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Genetic Diversity of the White Collar-2 (*wc-2*) Gene in Cereal *Phaeosphaeria* pathogens

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ABSTRACT

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The white collar-2 (*wc-2*) gene encodes a light responsive white collar-2 (*wc-2*) protein that forms a heterodimeric complex with white collar-1 protein, activates numerous light-dependent reactions including asexual sporulation and pathogenic aggressiveness, and maintains circadian clocks in ascomycete fungi. The structure of the *wc-2* gene and phylogenetic relationships based on the deduced polypeptide sequences in cereal *Phaeosphaeria* pathogens were investigated. The *wc-2* gene in 2 *Phaeosphaeria nodorum* (barley- (PN-b) and wheat- (PN-w) biotypes), 4 *Phaeosphaeria avenaria* (1 *P. a*. f. sp. *avenaria*, Paa and 3 *P. a*. f. sp. *triticea*, Pat1, Pat2 and Pat3), 1 *Phaeosphaeria* sp. from Polish rye (P-rye) and 1 *Phaeosphaeria* sp. from dallis grass (P-dg) contained two introns, transcribed to produce 1,410 bp mRNA and encoded a 469 amino acid polypeptide. Based on the deduced polypeptide sequences, *Phaeosphaeria* species were phylogetically closely related as a group, with the exception of Pat2 isolates from wild foxtail barley (*Hordeum jubatum* L.).

Keywords: white collar-2 protein, *Phaeosphaeria*, wheat

Phaeosphaeria nodorum (E. Müll.) Hedjar. [anamorph: *Stagonospora nodorum* (Berk.) E. Castell. & Germano] and *Phaeosphaeria avenaria* (G. F. Weber) O.E. Erikss. [anamorph: *Stagonospora avenae* (A. B. Frank) Bissett] are two important causal agents of Stagonospora leaf blotch diseases in cereals [19, 23]. The identification of these two pathogens is based largely on morphology of the anamorph and host pathogenicity [8, 9, 10]. Recently, genetic relatedness and differentiation of cereal *Phaeosphaeria* species have been examined at the molecular level. The phylogenetic relationships of *Phaeosphaeria* species have been discussed based on the nucleotide and amino acid sequences. In the glyceraldehyde-3-phosphate dehydrogenase (*gpd*), β-tubulin (*tubA*), β-glucosidase (*bgl1*) and white collar-1 (*wc-1*) genes, homothallic *P. avenaria* f.sp. *triticea* (T. Johnson) Shoemaker & C. E. Babc. (Pat1) is more closely related to the phylogenetic

clade containing *P. avenaria* f. sp. *avenaria* (Weber) O. E. Erikss. (Paa), *P. avenaria* f. sp. *triticea* from Washington State (Pat3) and barley-biotype *P. nodorum* (PN-b) than wheat- biotype *P. nodorum* (PN-w) [5, 20, 21, 26]. In the RNA polymerase II (*RPB2*) gene, homothallic Pat1 apparently is related to PN-w, heterothallic *P. avenaria* f. sp. *triticea* from foxtail barley (*Hordeum jubatum* L.) (Pat2) and *Phaeosphaeria* sp. from rye (P-rye) [18]. On the contrary, the trifunctional histidine biosynthesis (*his*) gene in Pat1 was not closely related to neither PN-w nor the clade including Paa, Pat3 and PN-b [27].

The white collar-2 (*wc-2*) gene encodes a light responsive protein (*wc-2*) that controls numerous light-dependent reactions and maintains circadian clocks in higher fungi [12, 15]. The *wc-2* protein is a transcription factor containing a single PER-ARNT-SIM (PAS) and putative GATA-type zinc finger (Znf) domains in ascomycete fungi. In *Neurospora crassa*, *wc-2* protein forms a heterodimeric white collar complex (WCC) with white collar-1 (*wc-1*) protein and maintains the steady-state level of *wc-1* protein [1]. The WC-2 protein induces transcription of the frequency (*frq*) gene through WCC [4, 6]. The frequency (frq)/WCC-based (FWC) oscillator is the core circadian oscillator for many observed circadian rhythms in *N. crassa* including rhythmic conidiation [3, 7, 15]. In addition, functional *wc-1* and *wc-2* are also reported to be required in another circadian frq-less oscillator (FLO), such as *ccg16* FLO, which is temperature compensated but not necessarily light regulated [11]. Several review papers on circadian clocks in fungi have been recently published [2, 13, 14, 17].

