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Genetic linkage map of *Phaeosphaeria nodorum*, the causal agent of stagonospora nodorum blotch disease of wheat

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Abstract A genetic linkage map of the fungal pathogen *Phaeosphaeria nodorum*, the causal agent of stagonospora nodorum blotch disease of wheat, was created. A total of 152 ascospore-derived progeny from a single pseudothecium, which resulted from a cross of two opposite mating type isolates, Sn37-1 and S-81-B13B, was analysed with AFLP, RAPD, ISSR, expressed sequence tag (EST)-derived microsatellite primers and sequence tagged site markers developed from specific genes. The genetic linkage map consisted of 276 molecular markers, and included markers developed from five genes [Glyceraldehyde 3-phosphate dehydrogenase (*gpd*), malate synthase (*Mls1*), mannitol 1-phosphate dehydrogenase (*Mpd1*), mating type (*MAT1*) and RNA polymerase II (*RPB2*)], which were assigned to 21 major linkage groups (LGs). The total length of the 21 major LGs was 1,932.1 centiMorgans (cM) with an average spacing of 6.88 cM between loci. The idiomorph mating type gene (*MAT1*) loci was placed in LG 2 and was closely linked to RAPD marker A4-680. On the other hand, 24 molecular markers and four gene loci [β-glucosidase (*bgl1*), histidinol dehydrogenase (*Hdh2*), mannitol 1-phosphate dehydrogenase (*Mpd2*), and xylanase (*Xyl 10-2*)] were dispersed in 11 minor LGs. The segregation ratio of the xylanase (*Xyl 10-1*) locus was distorted and not mapped. This is the first genetic linkage map reported for this important foliar pathogen of wheat. In combination with the genomic sequence of *P. nodorum* strain SN15 (www.broad.mit.edu), the availability of a genetic linkage map of this organism would be an important tool to investigate quantitative trait loci (QTL) of biologically important phenotypes and for positional cloning.

Keywords AFLP · Ascomycetes · ISSR · RAPD

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Introduction

Phaeosphaeria nodorum (syn. *Leptosphaeria nodorum*; anamorph: *Stagonospora nodorum*, syn. *Septoria nodorum*) is one of the most important fungal pathogens, causing stagonospora leaf and glume

blotch diseases of wheat, and other cereals and grasses. The fungal disease is present world-wide and causes significant economic losses by decreasing yield and seed quality (Bhathal et al. 2003). Air temperature and relative humidity are critical factors in the releases of *P. nodorum* ascospores and pycnidiospores from plant debris, which is the primary source of disease during the wheat growing season. Mycelium in the infected wheat seeds can spread to seedlings during germination and subsequently becomes the primary source of infection (Shah and Bergstrom 2000). Subsequently, pycnidiospores are splashed in rain drops which result in secondary multi-cycle infections.

Genetic linkage maps provide a fundamental basis for understanding genome organisation and evolution, and are essential for mapping, cloning and characterisation of genes and for quantitative trait loci (QTL) mapping (Young 1990). Recently, genetic linkage maps have been constructed for ascomycete pathogens causing canola blackleg (*Leptosphaeria maculans*) (Cozijnsen et al. 2000), septoria tritici wheat blotch (*Mycosphaerella graminicola*) (Goodwin et al. 2007), and banana black leaf streak (*Mycosphaerella fijiensis*) (Manzo-Sánchez et al. 2008) diseases. Based on linkage analysis, molecular markers associated with genetic loci conferring mating type (Kema et al. 2002; Kubisiak and Milgroom 2006; Kuhn et al. 2006), host specificity, vegetative incompatibility (Kubisiak and Milgroom 2006) and avirulence (Kema et al. 2002) have been reported.

In recent years, a great effort has been made to explore genome structures by sequencing as well as by comparative genetic evolution of pathogenicity and sporulation in necrotrophic fungi (Soanes et al. 2007). Several genes involved in *P. nodorum* pathogenicity and sporulation, such as those encoding malate synthase, ornithine decarboxylase, mitogen-activated protein kinase, Gα protein subunit, δ-amino laevulinic acid synthase, and mannitol 1-phosphate dehydrogenase have been identified (Bailey et al. 2000; Solomon et al. 2006 2004a, b, 2005a, b). A draft genome sequence of *P. nodorum* strain SN15 comprising 37.2 mega base pairs (Mbp) of nuclear DNA in 107 scaffolds and containing an estimated minimum of 10,762 genes was reported (Hane et al. 2007).

The objective of this study was to construct a high-density genetic linkage map using RAPD, AFLP and inter-simple sequence repeat (ISSR) markers, and to locate the mating type locus and several other gene loci of interest in *P. nodorum*. This is the first genetic linkage map reported for *P. nodorum*.

Materials and methods

Sexual crossing and progeny isolation

Phaeosphaeria nodorum Sn37-1 isolate with the *MAT1-2* mating type (Ueng et al. 2003a, b), collected from wheat in Szelejewo, Poland, was crossed with *P. nodorum* S-81-B13B isolate with the *MAT1-1* mating type collected from barley in Bledsoe, Georgia, USA and pseudothecia were induced *in vitro* according to the procedures previously outlined (Arseniuk et al. 1997; Halama and Lacoste 1991). A single pseudothecium was isolated and crushed in a few drops of sterile water. Ascospores were streaked on 2% water agar plates and transferred individually and grown on triticale agar medium (17 g of triticale grains were cooked with 1 l of water and filtered through cheesecloth; 30 g of agar was added). A total of 152 single ascospores of the F_1 population were incubated under 12 h near-UV light and 12 h dark at 20°C until pycnidiospores formed. All ascospore-derived cultures were lyophilised in glass ampoules and maintained at 4°C. Fungal cultures were grown in a liquid medium and genomic DNA was isolated as described previously (Ueng et al. 1992).

