 doi:10.20944/preprints201910.0147.v1

Article

Whole Genome Alignment Based Development of Molecular Marker for Detecting *Leptosphaeria maculans* and *Leptosphaeria biglobosa*, the Causal Agent of Blackleg Disease in Brassicas

Mostari Jahan Ferdous[1,†], Mohammad Rashed Hossain[1,2,†], Denison Michael Immanuel Jesse[1], Jong-In Park[1], Hee-Jeong Jung[1], Hoy-Taek Kim[1], Ill-Sup Nou[1,*]

[1]Department of Horticulture, Sunchon National University, Suncheon, Jeonnam, 57922, Republic of Korea
[2]Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensing-2202, Bangladesh
[†]Equal contributors

[*]**Correspondence:** nis@sunchon.ac.kr (I-S.N.), Tel: +82-61-750-3249 (ISN); Fax: +82-61-750-3208 (ISN).

Author's Email addresses: mjferdous@gmail.com (MJF); m.r.hossain@bau.edu.bd (MRH); michaelijesse@scnu.ac.kr (DMIJ); jipark@scnu.ac.kr (JIP); gml79wjd@scnu.ac.kr (HJJ); htkim@scnu.ac.kr (HTK); nis@scnu.ac.kr (ISN).

Running Title: Genome-alignment based molecular marker for blackleg fungus

Abstract:

Background: Accurate diagnosis of the differentially aggressive fungus *Leptosphaeria maculans* and *Leptosphaeria biglobosa* causing Blackleg in crucifers is crucial. Available markers were designed decades ago which may become ineffective due to the ever evolving nature of the fungus, requiring the development of more precise molecular markers.

Methods: The whole genomes of available isolates belonging to this two species were aligned using progressive MAUVE tool, species specific genomic regions were extracted and species specific primers were designed from the sequences that encode for effector proteins.

Results: Three (Lm1, Lm2 and Lm5) and two (Lb3 and Lb3') primer sets specifically detected the isolates of target species in PCR based assay, of which the primers Lm5 and Lb3' were multiplexed for detection of *Leptosphaeria maculans* and *Leptosphaeria biglobosa,* generating PCR amplicons of 230 and 834 bp, respectively from a single PCR reaction. The markers were highly sensitive and were able to amplify target species from crude 'pseudothecia and ascospores suspension' without requiring DNA extraction.

Conclusions: These markers, solitarily or in combination, designed from species specific genomic segments will serve as precise, sensitive and rapid detection of *Leptosphaeria maculans* and *Leptosphaeria biglobosa* species and will be helpful for surveillance, management and transboundary quarantine of the devastating disease.

Key words: Blackleg, Brassica, Diagnosis, Effector, Genome Alignment, *Leptosphaeria biglobosa*, *Leptosphaeria maculans,* Marker, PCR.

1. Introduction

Leptosphaeria maculans (Desm.) Ces. & de Not. (anamorph = *Plenodomus lingam*) and *Leptosphaeria biglobosa* Shoemaker & Brun (anamorph = *P. biglobosus*) are economically important dothideomycete fungal pathogen that cause the devastating blackleg or phoma stem canker on oilseed rape and other Brassica crops worldwide [1,2].

Leptosphaeria, one of the largest genera in the Pleosporales, having around 1600 described taxa, exhibits considerable species diversity [2,3]. As many as 12 species of Leptosphaeria have been identified from various cruciferous host crops and weeds [3], that includes *L. maculans* 'brassicae' and *L. biglobosa* 'brassicae' (from various *Brassica* crops), *L. biglobosa* 'erysimii' (*Erysimum* sp.), *L. maculans* 'lepidii ' (*Lepidium* sp.), *L. biglobosa* 'thlaspii' (*Thlaspi arvense*), *L. biglobosa* 'canadensis' (from Canada) and *L. biglobosa* 'australensis' (from Australia) etc. [2,4]. Among these, *L. maculans* is the most ubiquitous and aggressive pathogen, usually causes canker on stem base (blackleg) while the less aggressive *L. biglobosa* causes less damaging lesions higher up the stem base [1,5,6].

Besides, these two species have similar life cycles, common growth and morphological characteristics, mode of initial infections and favors similar ecological habitat and often co-exist in the same infected tissues of plants as observed in North America [6,7], Europe [1,8] and Australia [9,10]. *L. maculans* is the predominant cause of blackleg in North America, Europe and Australia and have substantial diversity in the pathogenicity and population genetic structures [7,11–13]. In Eastern Asia (China, Japan and Korea), one of the major growers of brassica crops including oilseed rape, cabbage and Chinese cabbage etc., however, the disease is caused by less aggressive *L. biglobosa* only [14–17].