In this report, the structure of the *wc-2* gene and phylogenetic relationships based on the deduced polypeptide sequences in cereal *Phaeosphaeria* pathogens were investigated.

The full-length *wc-2* gene sequence in PN-w isolate SN15 was retrieved by a protein BLAST search of *Stagonospora nodorum* isolate SN15 database (http: www.broad.mit.edu) with *wc-2* protein from *Neurospora crassa* (Accession no. P78714) as the query [16]. The *wc-2* gene in PN- w isolate SN15 (SNOG_14195) was 1,517 bp in length, contained two introns (nt22-nt80 and nt1357-nt1404) and encoded a 469 amino acid long polypeptide.

Procedures for fungal culture in a liquid medium and for genomic DNA (gDNA) isolation were described previously [25]. To determine the *wc-2* gene expression in PN-w, total RNA was isolated from a Sn37-1 culture grown under continuous fluorescent lights at room temperature (20±1℃) on sterile nitrocellulose membranes (BA-S 85, Schleicher & Schuell Inc., Keene N. H.) layered on V-8 juice agar (18% V8 juice, 0.2% calcium carbonate, and 2% agar) [5]. Total RNA purification and first strand cDNA synthesis followed the procedures described by Wang *et al.* [27]. Four primer sets (1A/1B, 2A/2B, 3A/3B and 3A/8B) designed from the 1,703 bp *wc-2* gene nucleotide sequence of PN-w isolate SN15, including a 1,517 bp coding sequence and a 186 bp partial 3' end sequence (Accession no. CH445354, nt 224083-nt225785), were used to amplify the *wc-2* gene from gDNA and 1x cDNA in PN-w isolate Sn37-1 (Table 1). The *wc-2* gene in PN-w isolate Sn37-1 was determined to contain 2 spliced introns, as annotated in SNOG_14195 (http: www.broad.

Table 1. Oligonucleotide primers used to amplify PCR products from the white collar-2 (*wc-2*) gene in cereal *Phaeosphaeria* species