Identification of polymorphism for RAPD and microsatellite markers

All PCR reactions used 1x reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0 at 25°C, 0.1% Triton X-100) and 0.5 U of *Taq* DNA polymerase from Promega (Madison, WI). The concentrations of other components in the reaction mixture varied with different amplification reactions.

For random amplification of polymorphic DNA (RAPD) analysis, 800 arbitrary primers (#1–#800) from University of British Columbia (UBC), Vancouver, Canada were used to screen for DNA polymorphisms between the two parental isolates, Sn37-1 and S-81-B13B. PCR was carried out in a 12.5 µl reaction mixture containing 1x reaction buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM arbitrary primer, 20 ng of genomic DNA, and 0.5 U of *Taq* DNA polymerase. Reaction parameters were: denaturation at 94°C for 3 min followed by

40 cycles of 94°C for 20 s, 35°C for 30 s, and 72°C for 1 min with final incubation at 72°C for 10 min. One hundred ISSR-targeted primers (#801–#900) from UBC were also used for genomic analysis in the two parental isolates. The reaction volume was 10 µl and contained 1x reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM ISSR primer, 16 ng of genomic DNA, and 0.5 U of *Taq* DNA polymerase. Reaction parameters were 94°C for 3 min followed by 40 cycles of 94°C for 30 s, 42°C for 45 s, and 72°C for 1 min with final incubation at 72°C for 10 min. Twelve expressed sequence tag (EST)-derived microsatellite primers were also used to detect potential polymorphism in the two parental isolates (Stukenbrock et al. 2005). PCR was performed following the concentrations and temperature settings described by the authors.

Amplification products were loaded on 1.5% SeaKem GTG agarose gel (Lonza, Rockland, ME) and run at 150 V for 3 h in 1x TBE buffer and stained with ethidium bromide for 30 min.

Identification of amplified fragment length polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP) analysis was used for genome mapping in wheat biotype *P. nodorum*. Two µg of genomic DNA was digested with 20 U of *Eco*RI and 10 U of *Mse*I endonucleases (New England Biolabs, Ipswich, MA) overnight at 37°C. Digested DNA was extracted with an equal volume of phenol/chloroform mixture (1:1), precipitated with ethanol, re-suspended in 50 µl of sterile water and ligated with a mixture of 5 µM *Eco*RI and 50 µM *Mse*I adaptors. The pre-selective PCR was performed in a 20 µl reaction mixture containing 1x reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM each of *Eco*RI N+0 and *Mse*I N+0 primers, 2 µl of ligated DNA, and 0.5 U of *Taq* DNA polymerase. The PCR was run with initial denaturation at 94°C for 2 min followed by 25 cycles of 94°C for 30 s, 35°C for 1 min, and 72°C for 1 min with final extension at 72°C for 5 min. Products of the pre-selective PCR were diluted 10X with water. Subsequently, selective PCR was carried out in a 20 µl reaction mixture containing 5 µl of diluted pre-selective reaction mixture as template and the same other components as used in the pre-selective PCR, except 0.5 µM of each *Eco*RI N+2 and *Mse*I N+2 primer were used. Reaction parameters were denaturation at 94°C for 2 min, followed by 12 cycles of denaturation at 94°C for 30 s, 65°C for 30 s with a 0.7°C decrease in each subsequent cycle and extension at 72°C for 1 min. This was followed by another 23 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min. The PCR-amplified samples were loaded on a 6% denaturing sequencing polyacrylamide gel and run on a Model S2 sequencing gel electrophoresis apparatus (Gibco BRL Life Technologies, Rockville, MD) at 60 W until blue dye reached the gel bottom. Gels were stained with SILVER SEQUENCE™ DNA staining reagents (Promega).

Identification and detection of nucleotide sequence variants

To position gene loci on the genetic map, nucleotide sequence variants between Sn37-1 and S-81-B13B from *P. nodorum* genes were first identified. Primer sets were designed to the regions with variants; two primer sets were selected based on each of the sequences from the two isolates, in the case of the histidinol dehydrogenase (*Hdh2*) gene, only one primer set was selected based on the sequence from one of the isolates. In the PCR process, with optimised PCR conditions, such as magnesium ion concentration and annealing temperature, primer sets specific to the sequence of one isolate will only amplify products from the progenies containing the identical genotype of the parental isolate from which the primers were designed. Thus, segregation corresponding to the presence or absence of the expected PCR fragment in the progeny would be observed on agarose gel and the resulting data were used for genetic mapping (Table 1).

To develop primer sets specific to the sequences of the genes encoding malate synthase (*Mls1*) and mannitol 1-phosphate dehydrogenase (*Mpd1* and *Mpd2*), sequences from GenBank accessions AY508881, AY547308 and AY587541 were used (Solomon et al. 2004a, b, 2005a, b). Genomic DNA amplification, fragment isolation, nucleotide sequencing, sequence alignment and variant identification followed the procedures previously described (Ueng et al. 2003a, b).