Previously, the more aggressive *L. maculans* has been reported to spread and cause significant increase in the severity of phoma stem canker in areas such as Poland, Mexico and Canada [18,19]. Since

both the species share common ecological-niche, and the agro-climatic factors are similar in Asian countries, there is a great risk that this invasive species may spread in Asian regions as well via inadvertent distribution of seeds and trade exchanges [6,16,19]. This constitutes a serious threat to the Asian canola and cabbage industry which is otherwise very sensitive to the *L. maculans*, and may cause further increase in the global loss by the disease [16]. Preventing the introduction of *L. maculans* is thus prioritized in these countries evidently by the recent impose of import restrictions [17,20,21]. This requires an accurate, efficient and rapid detection of the components of Leptosphaeria populations that can be readily employed for quarantine and disease surveillance purposes [16].

Common techniques used for detecting fungal pathogens include *in-vitro* growth and morphological traits; spectroscopic and imaging techniques, biochemical, serological and enzymological techniques; isozyme, soluble protein and toxin profiling; sirodesmin production and pathogenicity on differential cultivar set having various R-gene profiles etc. [2,11,22–24]. However, these techniques are often time consuming, laborious, insensitive and inconclusive, and are in-sufficient in detecting the closely related members of this multiple species complex. Polymerase Chain Reaction (PCR)-based assays that harness the polymorphisms in inherent DNA sequences, in this regard, are particularly advantageous as they offer accurate, sensitive and rapid detection of fungal species. Such PCR based assay for detection of Leptosphaeria species complex is limited and was based on sequence variation in internally transcribed spacer (ITS) region genes [25–27], which often are conserved among closely related species or isolates. However, whole genome sequences of several *L. maculans* and *L. biglobosa* isolates are now available [4,28,29], which are yet to be exploited for developing species specific molecular markers.

Here, we report the development of *L. maculans* 'brassicae' and *L. biglobosa* 'brassicae' specific PCR based novel molecular markers from the species specific effector encoding genes identified via whole genome alignment.

2. Results

2.1 Genome Alignment and Species Specific LCBs

The genome sizes of *Leptosphaeria maculans* isolates JN3 (23.1.3), Nz-T4 and WA74 ranged between 43.42-45.12 Mb which is comparatively larger than the *Leptosphaeria biglobosa* isolates B3.5 and G12-14 (31.78-34.95 Mb) (Table 1). In contrast, the GC content were slightly higher in *L. biglobosa* isolates (49.13-51.39%) compared to that of *L. maculans* isolates (45.30-46.50%). Alignment of the whole genomes of these isolates revealed that most nucleotide sequences are conserved among the isolates of same species (Figure 1A.B) (Figure 1C). However, there is considerable genomic re-arrangements, and isolate and species specific genomic segments (Figure 1 & 2). A total of 13 and 18 local colonial blocks (LCBs) of ≤3 Kb were extracted that were specific to *L. biglobosa* and *L. maculans* isolates, respectively (Figure S1).

2.2 Blast Hits and Effector Prediction

BLAST analysis of these LCB sequences identified which part of a particular LCB, if at all, encodes specific gene. Top BLAST hits of each LCBs were subjected to effector prediction analysis and five *L. maculans* specific LCBs namely, Lm_LCB9, Lm_LCB149, Lm_LCB247_2, Lm_LCB264 and Lm_LCB290, and five *L. biglobosa* specific LCBs namely, Lb_LCB38, Lb_LCB43, Lb_LCB47, Lb_LCB52 and Lb_LCB247 had genomic fragments that encodes for effector genes had been identified (Table 2; Figure S1). A total of five and seven *L. maculans* and *L. biglobosa* specific primers were designed in these species specific effector encoding nucleotide sequence (Table 2; Figure 1B and Figure S1).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 October 2019 doi:10.20944/preprints201910.0147.v1

Table 1. Genomic information of *Leptosphaeria maculans* and *Leptosphaeria biglobosa* isolates used for whole genome alignment.

