequences (Zhang & Gurr 2002).

c The incompletely specified bases (IUB) group codes are Y = T/C; M = A/C; W = T/A; R = A/G; N = G/A/T/C.

d The primer sets in parenthesis are used for DNA amplification of barley biotype *P. nodorum*, *P. a.* f.sp. *avenaria* (Paa) and *P. a.* f. sp. *triticea* (Pat1).

Table 2 – Isolates of *Phaeosphaeria* species used for analysis of the gene encoding the second largest protein subunit of RNA polymerase II (*RPB2*)

Species	Original host	Year	Geographic location	GenBank accession number
Phaeosphaeria nodorum (wheat biotype; PN-w)				
Sn37-1	Wheat	–	Szelejewo, Poland	DQ278491
9074	Wheat (*Triticum aestivum*)	1983	Gallatin County, MT, USA	(=DQ278491)
9076	Wheat	1986	Richland County, MT, USA	(=DQ278491)
8408	Wheat	1986	Mandan, ND, USA	(=DQ278491)
S-81-W15	Wheat	1981	Sheridan, OR, USA	(=DQ278491)
9506	Wheat	1987	Mandan, ND, USA	DQ499806
S-87-2	Wheat ('Oasis')	1987	Griffin, GA, USA	(=DQ499806)
Sn26-1	Wheat	-	Rzeszów, Poland	DQ499807
98-12981	Rye (*Secale cereale*)	1998	Mandan, ND, USA	(=DQ499807)
S-78-13	Wheat	1978	Toluca, Mexico	(=DQ499807)
S-80-301	Triticale (x*Triticosecale*)	1980	Williamson, GA, USA	(=DQ499807)
S-81-W12	Wheat	1981	Marion County, OR	(=DQ499807)
S-81-W13	Wheat	1981	Marion County, OR	(=DQ499807)
S-81-W16	Wheat	1981	Harrisburg, OR, USA	(=DQ499807)
S-74-20A (ATCC200806)	Wheat	1975	Griffin, GA, USA	DQ499808
Sn27-1	Wheat	-	Sieradz, Poland	DQ499809
S-81-B13B	Barley (*Hordeum vulgare*)	1981	Bledsoe, GA, USA	(=DQ499809)
Phaeosphaeria sp. (from Poland)				
Sn23-1	Winter rye	-	Bydgoszcz, Poland	DQ499804
Sn48-1	Winter rye	1995	Jelenia Góra, Poland	DQ499805
Phaeosphaeria nodorum (barley biotype; PN-b)				
S-80-603	Barley	1980	Williamson, GA, USA	DQ499803
S-80-611	Barley	1980	Laurinburg, NC, USA	(=DQ499803)
S-81-B9	Barley	1981	Clayton, GA, USA	(=DQ499803)
S-82-13 (ATCC200805)	Barley	1982	Senoia, GA, USA	(=DQ499803)
S-83-2 (ATCC200841)	Barley	1983	Tifton, GA, USA	(=DQ499803)
S-83-7	Barley	1983	Holland, VA, USA	(=DQ499803)
S-84-2	Barley	1984	Moultrie, GA, USA	(=DQ499803)
S-92-7	Barley	1992	Raleigh, NC, USA	(=DQ499803)
S-93-38	Barley	1993	Floyd County, GA, USA	(=DQ499803)
S-93-39	Barley	1993	Pike County, GA, USA	(=DQ499803)
Phaeosphaeria avenaria f. sp. *avenaria* (Paa)				
ATCC12277	Oat (*Avena sativa*)	-	USA	DQ499810
Sat002NY-84	Wheat	1984	New York, USA	DQ499811
1920WRS	Oat	2002	Manitoba, Canada	DQ499812
Saa001NY-85	Oat	1985	New York, USA	DQ499813
1919WRS	Oat	2002	Manitoba, Canada	(=DQ499813)
ATCC58582	Wheat	1984	New York, USA	DQ499814
ATCC58583	Wheat	1984	New York, USA	(=DQ499814)
5413	Oat	1983	Ontario, Canada	DQ499815
1921WRS	Oat	2002	Manitoba, Canada	(=DQ499815)
Sa37-2	Oat	2001	Radzików, Poland	DQ499816
Phaeosphaeria avenaria f. sp. *triticea* (Pat1)				
Sat24-1	Wheat	-	Warmińsko-Mazurskie, Poland	DQ499801
10052-2	Wheat	1988	Langdon, ND, USA	(=DQ499801)
12618	Wheat	1995	Dickinson, ND, USA	(=DQ499801)
12889	Wheat	1997	Mandan, ND, USA	(=DQ499801)
13050-2	Barley	1998	Dunn County, ND, USA	(=DQ499801)
13061	Barley	1998	Morton County, ND, USA	(=DQ499801)
13077-2	Barley	1998	Towner County, ND, USA	(=DQ499801)
Sa38-1	Oat	2001	Radzików, Poland	(=DQ499801)
Sa39-2	Oat	2001	Radzików, Poland	(=DQ499801)
Sat22-2	Rye	1995	Podkarpackie, Poland	(=DQ499801)
Sat23-2	Triticale	1995	Mazowieckie, Poland	(=DQ499801)
Sat23-8	Triticale	1995	Mazowieckie, Poland	(=DQ499801)
ATCC26374	Foxtail barley (*Hordeum jubatum*)	1972	Minnesota, USA	DQ499802
ATCC26375	Foxtail barley	1972	Minnesota, USA	(=DQ499802)
Phaeosphaeria avenaria f. sp. *triticea* (Pat2)				
ATCC26370	Foxtail barley	1972	Minnesota, USA	DQ499800
ATCC26377	Foxtail barley	1972	Minnesota, USA	(=DQ499800)
Phaeosphaeria avenaria f. sp. *triticea* (Pat3)				
S-81-W10	Wheat	1981	Washington, USA	DQ499799
Phaeosphaeria sp.				
S-93-48	Dallis grass (*Paspalum dilatatum*)	1993	Griffin, GA, USA	DQ499798