In two other genes, which encoded β-glucosidase (*bgl1*) (Accession no. AY683619) and glyceraldehyde 3-phosphate dehydrogenase (*Gpd*) (Accession no. AY364460) polypeptides, primer sets Bg4A/

Table 1 Primer sets used for PCR amplification of partial gene fragments as genetic markers for mapping in *Phaeosphaeria nodorum*

Gene [a]	*P. nodorum* isolate	Primer sets and their nucleotide (nt) positions [b]	Primer sequences (5′→3′)	PCR reaction parameters		Sizes (bp) [c] of PCR products
				Temp (°C)	$MgCl_2$ (mM)	
MAT1-2 [AY212019]	Sn37-1	Mat22GA/Mat22GB (nt2488-nt2509/nt2968-nt2945)	GGTGGCGCGGTTGCTGTTACTA/ GACTGTCGCTTCTTCTTCTCACCA	55	1.5	480
MAT1-1 [AY212018]	S-81-B13B	Mat12GA/Mat12GB (nt2603-nt2623/nt3011-nt2988)	GCTACGCGCCCAACAAGAACG/ TTTAGGTCGAAGGAGGGCAGAACG	55	1.5	408
bgl1	Sn37-1 [AY683619]	Bg4A2 [d]/Bg4B (nt76-nt97/nt591-nt570)	CCACGTCGAGTCGCCACGAAAA/ CTGTGCCGGTTGTGAGGTTGAC	70	1.25	516
	S-81-B13B	Bg4A1 [d]/Bg4B (nt76-nt97/nt591-nt570)	CCACGTCGAGTCGCCACGAAGC/ CTGTGCCGGTTGTGAGGTTGAC	70	1.25	516
Gpd	Sn37-1C [AY364460]	GPD-1A/GPD-4B (nt1016-nt1036/ nt1189-nt1169)	CACTGGTTAAAAAAAAAACAC/ CTCGTTGTCGTACCAGGAGAC	55	1.25	174
	S-81-B13B	GPD-2A [d]/GPD-4B	CACTGGTTAAGAAACACAAT/ CTCGTTGTCGTACCAGGAGAC	55	1.25	172
Hdh2 [e]	Sn37-1 [EU267784]	Hdh2 4A/Hdh2 4B(nt922-nt943/ nt1184-nt1163)	CAATAGCGTTCATTGACGAACT/ GATCAAATAAAGACATACCTTG	55	1.00	263
Mls1 [AY508881]	Sn37-1 [FJ151550]	MS12A/MS13B (nt1477-nt1498/ nt1624-nt1603)	GAGCCAGCTGTGGCAATGGACG/ CTCGGCAACGTGGAACTTGTTG	70	1.25	148
	S-81-B13B [FJ151551]	MS11A/MS11B (nt57-nt75/nt918-nt898)	CGACCAGAACCGTCACATC/ GAGTGGTCGCGAAGCTCGTAG	65	1.5	862
Mpd1 [AY547308]	Sn37-1 [FJ151548]	MPD1 2A/MPD1 2B (nt384-nt405/ nt768-nt747)	CAACGCCACAACCACATGGAGA/ GACAGCATCACGGACAATGTCA	70	1.25	385
	S-81-B13B [FJ151549]	MPD1 1A/MPD1 1B(nt384-nt405/ nt768-nt747)	CAACGCCACAACCACATGGAGG/ GACAGCGTCACGGACAATGTCG	70	1.00	385
Mpd2 [AY587541]	Sn37-1 [FJ151546]	MPD2 2A/MPD2 2B (nt168-nt189/ nt286-nt264)	CAATGCTGGCCGTGCGATGGAG/ GTTCGTCGCCAACGCGTATCGTG	70	1.00	119
	S-81-B13B [FJ151547]	MPD2 1A/MPD2 1B (nt168-nt189/ nt286-nt264)	CAATGCTGGCCGTGCGATGGAA/ GTTCGTCGCCAACGCGTATCGTA	70	1.00	119
RPB2	Sn37-1 [DQ278491]	RPB3A/RPB3B(nt1402-nt1420/ nt2489-2,470)RPB4A/RPB4B (nt204-nt222/nt1291-nt1272)	GAACACGCCCTACGACCCA/ GTCCTGCCAGATCCAGTCGT	60	1.25	1,088
	S-81-B13B [DQ499809]		GAACACGCCCTACGACCCC/ GTCCTGCCAGATCCAGTCGC	70	1.25	1,088
Xyl 10-1	Sn37-1 [FJ151542]	Xy4MA/Xy4MB (nt695-nt712/ nt1120-nt1103)	GACACACTACTGTGTGG**C**/ CTTGGTTTTGGCGTACGC	60	1.5	426
	S-81-B13B [FJ151543]	Xy4ZA/Xy4ZB (nt695-nt712/ nt1120-nt1103)	GACACACTACTGTGTGGT/ CTTGGTTTTGGCGTACGT	60	1.5	426
Xyl 10-2	Sn37-1 [FJ151544]	Xy3MA/Xy3MB (nt134-nt153/ nt711-nt691)	GTACTTCGTTGACGATCCGC/ CAGGATTGAACAAGCTTGACG	65	1.25	578
	S-81-B13B [FJ151545]	Xy3ZA/Xy3ZB (nt134-nt153/ nt711-nt691)	GTACTTCGTTGACGATCCGT/ CAGGATTGAACAAGCTTGACA	60	1.00	578

[a] The abbreviated gene names are as follows: *MAT1-2*=mating type 2; *MAT1-1*=mating type 1; *bgl1*=β-glucosidase; *gpd*=glyceraldehyde-3-phosphate dehydrogenase; *hdh2*=histidinol dehydrogenase 2; *Mls1*=malate synthase; *Mpd1* and *Mpd2*=mannitol 1-phosphate dehydrogenase 1 and 2; *RPB2*=the second largest protein subunit of RNA polymerase II; *Xyl 10-1*=xylanase 1; *Xyl 10-2*=xylanase 2.

[b] The nucleotide positions (nt) of the primer sequences are based on the complete gene sequences deposited in GenBank.