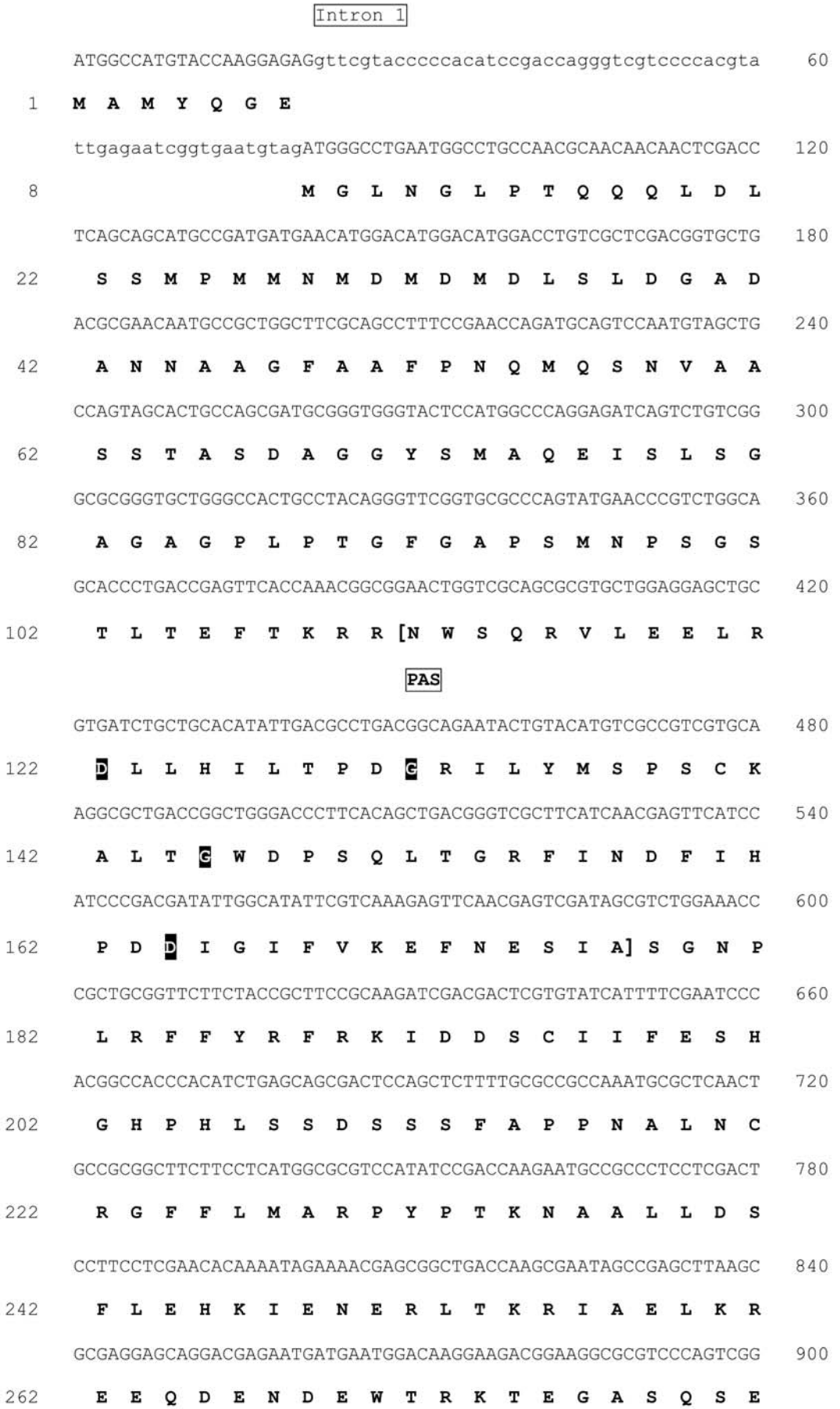
Primer sets	Nucleotide positions [1]	Sequences (5' →3')	*Phaeosphaeria* species [2]
1A/1B	1-21/765-745	ATGGCCATGTACCAAGGAGAG/ CATTCTTGGTCGGATATGGAC	All
2A/2B	626-647/1185-1164	CAAGATCGACGACTCGTGTATC/ CACGTATGAGGTTCGGACTTGC	All except Pat2
2AA/2BA	626-647/1185-1164	CAAGATTGATGATTCGTATCTCATC/ CGCGAATGAGGTTCGGACTCGC	Pat2
3A/3B	1086-1107/1517-1493	CATCTGGAGACGATTGAAATGC/ TCAGCTGCTACCGGTACTCGTATGC	All
3A/8B	1086-1107/1703-1681	CATCTGGAGACGATTGAAATGC/ CTAGTGGCCTCTAGCCTCGAGCT	All

[1] Nucleotide positions (nt) are in accord with the *wc-2*-like gene (SNOG_14195) reported in *Phaeosphaeria nodorum* SN15 isolate.
[2] *Phaeosphaeria* species include wheat-biotype *P. nodorum* (PN-w), barley-biotype *P. nodorum* (PN-b), *P. avenaria* f. sp. *avenaria* (Paa), *P. avenaria* f. sp. *triticea* (Pat1, Pat2 and Pat3), and *Phaeosphaeria* spp. from rye (P-rye) and dallis grass (P-dg).