DQ278491) containing the *RPB2* gene was obtained from the PN-w isolate Sn37-1 (Fig 2). Three programs were used to delineate the *RPB2* gene and its associated signals. Based on the Promoter Scan analysis (http://bimas.dcrt.nih.gov), the transcription start site (TSS) of the *RPB2* gene was detected at nt758. Additionally, several specific protein factor binding sequences required for the regulation of *RPB2* gene transcription were found in the upstream promoter region of the *RPB2* gene (Fig 2). The CArG binding site [CC (A+T rich)$_6$ GG] found in the promoter regions of human and mouse cardiac α-actin genes was present at nt728-nt737 (Miwa *et al.* 1987). Two activating transcription factor (ATF) sites, (G/T)(A/T)CGTCA, identical to several distinct nucleoprotein-binding sites in the early region 1A (E1A)-inducible adenovirus E3 promoter, were detected at nt261–nt267 and nt698–nt704 (Hurst & Jones 1987). Other sequences similar to the binding sites for yeast (*Saccharomyces cerevisiae*) autonomously replicating sequence-binding factor 1 (ABF1; ATCAN$_6$ACGA), general regulatory factor I (GRFI; RMACCCANNCAYY) and ribosomal protein genes box (RPG; ACACCCANNCA), were also found at nt115–nt128, nt550–nt562 and nt625–nt635, respectively (Fig 2) (Buchman *et al.* 1988). An analysis by Hamming-Clustering Methods for eukaryotic genes (HCtata and HCpolya programs) (http://www.itb.cnr.it/sun/webgene/) showed that the TATA signal was at nt731 and the Poly-A tailing signal was at nt5202. By using the FGENESH program (http://www.softberry.com) with *Aspergillus* as the organism parameter, two exons (nt1199–nt4821 and nt4874–nt5039) and one intron (nt4822–nt4873) were predicted. The intron position of the *RPB2* gene in PN-w was experimentally confirmed by cDNA sequencings.