[c] bp=base pairs.

[d] Primers Bg4A1 and Bg4A2 for the *bgl1* gene are derived from the PCR product sequences amplified with Bg4A (CGCGCCCGAGTGTTGAGAG)/Bg4B, and primer GPD-2A for the *gpd* gene is from the PCR product sequence amplified with primer set GPD-42A (GCACTGCGGCCCAGAACATCAT)/GPD-4B.

[e] Only one primer set was useful to detect the *Hdh2* gene fragment derived from Sn37-1 isolate.

The accession numbers of the genes are bracketed.

Bg4B (CGCGCCCGAGTGTTGAGAG / CTGTGCCGGTTGTGAGGTTGAC) and GPD-42A/GPD-4B (GCACTGCGGCCCAGAACATCAT / CTCGTTGTCGTACCAGGAGAC) were used to amplify the specific gene regions, respectively (Reszka et al. 2005; Ueng et al. 2003a, b). These two gene regions from the two parental isolates were sequenced to find nucleotide sequence diversities. The observed sequence variations were used for designing specific primer sets. In each gene, either $MgCl_2$ concentrations or annealing temperatures in reaction parameters were selected so that specific PCR products from one parental isolate, but not the other were amplified.

Sequence diversities were also observed in four full-length genes between two parental *P. nodorum* isolates, which included histidinol dehydrogenase (*Hdh2*) (Accession no EU267784), RNA polymerase II (*RPB2*) (Accession nos DQ278491 and DQ499809) and two families of 10 xylanases (*Xyl 10-1* and *Xyl 10-2*) (Accession nos FJ151542–FJ151545) (Malkus et al. 2006; Wang et al. 2008). The *Xyl 10* genes were isolated from *P. nodorum* by firstly amplifying the genomic DNA with two degenerate primers (TGGGAYGTNGTNAAYGA/TTRTARTCRTT DATRTANARYTT), derived from two peptide regions (WDVVNE and KLYINDYN) conserved in the family 10 xylanases in fungi. The complete xylanase gene sequences were further obtained with a 'step-down' PCR amplification technique (Zhang and Gurr 2002). As above, PCR primers were designed to permit amplification of a PCR product from one parental isolate and no amplification from the alternative parent.

Since the mating type genes in heterothallic *P. nodorum* were idiomorphs, which occupy a single locus in the chromosome, two primer sets were designed to amplify two completely different DNA fragments in the two parental isolates, Sn37-1 and S-81-B13B (Table 1).

Linkage and gene mapping analysis

The RAPD and ISSR arbitrary primer numbers assigned by UBC and the abbreviated names (EXXMYY) of *Eco*RI N+2 and *Mse*I N+2 primer combinations in AFLP were used as marker names. Amplified fragment sizes (bp), estimated using an Alpha Imager™ 3,400 (Alpha Innotech, San Leandro, CA) with GeneRuler™ 100 bp DNA Ladder Plus markers (Fermentas Inc, Hanover, MD), were listed after the marker names. Markers were also preceded with letters A or B indicating their presence in parental isolate Sn37-1 and isolate S-81-B13B, respectively.

Mapping analysis was done by using JoinMap® 4.0 software (Van Ooijen 2006). Markers with segregation ratios significantly different from the expected 1:1 ($P<0.01$) were discarded to avoid erroneous groupings of unlinked markers. Linkage analysis was first performed on the markers with LOD values ranging from 3.0 to 5.0. The final assembly of linkage groups was completed with the LOD value set at 3.5 and analysed with the Kosambi genome mapping function.

Results

Polymorphism of the RAPD, AFLP and ISSR markers between Sn37-1 and S-81-B13B

In the RAPD analysis, 134 of 800 arbitrary primers generated 203 reliable polymorphic bands that were easy to score in the two parental isolates. However, only 13 of 100 ISSR primers, which produced 17 polymorphic bands in the two parental isolates could be used for genetic map construction. After screening with 64 AFLP primer combinations, nine that gave clear and specific markers in the two parental isolates were chosen for analysis of the progeny. Of 96 AFLP markers, 49 produced a band from isolate Sn37-1 and 46 from isolate Sn81-B13B.

Polymorphism of the genes and EST-derived SSRs markers between Sn37-1 and S-81-B13B

Sequence variants among all ten genes were identified, and specific primers were designed and successfully amplified a product from one of the parental isolates and produced no product with the other. From these, two sets of primer pairs were selected for each of nine genes, and one primer set was selected for Hdh2. In addition, the gene sequences encoding MAP kinase (*Mak2*), trifunctional histidine biosynthesis (*his*) and β-tubulin (*tubA*) were analysed in two parental isolates (Malkus et al. 2005; Solomon et al. 2006; Wang et al. 2007). No nucleotide variation was identified in these three genes.

Table 2 The 32 linkage groups in the genetic map of wheat biotype *Phaeosphaeria nodorum*