Species	Strains (Isolate)	Assembly	Genome Size (Mb)	GC%	Source	Reference
Leptosphaeria maculans	JN3 (23.1.3)	GCA_000230375.1	45.12	45.30	Leptolife project	[28]
"	Nz-T4	GCA_900465115.1	43.42	45.70	Leptolife project	[28]
"	WA74	FO905058 - FO907085	44.2	46.50	EMBL-EBI database	[4]
Leptosphaeria biglobosa	B3.5	FO905058 - FO907085	31.78	51.39	EMBL-EBI database	[4]
"	G12-14	GCA_900465125.1	34.95	49.13	Leptolife project	[28]

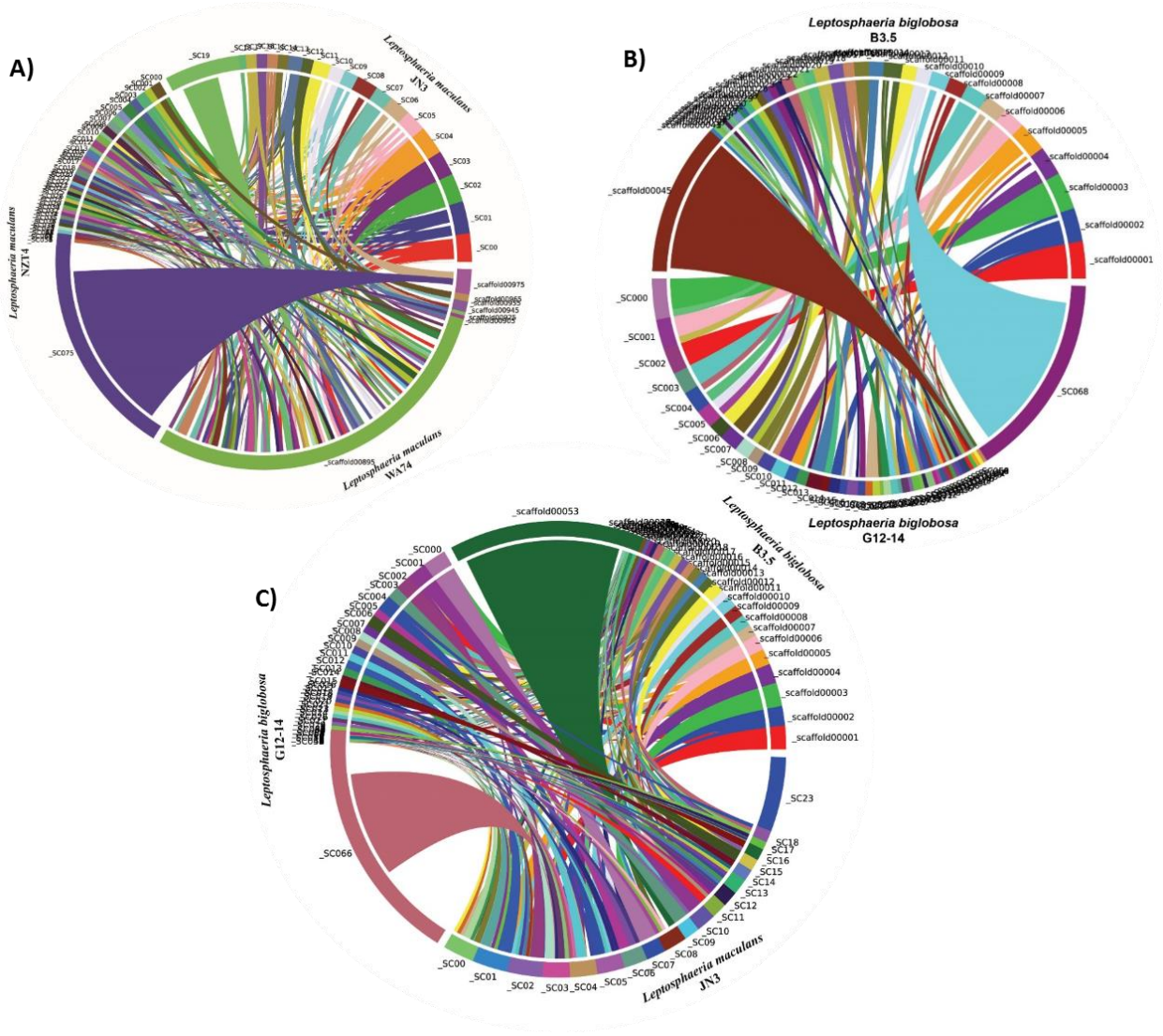
Table 2. Selected effector encoding *Leptosphaeria maculans* and *Leptosphaeria biglobosa* specific LCBs along with the corresponding blast hits and primer specifications.