The combined 3789 bp nucleotide sequence of two exons of the *RPB2* gene encoded a 1262 amino acid polypeptide (Fig 2). Like the C-terminal structures of the β subunit in prokaryotes and the RPB2 polypeptide in yeast RNA polymerase II, many conserved structural features were present in the C-terminal of RPB2 polypeptide in PN-w (Fig 2). They included a putative consensus zinc-binding motif ($CX_2CX_{15}CX_2C$) (aa1198–aa1220), a purine nucleotide-binding motif (GX_5GKT) (aa942–aa950), two amino acid sequence clusters (aa997–aa1014 and aa1129–aa1137) involved in the formation of the active site of RNA polymerase II, which contained two nucleotide-binding lysine (K) (aa1014 and aa1137) (James *et al.* 1991; Treich *et al.* 1991; Sweetser *et al.* 1987; Grachev *et al.* 1989). As in other eukaryotic RPB2 polypeptides, there was a conserved prokaryotic RNase-like domain in the *P. nodorum* peptide (aa323–aa426) (Fig 2) (Shirai & Gō 1991). There were 531 amino acids (42 % identity) in the full-length RPB2 polypeptides that are conserved in 11 ascomycetes (Fig 2).

The *RPB2* gene coding sequence amplified from cereal *Phaeosphaeria* species differed in length (Table 3). The *RPB2* gene from PN-w, homothallic Pat1, heterothallic Pat2, and *Phaeosphaeria* sp. from Polish ryes had the same length (3841 bp), while the same gene from PN-b, Paa, Pat3 and *Phaeosphaeria* sp. from dallis grass was 3840 bp in size. The size difference was due to variations in intron size (Table 3). Nucleotide substitutions were not found in ten PN-b isolates

Table 3 – Structure of RNA polymerase (*RPB2*) gene in cereal *Phaeosphaeria* species

Species	Isolate	Gene size (bp)[b]	Intron size (bp)	Nucleotide substitutions within the species	Substitutions as compared with wheat biotype *P. nodorum* Sn37-1 isolate Nucleotides	Amino acids
Phaeosphaeria nodorum (wheat biotype; PN-w)	Sn37-1 (5)[a]	3841	52	0		
	9506 (2)			1		
	Sn26-1 (7)			1		
	S-74-20A (1)			2		
	Sn27-1 (2)			7		
Phaeosphaeria sp. (from rye)	Sn23-1 (1)	3841	52	0	59	0
	Sn48-1 (1)			1	60	0
P. nodorum (barley biotype; PN-b)	S-84-2 (10)	3840	51	0	217	12
P. avenaria f. sp. *avenaria* (Paa)	ATCC12277 (1)	3840	51	0	220	15
	Sat002NY-84 (1)			3		
	1920WRS (1)			4		
	Saa001NY-85 (2)			5		
	ATCC58582 (2)			6		
	5413 (2)			8		
	Sa37-2 (1)			10		
P. avenaria f. sp. *triticea* (Pat1)	Sat24-1 (12)	3841	52	0	204	6
	ATCC26374 (2)			1	203	6
P. avenaria f. sp. *triticea* (Pat2)	ATCC26370 (2)	3841	52	0	361	18
P. avenaria f. sp. *triticea* (Pat3)	S-81-W10 (1)	3840	51	0	225	11
Phaeosphaeria sp. (from dallis grass)	S-93-48 (1)	3840	51	0	227	10

a Number of isolates with identical sequences are in parentheses (See Table 2).
b Fragment sizes are given in base pairs (bp).

and 12 homothallic Pat1 isolates from commercially cultivated cereals. However, one nucleotide substitution (nt3650) in the intron was found in two Pat1 isolates from wild barley. Nucleotide sequence diversity of the *RPB2* gene within the species was much less than inter-species. From one to ten substitutions were found in PN-w, *Phaeosphaeria* sp. from Polish ryes, and Paa (Table 3). In comparison with PN-w isolate Sn37-1, sequence differences in other *Phaeosphaeria* isolates were 59 to 361 bp (Table 3).