Linkage groups	Size (cM)	Total number of markers	Number of RAPD markers	Number of AFLP markers	Number of ISSR markers	Number of SSRs from ESTs	Positioned gene loci
1	187.5	35	21	11	3	-	-
2	173.2	20	12	8	-	-	*MAT1*
3	140.0	23	8	8	5	2	-
4	137.4	18	10	8	-	-	-
5	135.2	29	20	7	-	2	*Mls1*
6	121.6	31	21	9	1	-	*Mpd1*
7	119.4	14	9	5	-	-	-
8	117.2	13	13	-	-	-	-
9	108.9	8	5	3	-	-	*RPB2*
10	97.4	13	10	2	1	-	*Gpd*
11	78.8	4	4	-	-	-	-
12	71.6	9	7	1	1	-	-
13	68.5	9	8	1	-	-	-
14	65.8	11	4	3	3	1	-
15	59.1	7	3	4	-	-	-
16	54.2	7	5	2	-	-	-
17	53.8	7	5	2	-	-	-
18	52.5	6	5	1	-	-	-
19	49.6	4	4	-	-	-	-
20	38.3	4	2	2	-	-	-
21	2.1	4	1	3	-	-	-
Total	1,932.1	276	177	80	14	5	5
22	51.0	2	-	2	-	-	*Xyl 10-2*
23	33.5	3	2	-	1	-	-
24	28.6	3	2	1	-	-	-
25	17.7	2	1	1	-	-	*bgl1*
26	16.9	2	1	-	1	-	-
27	13.0	3	2	1	-	-	-
28	10.6	3	3	-	-	-	-
29	7.5	2	1	-	1	-	-
30	6.1	2	1	1	-	-	-
31	3.4	1	1	-	-	-	*Mpd 2*
32	2.1	1	1	-	-	-	*Hdh2*
Total	190.4	24	15	6	3	-	4

Among 12 microsatellite primer sets derived from ESTs, five loci amplified by three primer sets were polymorphic between the two parental isolates (Stukenbrock et al. 2005). Primer sets SNOD5 (Accession no. DR045205) and SNOD8 (Accession no. DR045843) produced polymorphic bands that distinguished the two parental isolates, and primer set SNOD26 (Accession no. DR045700) gave a single specific band for the S-81-B13B isolate. Primer sets SNOD11, SNOD21, SNOD22 (designed from Accession nos. DR045898, DR074925, DR045164) did not amplify a PCR product in either of the two parental isolates (Malkus *et al.*, unpublished data).

Genetic map

In total, 330 loci (203 RAPD, 17 polymorphic bands from 13 ISSR, 95 AFLP, 5 EST-SSR-derived markers and 10 gene loci) were analysed using JoinMap® 4.0 software with a LOD value of 3.5. Five marker loci, one RAPD (B305-529), and four AFLP (AEAGMAG1093, AEGGMTG561, BECAMTC1099, and BECTMTG106) and the *Xyl 10-1* gene deviated significantly from the expected 1:1 segregation ratio ($\chi^2 \geq 7.0$, $P<0.01$) and were omitted in further analysis. A total of 276 RAPD and AFLP markers and five gene loci was assigned to 21 major linkage groups (LGs), each of which had at least four loci (Fig. 1 and Table 2). The largest of the major LGs, LG1, was 187.5 centiMorgans (cM) in length and contained 35 RAPD and AFLP markers while the smallest, LG21, was only 2.1 cM in length and contained four markers. The 21 major LGs were a total of 1,932.1 cM in length with an average spacing of 6.88 cM between loci (Table 2). Eleven markers in LG17 and LG21 originated from isolate Sn37-1 while four markers in LG20 were associated with isolate S-81-B13B. Eight markers (9 RAPD, 6 AFLP) were unlinked. The idiomorph mating type gene (*MAT1*) was placed in LG 2 and was closely linked to RAPD marker A4-680 (Fig. 1).

A total of 24 RAPD and AFLP markers and four gene loci were dispersed in 11 minor genetic linkage groups containing 1–3 loci ranging in length from 2.1 to 51 cM. The *Mpd2* and *Hdh2* gene markers were each linked with only one marker, whereas the *Xyl 10-2* and *bgl1* gene markers were each in linkage groups with two other markers.