Species specific genomic segment		Blast Hit	Bit-Score (E-Value)	Description	Primers	Forward primer	Product (bp)
LCB	Position (bp)						
Lm_LCB9	1829,3484-6837	OWY46227	238.42/ (1.92E-61)	Actin-like ATPase-like protein [*Alternaria alternata*]	Lm1	F: ATTCCCACTCTCCCTAAACTCC R: TCGCACTATACCACTCCTTCAA	248
Lm_LCB149	1817,1471-2022	OCK82192	169.86 (1.49E-44)	GIY-YIG-domain-containing protein [*Lepidopterella palustris*]	Lm2	F: TGAAGAATCTGCATCATCTGCT R: ATCTCACTGTCCACATCTGGTG	196
Lm_LCB247_2	3326,0973-1293	XP_003834629	207.99 (3.07E-101)	Hypothetical protein LEMA_P067720.1 [*Leptosphaeria maculans* JN3]	Lm3	F: AACACTTATCGCCAGCTTTGAC R: GTGCACCGTCACTGTCTATGTT	167
Lm_LCB264	148,610-861	EKG16596	67.40 (4.65E-12)	Carboxyl transferase [*Macrophomina phaseolina* MS6]	Lm4	F: CAGCTATTCACAAGAGCAAGGA R: GTCTGTAGGATTCCCATCGAAC	240
Lm_LCB290	1579,3604-4337	RYF49765	192.2 (2.47E-53)	Hypothetical protein EOO38_07195 [*Cytophagaceae bacterium*]	Lm5	F: TTGGTAGTAGCAAGGTCCATGA R: GTTGATAAAGCCCAACCAAGAG	230
Lb_LCB38	28,0553-3706	RMY82212	98.21 (1.01E-27)	Hypothetical protein D0861_07991 [*Hortaea werneckii*]	Lb1	F: GTTTCTCGATTTTTCGCTGAGT R: AATACAAACCACCCAGATCACC	163
Lb_LCB43	3,88838-91356	PWO12528	62.00 (1.19E-19)	Hypothetical protein PtrM4_07871 [*Pyrenophora tritici-repentis*]	Lb2	F: CCAACACAGGCTTAAGAAATCC R: GATATTATCTCGGGCGTTTTCA	112
Lb_LCB43	3,88838-91356	CCT61205	85.11 (8.16E-52)	Predicted protein [*Leptosphaeria maculans* JN3]	Lb3 Lb3'	F: AGTTTCGCTTTCTTGTGCAGAT R: GCTCCTTGATTACAAGGCTGAT R': CCAGCAGCCGTTAATATACACA	243 834
Lb_LCB47	47,6979-9639	RDL36686	42.36 (2.44E+00)	Clathrin heavy chain [*Phialophora* cf. *hyalina* BP 5553]	Lb4	F: ACACTCCCACTTCATCTCGACT R: TCTAGGATATGCTGCGTACACC	185
Lb_LCB52	58,6888-9751	KNG51546	622.47 (0)	Dimethylaniline monooxygenase [*Stemphylium lycopersici*]	Lb5	F: TGCACCCTTTAGCTCTTCTAGG R: TAGTCTCTCGCATACGTCTCCA	168
Lb_LCB247	35,49709-43263-	KNG45253	40.43 (4.54E+00)	Hypothetical protein TW65_08089 [*Stemphylium lycopersici*]	Lb6	F: CTGTGAGCAAGTATGAGCGATG R: CATCCCAGCATGAACGAGAT	159

LCBs. local colonial blocks; Lm. *Leptosphaeria maculans*; Lb. *Leptosphaeria biglobosa*. Positions of Lm and Lb specific LCBs are based on JN3 and B3.5 genomes, respectively; Primer set Lb3 = Lb3F + Lb3R; Primer set Lb3' = Lb3F + Lb3R'. LCB segments are shown in Figure 2. Amplification of the primers that perfectly detected species specific isolates are shown in Figure 3 and primers that showed non-specific amplifications are shown in Figure S3.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 October 2019 doi:10.20944/preprints201910.0147.v1

125



126

127 **Figure 1.** The synteny plot among the genomes of *Leptosphaeria maculans* (A), *Leptosphaeria biglobosa* (B) isolates and
128 between the selected isolates of both species (C); generated with SyMap v4.2. The circular plot shows all syntenic blocks
129 between the scaffolds of the genomes joined with lines of same color.