Many of the nucleotide changes occurred in either intron or the third position of amino acid coding triplets and did not affect the amino acid composition in RPB2 polypeptides. There were five nucleotide changes in the intron and 54–55 changes in exons in the *RPB2* gene between PN-w isolate Sn37-1 and two *Phaeosphaeria* sp. from Polish ryes, but no amino acid changes were found in their RPB2 polypeptides (Table 3). There were six to 18 substitutions in the deduced amino acid sequences of RPB2 polypeptides of other *Phaeosphaeria* species as compared with PN-w (Table 3). It appears that the RPB2 polypeptide in two heterothallic Pat2 isolates from wild barley is the most diversified among the cereal *Phaeosphaeria* species (Table 3). All amino acid divergences were present in two regions of the RPB2 polypeptide, which were from aa3 to aa299 and from aa572 to aa802. Two other amino acid substitutes (V ↔ I), which occurred at aa345 and aa1046, were found in two heterothallic Pat2 isolates. The structural features conserved in the C-terminal and the prokaryotic RNase-like domains in the N-terminal were not affected.

The phylogenetic relationships based on the deduced RPB2 amino acid sequence was the same as that based on the *RPB2* gene nucleotide sequence in *Phaeosphaeria* species (Fig 3). It appears that homothallic Pat1, heterothallic Pat2, PN-w and *Phaeosphaeria* sp. from rye are closely related, and PN-b, Paa, Pat3 and *Phaeosphaeria* sp. from dallis grass form a single clade (Fig 3).

Discussion

The phylogenetic positions of organisms at the primary kingdoms of life might be misplaced due to the usage of genes with