Discussion

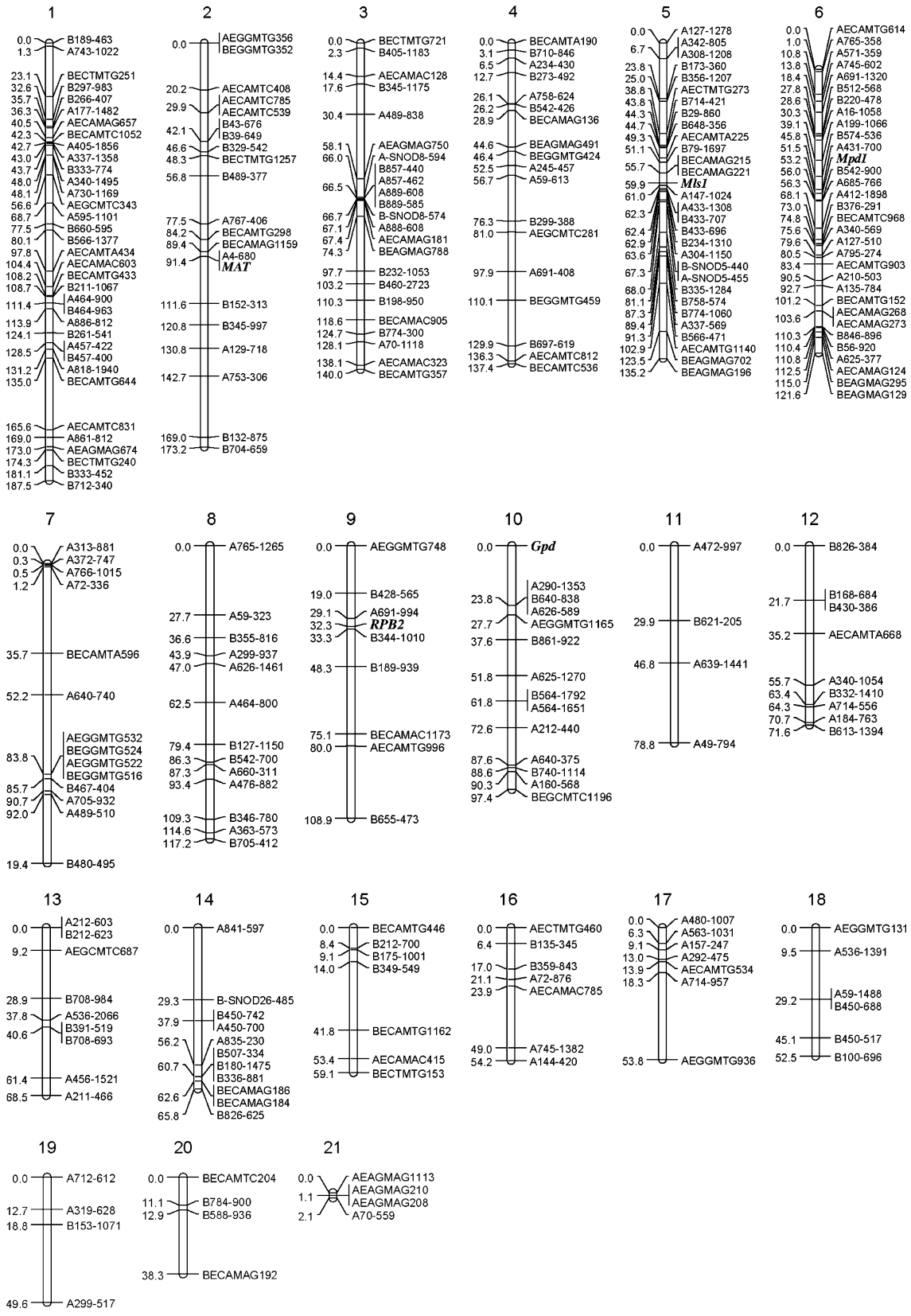
A single pseudothecium of *P. nodorum* is reported to produce from 120 to 200 ascospores (Scharen 1999). In this study, we successfully crossed two *P. nodorum* isolates with opposite mating types, and obtained a large number (152) of isolates from a single pseudothecium. To our knowledge this is the largest reported population generated *in vitro* for this pathogen, and larger than the population sizes commonly used for genetic mapping in other plant pathogenic fungi by other investigators. For example, 68 isolates were used for mapping in *M. graminicola* (Kema et al. 2002), 61 in *M. fijiensis* (Manzo-Sánchez et al. 2008), 98 in *L. maculans* (Kuhn et al. 2006), and 58 in *Magnaporthe grisea* (Kaye et al. 2003). It is understood that a larger population size provides a more precise map in terms of resolving the order of closely linked markers and of providing superior estimates of genetic distances.

The genetic linkage map of *P. nodorum* contains 276 marker loci and five known gene loci within 21 major linkage groups (LGs), which have a total length of 1,932.1 cM (Table 2). A large number of LGs was also reported in *L. maculans* (15 LGs with 443 loci) (Kuhn et al. 2006) and in *M. graminicola* (23 LGs with 280 loci) (Kema et al. 2002). The number of major LGs in *P. nodorum* estimated in this study was approximately equal to the number of chromosomes (14–19) in *P. nodorum* as visualised by pulsed-field electrophoresis (Cooley and Caten 1991).

On the other hand, there are 24 markers and four known gene loci assigned to 11 small LGs as well as eight unlinked markers (Table 2), which may be merged with the major LGs when more markers are available for segregation analysis. Using the EST database of *Mycosphaerella graminicola*, 23 new polymorphic SSR markers were added to the existing genetic linkage map previously described by Kema et al. (2002), which resulted in the merging of LG5 with LG10 and LG11 with LG20 (Goodwin et al. 2007). Likewise, the EST database generated from *P. nodorum* isolate N15 (http://www.broad.mit.edu/annotation/genome/stagonospora_nodorum/Home.html) should be a valuable source for SSR marker development. In our work, five of the 12 EST-derived microsatellite loci selected by Stukenbrock et al. (2005) were incorporated into the current linkage map.

The mating type locus was positioned on major LG2, and was closely linked and co-segregated with marker A4-680. As previously reported, the mating type loci were also successfully mapped in three other fungal pathogens, *Cryphonectria parasitica* (Kubisiak and Milgroom 2006), *L. maculans* (Cozijnsen et al. 2000; Kuhn et al. 2006), and *M. graminicola* (Kema et al. 2002).

The genetic linkage map is an important tool to investigate QTL controlling biologically important phenotypic traits (Young 1990). In fungal pathogens, pathogenicity and sporulation are generally regulated by multiple genes. Recently, QTL associated with aggressiveness and pathogenicity in *Gibberella zeae*, a major causal agent of Fusarium head blight of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◀ **Fig. 1** The genetic linkage map of *Phaeosphaeria nodorum*. A total of 276 molecular markers and five genes encoding mating type (*MAT*), malate synthase (*Mls1*), mannitol 1-phosphate dehydrogenase (*Mpd1*), RNA polymerase II (*RPB2*) and glyceraldehyde 3-phosphate dehydrogenase (*Gpd*) was included in the 21 major linkage groups analysed with LOD value set at 3.5. Marker distance (cM) derived with Kosambi genome mapping function is indicated on the left of each linkage group. RAPD, ISSR, AFLP and EST-derived microsatellite markers are denominated as in Materials and methods. The total length of the genetic linkage map is 1,932.1 cM

wheat, were successfully mapped (Cumagun et al. 2004). In tetrad analysis of sexual crossing products, the aggressiveness in *P. nodorum* seems to be a quantitative trait (Halama et al. 1999). An analysis of the segregation of phenotypic and genetic markers to determine the QTL associated with fungal aggressiveness would be a logical follow-up to the current study.