130

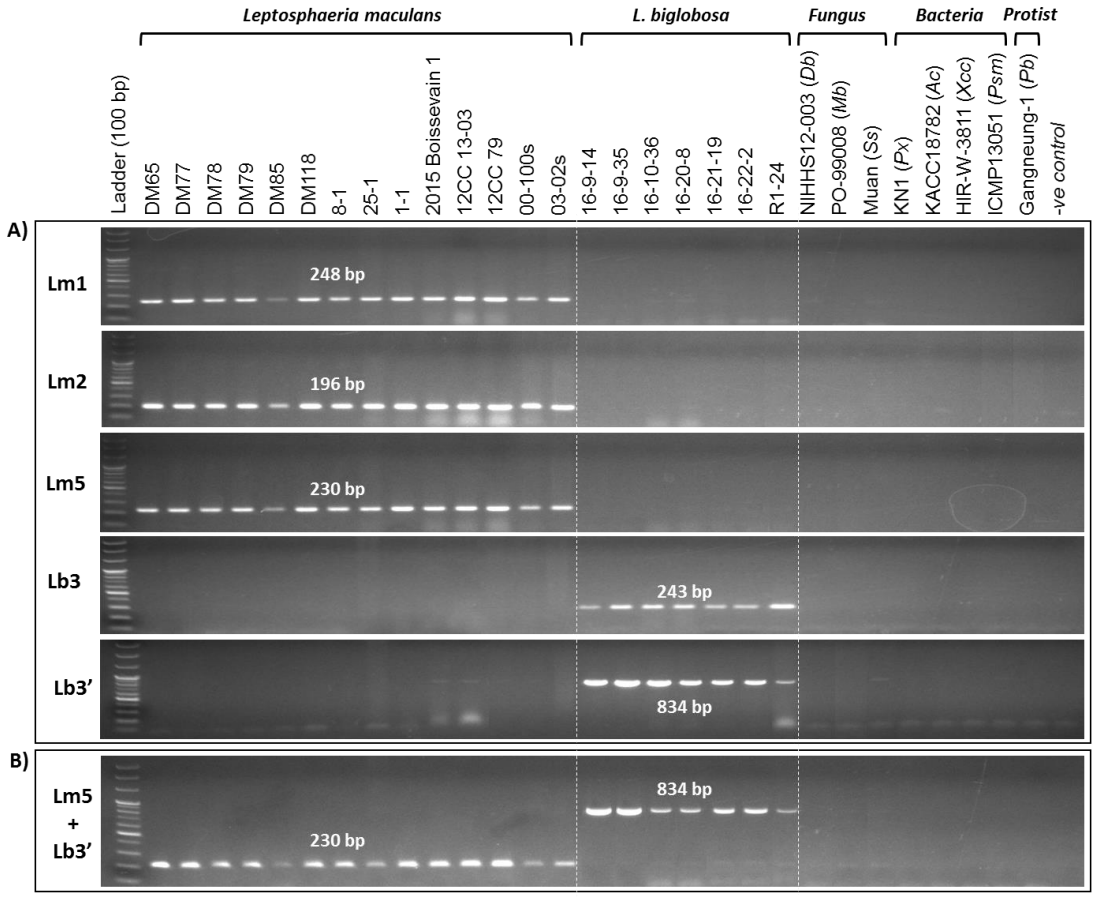
Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 October 2019 doi:10.20944/preprints201910.0147.v1

Figure 2. Progressive MAUVE alignment of the whole genomes of *L. maculans* and *L. biglobosa* isolates (A) and Segments of the alignment where species specific primers were designed (B).
In Figure A, the homologous blocks (termed as the local colonial blocks, LCBs) shared among the genomes are indicated as colored rectangles which are connected with lines of similar colors, indicating corresponding positions of the homologous blocks among the genomes in a way to visualize the genomic re-arrangements. Blocks below the center line indicate regions that align in the reverse complement (inverse) orientation and segments unique to a particular genome are indicated by white areas. In Figure B, three parallel lines indicate presence and single/no line indicates absence of genomic segment in a particular genome, respectively (as shown within red box in second top segment from left, as an example). Primer names are indicated in parenthesis after the LCB names under each segment, e.g., Lm-LCB9 (Lm1) indicates *L. maculans* specific primer Lm1, designed on LCB9. Lm. *Leptosphaeria maculans*, Lb. *Leptosphaeria biglobosa*. LCB positions and primers are shown in Table 1.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 October 2019 doi:10.20944/preprints201910.0147.v1