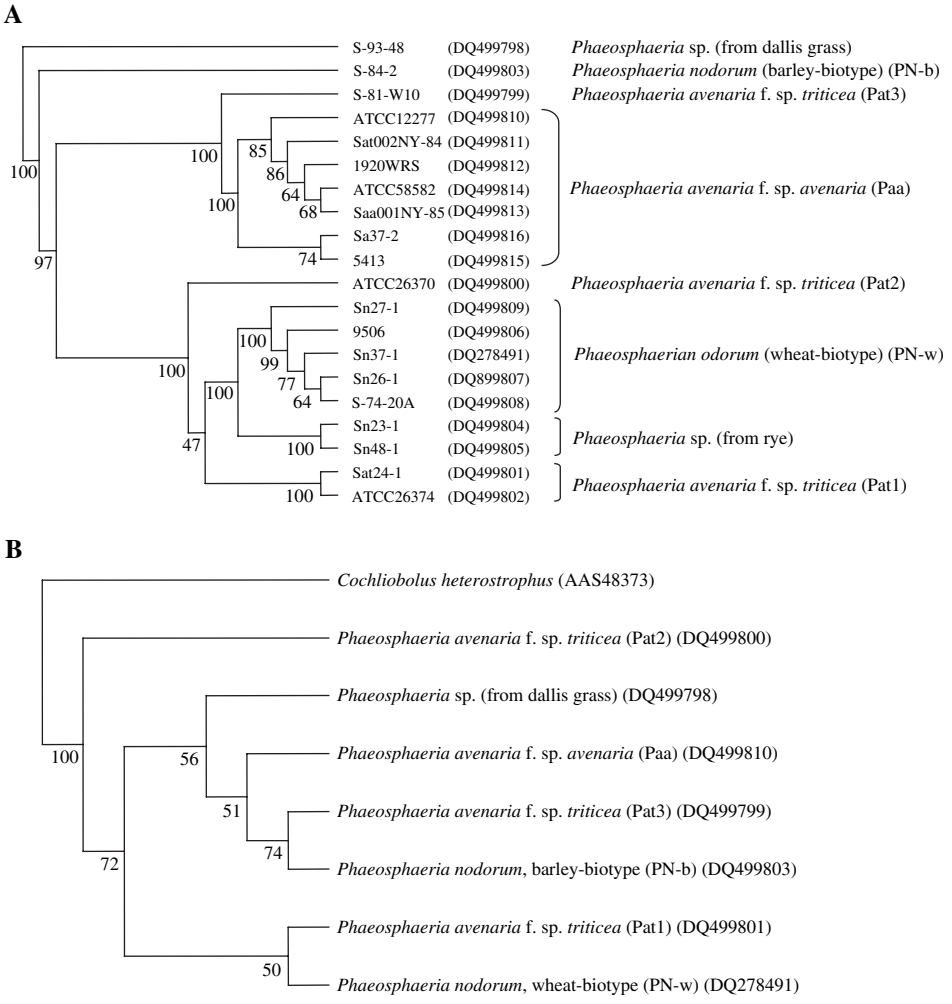

Fig 3 – Phylogenetic relationship based on the full-length nucleotide (A) and its deduced amino acid (B) sequences of the second largest subunit of RNA polymerase II (*RPB2*) gene in cereal *Phaeosphaeria* species. GenBank accession numbers are given in parentheses. The maize pathogen, *Cochliobolus heterostrophus*, was used as an out-group in B. BS values (with 1K replications) of the internal branches are indicated. The amino acid sequences of the *RPB2* gene in PN-w and *Phaeosphaeria* sp. from rye are identical.

an unusually high rate of sequence divergence for analysis. The *RPB2* gene encodes a protein with a modest rate of evolutionary change, and its polypeptide sequence has been used for phylogenetic studies in green plants (Denton *et al.* 1998), archaebacteria (Iwabe *et al.* 1991), red algae (Stiller & Hall 1997), and ascomycetes, including lichen-forming taxa (Liu *et al.* 1999; Liu & Hall 2004; Reeb *et al.* 2004). The sequence diversities in the *RPB2* gene also provide useful phylogenetic relationships at inter- and intra-specific levels in the genera *Trichoderma/Hypocrea* and *Leotia* (Chaverri *et al.* 2003; Zhong & Pfister 2004).

In order to understand the phylogenetic relationships of PN-w with other *Loculoascomycetes*, ten other full-length RPB2 polypeptide sequences (1208–1287aa in length) of ascomycetes deposited in the GenBank were used for phylogenetic analysis. It appears that *Cochliobolus heterostrophus* and PN-w are more closely related to each other than to the other ascomycetes (Fig 4). By using the FGENESH program with *Aspergillus* as the organism parameter, the 3852 bp *RPB2* gene coding sequence (nt794–nt4645) in *C. heterostrophus* (accession no. AY533025) contains a 63 bp intron and 3789 bp nucleotide sequence in two exons, which encoded a protein of 1262 amino acid residues. The RPB2 polypeptides in both *C. heterostrophus* and PN-w were the same length and had a 90.3 % amino acid identity (Ueng, unpublished data). The identities of RPB2 polypeptides between PN-w and the other nine ascomycetes listed in Fig 4 were as low as 63.6–66.9 %. In previous studies, high identities between *C. heterostrophus* and PN-w were reported in the mating-type gene-encoded proteins and glyceraldehyde-3-phosphate dehydrogenase (GPD) (EC1.2.1.12) enzyme (Bennett *et al.* 2003; Ueng *et al.* 2003b). The close relationship between these two organisms was re-confirmed with RPB2 polypeptide sequence in this study (Fig 4).

A *P. nodorum* gene encoding a hypothetical protein similar to DNA-dependent RNA polymerase II RPB140 of *Curvularia brachyspora* (accession no. AAF19075) was recently reported (www.broad.mit.edu/annotation/genome; SNOG_11456.1). The 3842 bp coding sequence was predicted to have three introns (42, 193 and 100 bp in size) and four exons, and encoded an 1168 amino acid polypeptide. By using the FGENESH program with *Aspergillus* as the organism parameter, this 3842 bp coding sequence (SNOG_11456.1) was predicted to have two introns (130 and 52 bp in size) and three exons, and encoded a 1216-amino acid polypeptide. As compared with the *RPB2* gene genomic sequence in PN-w Sn37-1 isolate (accession no. DQ278491), there was one nucleotide substitution at nt876 (from 'C' to 'T') and one extra 'T' nucleotide between nt1188 and nt1189 in this gene coding sequence (SNOG_11456.1). As the intron position and the coding sequence size of the *RPB2* gene were confirmed experimentally by genomic and cDNA sequencing in this study, the prediction that it contains one intron using the FGENESH program appears to be correct. The presence of an extra 'T' nucleotide in the *P. nodorum* gene (SNOG_11456.1) results in the prediction of a different gene structure using the FGENESH program.