Table 2. Isolates of *Phaeosphaeria* species used for analysis of the white collar-2 (*wc-2*) gene

Species	Original host	Year	Geographic location	GenBank accession number
Phaeosphaeria nodorum (wheat-biotype) (PN-w)				
Sn37-1	Wheat	-	Szelejewo, Poland	GQ254704
8408	Wheat	1986	Mandan, ND, USA	GQ254705
Sn27-1	Wheat	-	Sieradz, Poland	GQ254706
S-79-1	Triticale (*xTriticosecale*)	1979	Tifton, GA, USA	(=GQ254706)
9074	Wheat (*Triticum aestivum* L.)	1983	Gallatin County, MT, USA	GQ254707
9076	Wheat	1986	Richland County, MT, USA	(=GQ254707)
Sn26-1	Wheat	-	Rzeszów, Poland	GQ254708
S-78-13	Wheat	1978	Toluca, Mexico	(=GQ254708)
S-81-B13B	Barley (*Hordeum vulgare* L.)	1981	Bledsoe, GA, USA	(=GQ254708)
Phaeosphaeria sp. (From Poland) (P-rye)				
Sn48-1	Winter rye (*Secale cereale* L.)	1995	Jelenia Góra, Poland	GQ254709
Sn23-1	Winter rye	-	Bydgoszcz, Poland	(=GQ254709)
Phaeosphaeria nodorum (barley-biotype) (PN-b)				
S-83-2 (ATCC200841)	Barley	1983	Tifton, GA, USA	GQ254700
S-80-603	Barley	1980	Williamson, GA, USA	GQ254701
S-82-13 (ATCC200805)	Barley	1982	Senoia, GA, USA	(=GQ254701)
S-80-611 (ATCC200842)	Barley	1980	Laurinburg, NC, USA	GQ254702
S-81-B9	Barley	1981	Clayton, GA, USA	GQ254703
Phaeosphaeria avenaria f. sp. *avenaria* (Paa)				
5413	Oat (*Avena sativa* L.)	1983	Ontario, Canada	GQ254691
ATCC12277	Oat	-	USA	GQ254692
ATCC58582	Wheat	1984	New York, USA	GQ254693
ATCC58583	Wheat	1984	New York, USA	(=GQ254693)
Sa37-2	Oat	2001	Radzików, Poland	GQ254694
Phaeosphaeria avenaria f. sp. *triticea* (Pat1)				
Sat24-1	Wheat	-	Warmińsko-Mazurskie, Poland	GQ254695
10052-2	Wheat	1988	Langdon, ND, USA	(=GQ254695)
12618	Wheat	1995	Dickinson, ND, USA	(=GQ254695)
ATCC26374	Foxtail barley (*Hordeum jubatum* L.)	1972	Minnesota, USA	(=GQ254695)
ATCC26375	Foxtail barley	1972	Minnesota, USA	(=GQ254695)
Phaeosphaeria avenaria f. sp. *triticea* (Pat2)				
ATCC26370	Foxtail barley	1972	Minnesota, USA	GQ254696
ATCC26377	Foxtail barley	1972	Minnesota, USA	GQ254697
Phaeosphaeria avenaria f. sp. *triticea* (Pat3)				
S-81-W10	Wheat	1981	Washington, USA	GQ254698
Phaeospharia sp. (P-dg)				
S-93-48	Dallis grass (*Paspalum dilatatum* Poir.)	1993	Griffin, GA. USA	GQ254699

mit.edu). A third intron (nt1465-nt1557) suggested to be spliced in the 1703 bp *wc-2* gDNA sequence (SNOG_14196, GenBank accession no. XM_001804342 for mRNA) was not found (Fig. 1). Therefore, the deduced polypeptide of the *wc-2* protein in the PN-w isolates should be 469 amino acids long, instead of 500 amino acids as reported for SNOG_14196 (Accession no. EAT78433) (Fig. 1).