146 *2.3 Species specificity of Primers in PCR and Multiplex PCR assay*

147 For specificity testing of the designed *L. maculans* and *L. biglobosa* specific primer sets in PCR assay,
148 14 and 7 isolates (Table 3) belonging to *L. maculans* and *L. biglobosa*, respectively were used. In addition, one
149 isolate each of four other fungi and one strain each of three bacteria and one portist were used as control.
150 Of the five *L. maculans* specific primer sets, three sets namely, Lm1, Lm2 and Lm5 produced specific
151 amplicons of desired size (248, 167 and 230 bp, respectively) for all *L. maculans* isolates, whereas no such
152 amplicons were produced for any of the *L. biglobosa* isolates and other fungi, bacteria and protist used
153 (Figure 3A). Alternately, only two of the seven *L. biglobosa* specific primer sets, namely, Lb3 and Lb3', specifically detected all isolates of *L. biglobosa* isolates (amplicon size of 243 and 834 bp, respectively),
154 whereas none of the isolates of *L. maculans* and other fungi, bacteria and protist was amplified (Figure 3A).
155 The remaining two *L. maculans* specific (Lm3 and Lm4) and five *L. biglobosa* specific (Lb1, Lb2, Lb4, Lb5 and
156 Lb6) primer sets did not produce such exclusive species specific amplicons as evident either by non-
157 amplification of at least one of the target isolates or by amplification of non-target isolates by these primer
158 sets (Figure S2).
159



160

161 **Figure 3.** Specific detection of *Leptosphaeria maculans* and *Leptosphaeria biglobosa* isolates using the developed species
162 specific primers via PCR (A) and multiplex PCR (B) assay.
163 Genomic DNA (~100 ng/µL) of the fungal isolates was used as template. Primer sets Lm1, Lm2, Lm5 specifically
164 detected all *Leptosphaeria maculans* isolates and primer set Lb3 detected all *Leptosphaeria biglobosa* isolates. *Db. Didymella*
165 *bryoniae; Mb. Mycosphaerella brassicicola; Ss. Sclerotinia sclerotiorum; Px. Podosphaera xanthii; Ac. Acidovorax citrulli; Xcc. X.*
166 *campestris pv. campestris; Psm. Pseudomonas syringae pv. maculicola; Pb. Plasmodiophora brassicae*.
167

 doi:10.20944/preprints201910.0147.v1

168 **Table 3.** List of *Leptosphaeria maculans* and *Leptosphaeria biglobosa* isolates and other plant pathogenic agents.

Species	Strains/Isolate	Host	Sources	Media	Disease
Leptosphaeria maculans	DM65	Brassica	UoM, Canada	V8 agar	Blackleg of Brassica
"	DM77	Brassica	"	"	"
"	DM78	Brassica	"	"	"
"	DM79	Brassica	"	"	"
"	DM85	Brassica	"	"	"
"	DM118	Brassica	"	"	"
"	8-1	Brassica	"	"	"
"	25-1	Brassica	"	"	"
"	1-1	Brassica	"	"	"
"	Boissevain 1	Brassica	AAFC, Canada	"	"
"	12CC 13-03	Brassica	"	"	"
"	12CC 79	Brassica	"	"	"
"	00-100s	Brassica	"	"	"
"	03-02s	Brassica	"	"	"
Leptosphaeria biglobosa	16-9-14	Brassica	UoM, Canada	PDA	"
"	16-9-35	Brassica	"	"	"
"	16-10-36	Brassica	"	"	"
"	16-20-8	Brassica	"	"	"
"	16-21-19	Brassica	"	"	"
"	16-22-2	Brassica	"	"	"
"	R1-24	Brassica	AAFC, Canada	"	"
Didymella bryoniae	NIHHS12-003	*Cucumis melo*	NIHHS, South Korea	PDA	Gummy stem blight
Mycosphaerella brassicicola	PO-99008	-	DLO, The Netherlands	V8 agar	Ringspot of cabbage
Sclerotinia sclerotiorum	Muan	*Brassica oleracea*	South Korea	PDA	Sclerotinia stem rot (white mold)
Podosphaera xanthii	KN1	*Cucumis melo*	Hadong, South Korea	Melon leaves	Powdery Mildew in cucurbits
*Acidovorax citrulli**	KACC18782	Melon	KACC, South Korea	King's B	Bacterial Fruit Blotch of Cucurbits
*Xanthomonas campestris pv. campestris**	HRI-W-3811	*B. oleracea*	HRI-W, UK	King's B	Black rot of crucifers
*Pseudomonas syringae pv. maculicola**	ICMP13051	-	ICMP, New Zealand	King's B	bacterial leaf spot of crucifer
*Plasmodiophora brassicae***	Gangneung-1	*B. rapa*	South Korea	*B. rapa* plants	club root disease of crucifers