References

Arseniuk, E., Czembor, P. C., & Cunfer, B. M. (1997). Segregation and recombination of PCR-based markers in progenies of *in vitro* mated isolates of *Phaeosphaeria nodorum* (Müller) Hedjaroude. *Phytopathology*, *87*(Supplement), S5.

Bailey, A., Mueller, E., & Bowyer, P. (2000). Ornithine decarboxylase of *Stagonospora* (*Septoria*) *nodorum* is required for virulence toward wheat. *The Journal of Biological Chemistry*, *275*, 14242–14247. doi:10.1074/jbc.275.19.14242.

Bhathal, J. S., Loughman, R., & Speijers, J. (2003). Yield reduction in wheat in relation to leaf disease from yellow (tan) spot and septoria nodorum blotch. *European Journal of Plant Pathology*, *109*, 435–443. doi:10.1023/A:1024277420773.

Cooley, R. N., & Caten, C. E. (1991). Variation in electrophoretic karyotype between strains of *Septoria nodorum*. *Molecular Genetics and Genomics*, *228*, 17–23.

Cozijnsen, A. J., Popa, K. M., Purwantara, A., Rolls, B. D., & Howlett, B. J. (2000). Genome analysis of the plant pathogenic ascomycete *Leptosphaeria maculans*; mapping mating type and host specificity loci. *Molecular Plant Pathology*, *1*, 293–302. doi:10.1046/j.1364-3703.2000.00033.x.

Cumagun, C. J. R., Bowden, R. L., Jurgenson, J. E., Leslie, J. F., & Miedaner, T. (2004). Genetic mapping of pathogenicity and aggressiveness of *Gibberella zeae* (*Fusarium graminearum*) toward wheat. *Phytopathology*, *94*, 520–526. doi:10.1094/PHYTO.2004.94.5.520.

Goodwin, S. B., Van der Lee, T. A. J., Cavaletto, J. R., Te Lintel Hekkert, B., Crane, C. F., & Kema, G. H. J. (2007). Identification and genetic mapping of highly polymorphic microsatellite loci from an EST database of the septoria tritici blotch pathogen *Mycosphaerella graminicola*. *Fungal Genetics and Biology*, *44*, 398–414. doi:10.1016/j.fgb.2006.09.004.

Halama, P., & Lacoste, L. (1991). Déterminisme de la reproduction sexuée du *Phaeosphaeria* (*Leptosphaeria*) *nodorum*, agent de la septoriose du blé. I. hétérothallisme et rôle des microspores. *Canadian Journal of Botany*, *69*, 95–99.

Halama, P., Skajennikoff, M., & Dehorter, B. (1999). Tetrad analysis of mating type, mutations, esterase and aggressiveness in *Phaeosphaeria nodorum*. *Mycological Research*, *103*, 43–49. doi:10.1017/S0953756298006728.

Hane, J. K., Lowe, R. G. T., Solomon, P. S., Tan, K. C., Schoch, C. L., Spatafora, J. W., et al. (2007). Dothideomycete-plant interactions illuminated by genome sequencing and EST analysis of the wheat pathogen *Stagonospora nodorum*. *The Plant Cell*, *19*, 3347–3368. doi:10.1105/tpc.107.052829.

Kaye, C., Milazzo, J., Rozenfeld, S., Lebrun, M. H., & Tharreau, D. (2003). The development of simple sequence repeat (SSR) markers for *Magnaporthe grisae* and their integration into an established genetic linkage map. *Fungal Genetics and Biology*, *40*, 207–214. doi:10.1016/j.fgb.2003.08.001.

Kema, G. H. J., Goodwin, S. B., Hamza, S., Verstappen, E. C. P., Cavaletto, J. R., Van der Lee, T. A. J., et al. (2002). A combined amplified fragment length polymorphism and randomly amplified polymorphism DNA genetic linkage map of *Mycosphaerella graminicola*, the Septoria tritici leaf blotch pathogen of wheat. *Genetics*, *161*, 1497–1505.

Kubisiak, T. L., & Milgroom, M. G. (2006). Markers linked to vegetative incompatibility (*vic*) genes and a region of high heterogeneity and reduced recombination near the mating type locus (*MAT*) in *Cryphonectria parasitica*. *Fungal Genetics and Biology*, *43*, 453–463. doi:10.1016/j.fgb.2006.02.002.

Kuhn, M. L., Gout, L., Howlett, B. J., Melayah, D., Meyer, M., Balesdent, M. H., et al. (2006). Genetic linkage maps and genomic organization in *Leptosphaeria maculans*. *European Journal of Plant Pathology*, *114*, 17–31. doi:10.1007/s10658-005-3168-6.

Malkus, A., Reszka, E., Chang, C. J., Arseniuk, E., Chang, P. F. L., & Ueng, P. P. (2005). Sequence diversity of β-tubulin (*tubA*) gene in *Phaeosphaeria nodorum* and *P. avenaria*. *FEMS Microbiology Letters*, *249*, 49–56. doi:10.1016/j.femsle.2005.05.049.

Malkus, A., Chang, P. F. L., Zuzga, S. M., Chung, K. R., Shao, J., Cunfer, B. M., et al. (2006). RNA polymerase II gene *(RPB2)* encoding the second largest protein subunit in *Phaeosphaeria nodorum* and *P. avenaria*. *Mycological Research*, *110*, 1152–1164. doi:10.1016/j.mycres.2006.07.015.