The intron position within the *RPB2* gene may reflect its gain and/or loss during fungal evolution, but it might not be reliable as a phylogenetic character (Liu *et al.* 1999). However, the intron position of the *RPB2* gene in PN-w is the same as in three other high ascomycetes. The intron is inserted between the second and third codon positions of alanine (A) in aa1202 of *Aspergillus fumigatus* (accession no. EAL84702), aa1208 of *Cochliobolus heterostrophus* (accession no. AAS48373), aa1234 of *Neurospora crassa* (accession no. CAD70445) and aa1208 of PN-w (accession no. DQ278491). The only lower ascomycete reported to have an intron in the *RPB2* gene is fission yeast, *Schizosaccharomyces pombe* (accession no. BAA02600). The intron is inserted between the first and second codon positions of aa6 tyrosine (Y).

Stagonospora blotch diseases of cereals are a disease complex caused by a number of fungi that are not always closely related (Scharen & Sanderson 1985; Sprague 1950). Application of molecular approaches may complement the morphologically based classification and facilitate the identification of

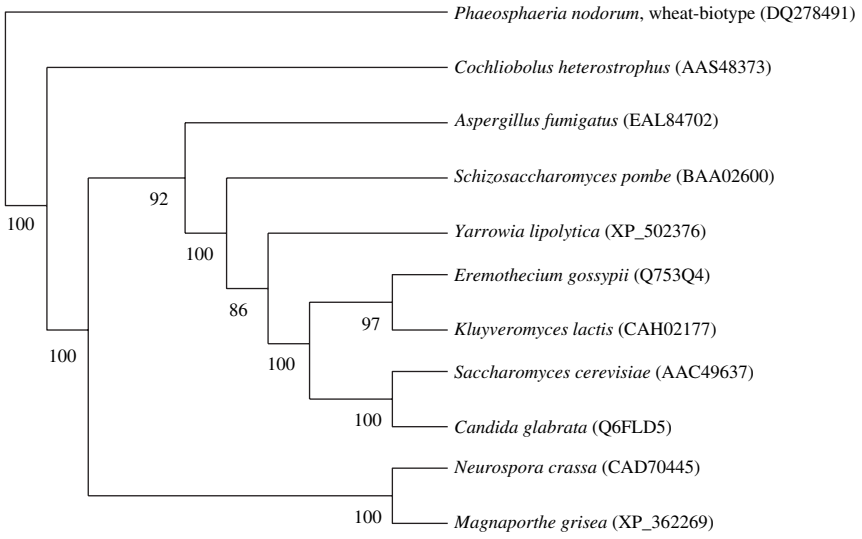

Fig 4 – Phylogenetic relationship based on the deduced polypeptide sequences of the second largest subunit of RNA polymerase II (RPB2) in ascomycetes. GenBank accession numbers are given in parentheses. BS values (with 1K replications) of the internal branches are indicated.

Table 4 – Comparison of endonuclease restriction of PCR-amplified RNA polymerase (*RPB2*) gene products in *Phaeosphaeria* leaf blotch pathogens