169 UoM. University of Manitoba, Canada; AAFC. Agriculture and Agri-Food Canada; NIHHS. National Institute of Horticultural and Herbal Science, Korea; ; PDA, Potato Dextrose Agar;
170 DLO. Stichting Dienst Landbouwkundig Onderzoek, Research institute Praktijkonderzoek Plant & Omgeving/Plant Research International,Wageningen, The Netherlands; KACC.
171 Korean Agricultural Culture Collection, Korea; HRI-W, Horticulture Research International, Wellesbourne, The University of Warwick,UK; ICMP, The International Collection of
172 Microorganisms from Plants, Landcare Center, Auckland, New Zealand; * Bacteria; **Protist. Growth patterns in culture plates of these isoalates are shown in Figure S3.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 October 2019 doi:10.20944/preprints201910.0147.v1

One each of the *L. maculans* and *L. biglobosa* specific primer sets namely, Lm5 and Lb3', respectively having distinctively different amplicon sizes, were multiplexed for detection of target species from a single PCR reaction. These primers too amplified the target isolates and did not amplified the non-target isolates and other fungi, bacteria and protist (Figure 3B), indicating their suitability to be used in multiplex PCR.

2.4 PCR Sensitivity

The minimum concentrations of DNA required for detectable amplification of the target species by the developed primers in PCR assay was tested using a dilution series of the DNA of *Leptosphaeria maculans* isolate 12CC 13-03. The least concentration of genomic DNA of the isolate 12CC 13-03 that was detectibly amplified by the tested primers was 0.01 ng/µL (Figure 4), indicating high sensitivity of the designed *L. maculans* and *L. biglobosa* specific primers. These results were achieved using 30 cycles of PCR amplification. Increasing the number of PCR cycles may detect target isolates from further lower concentration of template DNA.

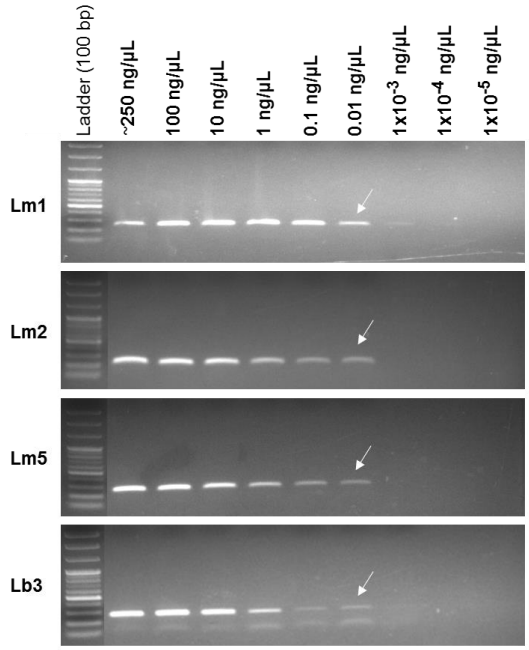


Figure 4. Determination of PCR sensitivity using selected *Leptosphaeria maculans* and *Leptosphaeria biglobosa* species specific primers.
A 10-fold dilution series of genomic DNA of *Leptosphaeria maculans* isolate 12CC 13-03 was used as template DNA.

2.5 Direct implication of target isolates

The efficacy of the developed primers for direct detection of the target speceis without requiring DNA extraction were further tested using 'fungal pseudothecia and ascospores suspension' of two selected *L. maculans* (DM65 and 12CC-13-03) and *L. biglobosa* (16-9-35 and 16-22-2) isolates as template sample instead of genomic DNA in PCR assay. All the designed primers, including the multiplexed primer sets effectively detected the target species (Figure 4), indicating the suitability of the developed primers in direct detection of the target species.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 13 October 2019 doi:10.20944/preprints201910.0147.v1