Intron 1
ATGGCCATGTACCAAGGAGAGgttcgtacccccacatccgaccagggtcgtccccacgta 60
1 M A M Y Q G E
ttgagaatcggtgaatgtagATGGGCCTGAATGGCCTGCCAACGCAACAACAACTCGACC 120
8 M G L N G L P T Q Q Q L D L
TCAGCAGCATGCCGATGATGAACATGGACATGGACATGGACCTGTCGCTCGACGGTGCTG 180
22 S S M P M M N M D M D M D L S L D G A D
ACGCGAACAATGCCGCTGGCTTCGCAGCCTTTCCGAACCAGATGCAGTCCAATGTAGCTG 240
42 A N N A A G F A A F P N Q M Q S N V A A
CCAGTAGCACTGCCAGCGATGCGGGTGGGTACTCCATGGCCCAGGAGATCAGTCTGTCGG 300
62 S S T A S D A G G Y S M A Q E I S L S G
GCGCGGGTGCTGGGCCACTGCCTACAGGGTTCGGTGCGCCCAGTATGAACCCGTCTGGCA 360
82 A G A G P L P T G F G A P S M N P S G S
GCACCCTGACCGAGTTCACCAAACGGCGGAACTGGTCGCAGCGCGTGCTGGAGGAGCTGC 420
102 T L T E F T K R R [N W S Q R V L E E L R
PAS
GTGATCTGCTGCACATATTGACGCCTGACGGCAGAATACTGTACATGTCGCCGTCGTGCA 480
122 D L L H I L T P D G R I L Y M S P S C K
AGGCGCTGACCGGCTGGGACCCTTCACAGCTGACGGGTCGCTTCATCAACGAGTTCATCC 540
142 A L T G W D P S Q L T G R F I N D F I H
ATCCCGACGATATTGGCATATTCGTCAAAGAGTTCAACGAGTCGATAGCGTCTGGAAACC 600
162 P D D I G I F V K E F N E S I A] S G N P
CGCTGCGGTTCTTCTACCGCTTCCGCAAGATCGACGACTCGTGTATCATTTTCGAATCCC 660
182 L R F F Y R F R K I D D S C I I F E S H
ACGGCCACCCACATCTGAGCAGCGACTCCAGCTCTTTTGCGCCGCCAAATGCGCTCAACT 720
202 G H P H L S S D S S S F A P P N A L N C
GCCGCGGCTTCTTCCTCATGGCGCGTCCATATCCGACCAAGAATGCCGCCCTCCTCGACT 780
222 R G F F L M A R P Y P T K N A A L L D S
CCTTCCTCGAACACAAAATAGAAAACGAGCGGCTGACCAAGCGAATAGCCGAGCTTAAGC 840
242 F L E H K I E N E R L T K R I A E L K R
GCGAGGAGCAGGACGAGAATGATGAATGGACAAGGAAGACGGAAGGCGCGTCCCAGTCGG 900
262 E E Q D E N D E W T R K T E G A S Q S E