Manzo-Sánchez, G., Zapater, M. F., Luna-Martínez, F., Conde-Ferráez, L., Carlier, J., James-Kay, A., et al. (2008). Construction of a genetic linkage map of the fungal pathogen of banana *Mycosphaerella fijiensis*, causal agent of black leaf streak disease. *Current Genetics*, *53*, 299–311. doi:10.1007/s00294-008-0186-x.

Reszka, E., Chung, K. R., Tekauz, A., Malkus, A., Arseniuk, E., & Krupinsky, J. M. (2005). Presence of β-glucosidase (*bgl1*) gene in *Phaeosphaeria nodorum* and *Phaeosphaeria avenaria* f.sp. *triticea*. *Canadian Journal of Botany*, *83*, 1001–1014. doi:10.1139/b05-052.

Scharen, A. L. (1999). Biology of the *Septoria*/*Stagonospora* pathogens: An overview. In M. van Ginkel, A. McNab & J. Krupinsky (Eds.), *Septoria and Stagonospora diseases of cereals: a compilation of global research*, pp. 19–22. Mexico, D.F: CIMMYT.

Shah, D. A., & Bergstrom, G. C. (2000). Temperature dependent seed transmission of *Stagonospora nodorum* in wheat. *European Journal of Plant Pathology*, *106*, 837–842. doi:10.1023/A:1008723823196.

Soanes, D. M., Richards, T. A., & Talbot, N. J. (2007). Insights from sequencing fungal and oomycete genomes: what can we learn about plant disease and the evolution of pathogenicity. *The Plant Cell*, *19*, 3318–3326. doi:10.1105/tpc.107.056663.

Solomon, P. S., Lee, R. C., Wilson, T. J. G., & Oliver, R. P. (2004a). Pathogenicity of *Stagonospora nodorum* requires malate synthase. *Molecular Microbiology*, *53*, 1065–1073. doi:10.1111/j.1365-2958.2004.04178.x.

Solomon, P. S., Tan, K. C., Sanchez, P., Cooper, R. M., & Oliver, R. P. (2004b). The disruption of a Gα subunit sheds new light on the pathogenicity of *Stagonospora nodorum* on wheat. *Molecular Plant-Microbe Interactions*, *17*, 456–466. doi:10.1094/MPMI.2004.17.5.456.

Solomon, P. S., Tan, K. C., & Oliver, R. P. (2005a). Mannitol 1-phosphate metabolism is required for sporulation in planta of the wheat pathogen *Stagonospora nodorum*. *Molecular Plant-Microbe Interactions*, *18*, 110–115. doi:10.1094/MPMI-18-0110.

Solomon, P. S., Waters, O. D. C., Simmonds, J., Cooper, R. M., & Oliver, R. P. (2005b). The *Mak2* MAP kinase signal transduction pathway is required for pathogenicity in *Stagonospora nodorum*. *Current Genetics*, *48*, 60–68. doi:10.1007/s00294-005-0588-y.

Solomon, P. S., Jörgens, C. I., & Oliver, R. P. (2006). δ-aminolaevulinic acid synthesis is required for virulence of the wheat pathogen *Stagonospora nodorum*. *Microbiology*, *152*, 1533–1538. doi:10.1099/mic.0.28556-0.

Stukenbrock, E. H., Banke, S., Zala, M., McDonald, B. A., & Oliver, R. P. (2005). Isolation and characterization of EST-derived microsatellite loci from the fungal wheat pathogen *Phaeosphaeria nodorum*. *Molecular Ecology Notes*, *5*, 931–933. doi:10.1111/j.1471-8286.2005.01120.x.

Ueng, P. P., Arseniuk, E., Cunfer, B. M., & Song, Q. J. (2003a). Transposition and aggressiveness in a *Phaeosphaeria nodorum* sexual cross. *Plant Pathology Bulletin*, *12*, 149–156.

Ueng, P. P., Reszka, E., Chung, K. R., Arseniuk, E., & Krupinsky, J. M. (2003b). Comparison of glyceraldehyde-3-phosphate dehydrogenase genes in *Phaeosphaeria nodorum* and *P. avenaria* species. *Plant Pathology Bulletin*, *12*, 255–268.

Ueng, P. P., Bergstrom, G. C., Slay, R. M., Geiger, E. A., Shaner, G., & Scharen, A. L. (1992). Restriction fragment length polymorphisms in the wheat glume blotch fungus, *Phaeosphaeria nodorum*. *Phytopathology*, *82*, 1302–1305. doi:10.1094/Phyto-82-1302.

Van Ooijen, J. W. (2006). *JoinMap® 4, Software for the calculation of genetic linkage maps in experimental populations*. Wageningen, Netherlands: Kyazma BV.

Wang, C. L., Malkus, A., Zuzga, S. M., Chang, P. F. L., Cunfer, B. M., Arseniuk, E., et al. (2007). Diversity of the trifunctional histidine biosynthesis gene (*his*) in cereal *Phaeosphaeria* species. *Genome*, *50*, 595–609. doi:10.1139/G07-038.

Wang, C. L., Lin, Y. H., Chiu, E. Y. H., & Ueng, P. P. (2008). Evolution of a histidinol dehydrogenase (Hdh2) pseudogene in wheat-biotype *Phaeosphaeria nodorum*. *Plant Pathology Bulletin*, *17*, 221–232.

Young, N. D. (1990). Potential applications of map-based cloning to plant pathology. *Physiological and Molecular Plant Pathology*, *37*, 81–94. doi:10.1016/0885-5765(90)90001-E.

Zhang, Z., & Gurr, S. J. (2002). A "step down" PCR-based technique for walking into and the subsequent direct-sequence analysis of flanking genomic DNA. *Methods in Molecular Biology (Clifton, N.J.)*, *192*, 343–350.