Endonulcease enzyme	Restriction site (no.)	PN-w (332 bp)	PN-rye (332 bp)	PN-b (331 bp)	Paa (331 bp)	Pat			P-93-48 (331 bp)
						Pat1 (332 bp)	Pat2 (332 bp)	Pat3 331 bp)	
*Ava*II	301 (302)	-	30, 302	30, 301	30, 301	30, 302	-	30, 301	30, 301
*Ban*II	107	-	-	-	-	-	107, 225	-	-
*Bgl*II	164	-	-	-	-	-	-	-	164, 167
*Dpn*I	167	-	-	-	-	165, 167	-	-	-
*Hae*II	46	-	-	46, 285	46, 285	46, 286	46, 286	46, 285	46, 285
*Hin*fI	103 (104)	103, 229	103, 229	103, 228	104, 227	-	-	103, 228	103, 228
*Ssp*I	280	-	-	-	51, 280	-	-	-	-
*Taq*I	140 (145)	140, 192	140, 192	-	-	-	145, 187	-	-
*Xba*I	161	-	-	-	161, 170	-	-	161, 170	-

An m6A/m10B primer set was used to amplify the 331–332 bp fragments from wheat biotype *Phaeosphaeria nodorum* (PN-w), isolates from Polish ryes (PN-rye), barley biotype *P. nodorum* (PN-b), *P. avenaria* f.sp. *avenaria* (Paa), *P. avenaria* f.sp. *triticea* (Pat) including homothallic isolates (Pat1), heterothallic isolates (Pat2) and S-81-W10 isolate from state of Washington (Pat3), and P-93-48 isolate from dallis grass. Protocols for PCR amplification were the same as those for genomic DNA gene amplification in Materials and Methods. After PCR reaction, a 4 μl sample was used for enzymatic restriction following the standard procedures (Promega, Madison, Wisconsin, USA). Fragment sizes are given in base pairs (bp). -, Not cut by endonuclease enzymes.

the Stagonospora complex in cereal blotch diseases. Recently, PCR-based methods have been used to distinguish plant pathogens in a mixed infection and to detect genetic variation in a pathogen population (Fraaije *et al.* 2001; McCartney *et al.* 2003). In addition to previous studies using ITS region sequence, the *mat1* gene conserved region, and the partial *gpd*, *bgl1* and *tub*A genes were used to distinguish cereal and other unidentified *Phaeosphaeria* species (Malkus *et al.* 2005; Reszka *et al.* 2005; Ueng *et al.* 1998, 2003a,b). In this study, enzymatic restriction patterns of the *RPB2* gene fragments amplified with the m6A/m10B primer set are proposed (Table 4). The endonuclease restriction sites were deduced from the sequencing data and were experimentally demonstrated by PCR amplification, enzymatic restriction and agarose gel electrophoresis (Ueng, unpublished data). Nucleotide substitutions occurring in the amplified fragments did not affect the enzymatic restrictions and the banding patterns in agarose electrophoresis gels (Ueng, unpublished data). It appears that specific digestions by *Ban*II, *Bgl*II, *Dpn*I and *Ssp*I can identify heterothallic Pat2, P-93-48 isolate from dallis grass, homothallic Pat1 and Paa, respectively. The *Ava*II enzymatic restriction can separate PN-w from *Phaeosphaeria* sp. of Polish rye. Digestion with a combination of three other enzymes (*Hae*II, *Taq*I and *Xba*I) may distinguish PN-b and Pat3 from other *Phaeosphaeria* species tested.

The polypeptide sequences encoded by the *gpd* and *tub*A genes are identical in cereal *Phaeosphaeria* species tested, with the exception of heterothallic Pat2 isolates from wild barley (Malkus *et al.* 2005; Ueng *et al.* 2003b). This indicates that the cereal *Phaeosphaeria* species are closely related phylogenetically. However, based on the nucleotide sequences, the correlation between homothallic Pat1 and PN-w are different in different genes. In the *gpd*, *tub*A and *bgl1* genes, homothallic Pat1 is more closely related to the phylogenetic clade containing Paa, Pat3 and PN-b than to PN-w (Malkus *et al.* 2005; Reszka *et al.* 2005; Ueng *et al.* 2003b). Homothallic Pat1 apparently is closely related to PN-w, Pat2 and *Phaeosphaeria* sp. from rye in the *RPB2* gene (Fig 3A). To further understand the phylogenetic relationships in cereal *Phaeosphaeria* species, more sequence data from numerous genes are required for comparison.

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