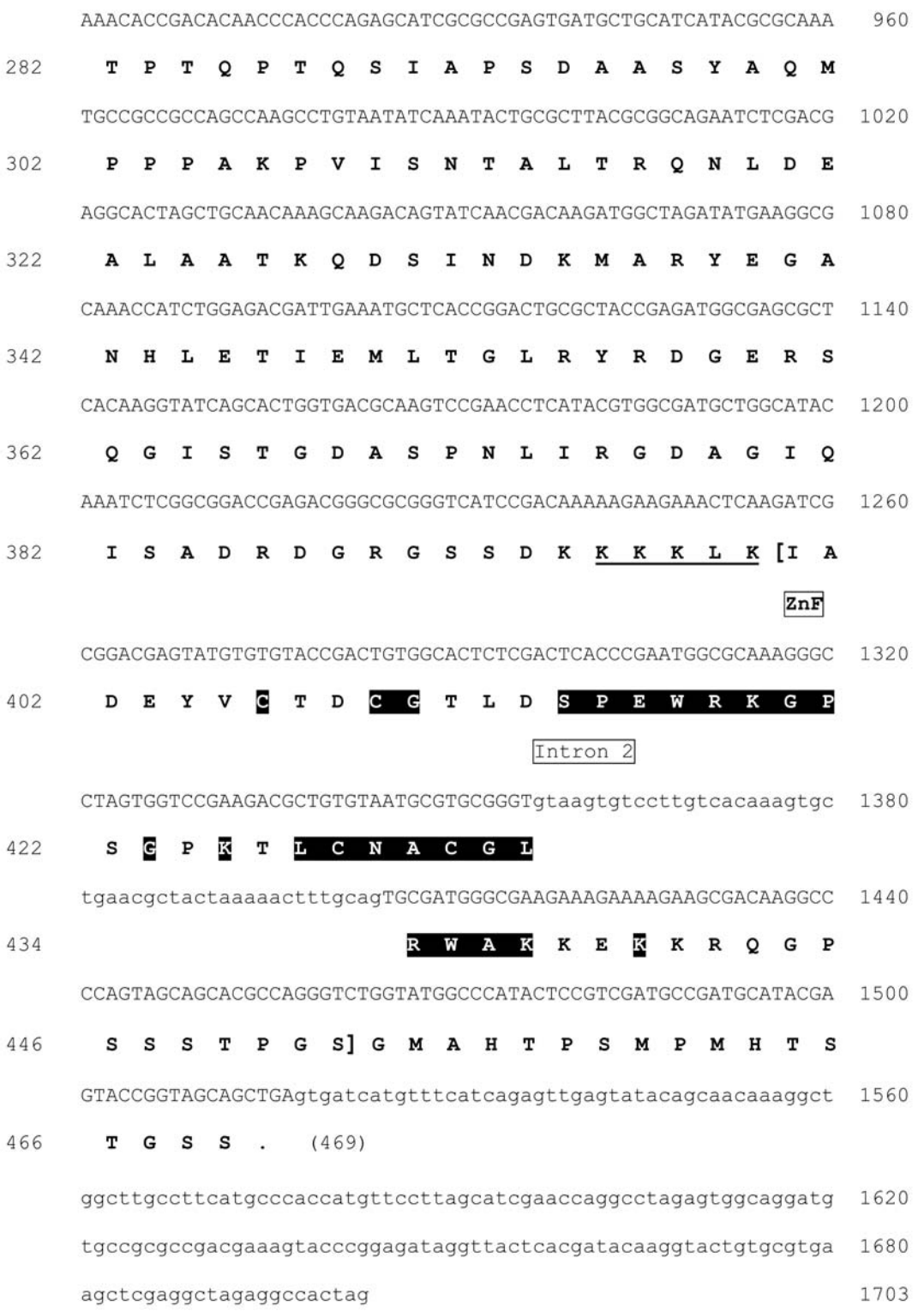


Fig. 1. Nucleotide and deduced amino acid sequences of the white collar-2 (*wc-2*) gene (accession no. GQ254704) in wheat-biotype *Phaeosphaeria nodorum* isolate Sn37-1. The numbers 60 to 1703 (right column) designate nucleotides and the numbers 1 to 466 (left column) designate amino acids. Lower case letters represent 2 introns and the 3' end untranslated flanking sequence, and the upper case letters represent the nucleotide coding sequence. A PAS motif (aa111 - aa177) and 1 GATA-type zinc finger (Znf) domain (aa400 - aa452) are bracketed. Amino acids identical in PAS1 and Znf domains in *Phaeosphaeria* and other ascomycetes are dark boxed. The putative nuclear targeting sequence (KKKLK) is underlined.

With four primer sets described above, the full-length *wc-2* gene sequence was amplified from 9 isolates of PN-w, 2 of P-rye, 5 of PN-b, 5 of Paa, 5 of Pat1, 2 of Pat2, 1 of Pat3 and 1 *Phaeosphaeria* sp. from dallis grass (*Paspalum dilatatum* Poir.) (P-dg) (Table 2). Primer set 2AA/2BA was substituted for 2A/2B for the *wc-2* gene amplification in two Pat2 isolates (Table 1). In this study, the size of the *wc-2* gene was 1,517 bp in all *Phaeosphaeria* species, with the exception of those in Pat2 and P-dg (Table 3). The size differences of the *wc-2* gene in Pat2 and P-dg (1,524 bp and 1,518 bp, respectively) were attributed to intron 1 nucleotide sequences, which was 66 bp and 60 bp, respectively. The full length sequence of the predicted *wc-2* mRNA in all *Phaeosphaeria* species was 1,410 bp encoding a 469 amino acid size polypeptide.

Similar to *wc-2* proteins in other ascomycetes, the *wc-2* polypeptides in *Phaeosphaeria* species contained a PAS motif, which associates with the 'PAS A' motif of *wc-1* protein to form heterodimerically a WCC (Fig. 1). The single putative GATA-type zinc finger (Znf) domain in wc-2 polypeptide of *Phaeosphaeria* and other ascomycetes were well conserved and belonged to zinc finger type IVb ($C\text{-}x_2\text{-}C\text{-}x_{18}\text{-}C\text{-}x_2\text{-}C$) [24]. A putative nuclear targeting sequence (KKKLK) upstream (aa395 - aa399) of the Znf domain was also found (Fig. 1) [16].

Nucleotide substitutions of the *wc-2* gene were fewer (from 1 to 17) within the species in PN-w, PN-b, Paa and Pat2 than between *Phaeosphaeria* species (Table 3). In

Table 3. Structure of the white collar-2 (*wc-2*) gene in *Phaeosphaeria* species

Species/Isolates	Gene size (bp)	# of nucleotide substitutions		# of amino acid substitutions	
		Intra-species[c]	Inter-species[d]	Intra-species[c]	Inter-species[d]
Phaeosphaeria nodorum (wheat-biotype) (PN-w)					
Sn37-1	1517	-		-	
SN15[a] (3)[b]		1		1	
Sn27-1 (1)		16		1	
8408		4		0	
9074 (1)		17		1	
Phaeosphaeria sp. (from Poland) (P-rye)					
Sn48-1 (1)	1517	-	21	-	3
Phaeosphaeria nodorum (barley-biotype) (PN-b)					
S-83-2	1517	-	87	-	8
S-80-603 (1)		1		1	
S-80-611		1		1	
S-81-B9		2		2	
Phaeosphaeria avenaria f.sp. *avenaria* (Paa)					
ATCC12277	1517	-	82	-	5
ATCC58582 (1)		9		0	
5413		5		0	
Sa37-2		2		0	
Phaeosphaeria avenaria f.sp. *triticea* (Pat1)					
Sat24-1 (4)	1517	-	85	-	11
Phaeosphaeria avenaria f.sp. *triticea* (Pat2)					
ATCC26370	1524	-	166	-	17
ATCC26377		1		1	
Phaeosphaeria avenaria f.sp. *triticea* (Pat3)					
S-81-W10	1517	-	85	-	5
Phaeosphaeria sp. (P-dg)					
S-93-48	1518	-	71	-	4

[a] The hypothetical polypeptide sequence (SNOG_14195) for Australian isolate SN15 was used.
[b] Number of isolates with identical sequences is in parentheses (See Table 2).
[c] Substitutions as compared with the first isolate within the species.
[d] Substitutions as compared with PN-w isolate Sn37-1.

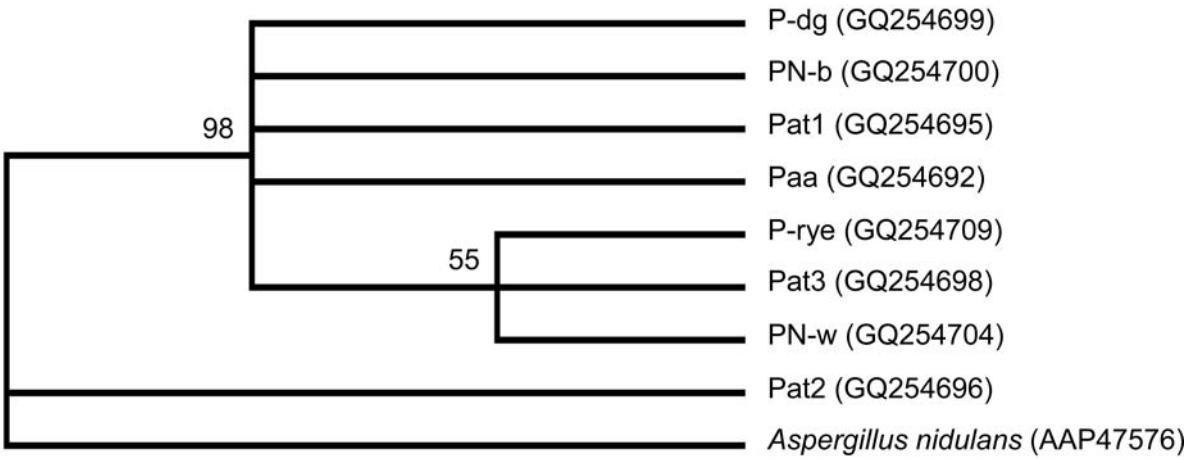


Fig. 2. Phylogenetic relationships based on the deduced amino acid sequences of the white collar-2 (*wc-2*) gene of cereal *Phaeosphaeria* pathogens. The 469 amino acid *wc-2* polypeptide sequences deduced from *Phaeosphaeria* pathogens are aligned and analyzed. The GATA factor of *Aspergillus nidulans* (Accession no. AAP47576) is used as the out-group in the analysis. GenBank Accession numbers for nucleotide sequences encoding the *wc-2* polypeptides of *Phaeosphaeria* pathogens are shown in parentheses. Bootstrap values with 1,000 replications of the internal branches are indicated.

comparison with PN-w Sn37-1 isolate, there were 21-166 nucleotide and 3-17 amino acid sequence differences in other *Phaeosphaeria* species (Table 3). Based on the analysis of deduced *wc-2* polypeptide sequences by using Mega Version 4.0 (http://www.megasoftware.net/index.html), all *Phaeosphaeria* species with the exception of Pat2 were closely related (Fig. 2). In addition to the *wc-2* gene, diversities of the nucleotides and their deduced polypeptide sequences in numerous genes including *bgl1*, *gpd*, *his*, *RPB2*, *tubA* and *wc-1* [5, 18, 20, 21, 26, 27] suggested that Pat2 evolved separately from other cereal *Phaeosphaeria* species. The Pat2 isolates used in this study were first isolated from wild foxtail barley in Minnesota, USA and reported to be highly virulent to commercially cultivated wheat [22].

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摘 要

邱燕欣[1]、張碧芳[2]、高凌岩[3]、周俊吉[4]、翁溥[5, 6]. 2009. 穀類葉枯病菌白圈環 -2 基因之多型性. 植病會刊 18: 175-183. ([1]臺中新社 農委會種苗改良繁殖場；[2]臺中 國立中興大學植物病理學系；[3]中國內蒙古農業大學生態與環境科學院；[4]臺南微兆發生命科學公司；[5]美國農部植物科學所分子植物病理研究室；[6]聯絡作者，電子郵件：ppuuueng@gmail.com；傳眞：+1-301-504-5449)

白圈環 -2 [white collar-2 (*wc-2*)] 基因編碼一個對光有反應的白圈環 -2 蛋白 (*wc-2*)，白圈環 -2 蛋白與白圈環 -1 蛋白形成異質二元複合體 (heterodimeric complex)，活化許多需光反應，包括：子囊菌的無性產孢與病原性強弱，並可維持其日週期時鐘 (circadian clocks)。本研究根據穀類葉枯病菌的白圈環 -2 基因之推演蛋白質序列，分析其基因結構與譜系關係 (phylogenetic relationships)。在二株 *Phaeosphaeria nodorum* (大麥生物型 PN-b 與小麥生物型 PN-w)、四株 *Phaeosphaeria avenaria* (一株 *P. a*. f. sp. *avenaria* (Paa) 與三株 *P. a*. f. sp. *triticea* (Pat1、Pat2 及 Pat3)), 一株由波蘭裸麥分得之 *Phaeosphaeria* sp. 菌株 (P-rye) 與一株由達利雀稗 (dallis grass) 分得之 *Phaeosphaeria* sp. 菌株 (P-dg) 當中，白圈環 -2 基因包含二個內含子 (intron)，轉錄產物爲一帶著 1,410-bp 開放解讀框的 mRNA，編碼 469 個胺基酸。根據這些推演的多胜肽序列所進行的類緣關係分析顯示，除了由狐尾大麥 (*Hordeum jubatum* L.) 分離所得的 Pat2 菌株外，*Phaeosphaeria* 屬菌株在譜系關係中非常相近而爲同一群。

關鍵詞：白圈環 -2 蛋白、*Phaeosphaeria* 屬、小麥