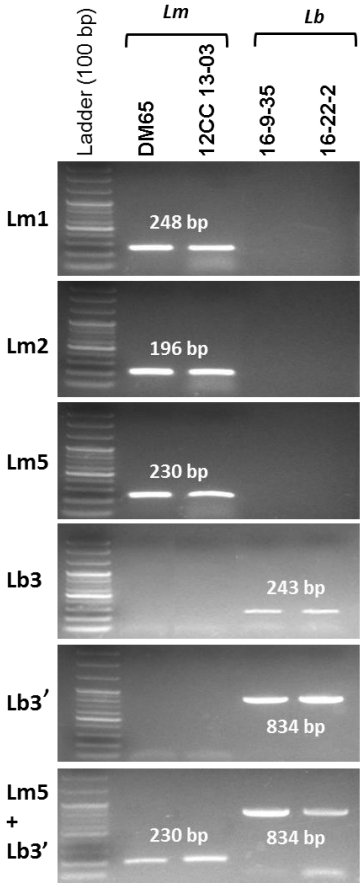


Figure 5. Specific detection of selected *Leptosphaeria maculans* and *Leptosphaeria biglobosa* isolates using crushed pseudothecia and ascospore suspension as template DNA in PCR assays.
Lm. Leptosphaeria maculans; *Lb. Leptosphaeria biglobosa*; Primer set Lm5 + Lb3' were used for multiplex PCR.

3. Discussion

Here, we developed novel PCR based molecular markers for detecting two prevalent Leptosphaeria species, *L. maculans* and *L. biglobosa* that specializes on Brassica crops from the species specific effector protein encoding nucleotide sequences identified by aligning whole genomes of available isolates of these two species.

Among the Leptosphaeria species that specializes on Brassica crops and weeds, especially *L. maculans* and *L. biglobosa* that causes devastating economic loss to the global oilseed rape and other brassica crops are of particular interest. These two species has long been considered as single species [3]. However, genetic barrier due to mating incompatibility [30], differential virulence on Brassica hosts, variable profiles of sirodesmin, phomenoic acid and esterase [23,31,32] and distinctive polymorphism in 5.8s rRNA and internal transcribed spacers (ITS1 and ITS2) sequences [33,34] eventually identified *L. maculans* and *L. biglobosa* as two distinct species. Among these two co-evolved species, *L. biglobosa* is believed to be evolved earlier than *L. maculans* [35,36]. Since, these two differentially aggressive species favor similar ecological niche and co-exist in infected plants, effective detection is necessary for diagnosis and management of the disease and for international quarantine.

 doi:10.20944/preprints201910.0147.v1

In the pre-genomic era, these species were detected by cultural characteristics and production of toxic secondary metabolites [23,31], isozyme and soluble protein profiles [2] and virulence studies on Brassica species [22,23,37,38] etc. However, the difficulty posed by these techniques were soon overcome by DNA based techniques that harnessed the inherent polymorphism in the repeated element [39], 5.8S rDNA [2,7,10], ribosomal internal transcribed spacer (ITS) region [40,41] and *actin* and *b*-tubulin genes [42] etc. Several PCR based techniques such as RAPD [43], AFLP [44], RFLP [23,31,33] and Rep-PCR [37] etc. were used for characterization of Leptosphaeria species complex. The highly conserved nature of the ITS sequences within a fixed species are useful for studying population structure and species distinction [45]. However, there is report of high level of polymorphism within *L. maculans* species that raises concern for their future use [2], particularly in the face of ever evolving fungal populations [4]. Use of genealogies of multiple additional gene or non-coding sequences were thus suggested for detection of *L. maculans* [2,39].

The current genomic-era provides the opportunity to exploit the available whole genome sequences of a number of Leptosphaeria species. We focused on detecting species that are devastating to Brassica crops and hence, used the recent genomes of three *L. maculans* and two *L. biglobosa* isolates [28,46,47]. The differences in the genome size of these two species clearly indicated that there is species specific genomic segments (Table 1). Synteny analysis of these whole genome identified that there is extensive genomic rearrangement between these two species (Figure 1), which was clearly visualized in the progressive MAUVE alignment view (Figure 2). The species specific genomic fragments were extracted and species specific primers were designed on the sequences that encoded effector proteins. The effector proteins were targeted since the differential virulence of these two species is mainly manifested by the avirulence gene profiles which are essentially the small-secreted proteins (SSPs) or effector proteins localized in transposon-rich AT-isochores [29,48,49]. Among the designed primers, three (Lm1, Lm2 and Lm5) and two (Lb3 and Lb3′) *L. maculans* and two *L. biglobosa* specific primers, respectively detected respective isolates collected from diverse global sources precisely (Figure 3). Two of these primers namely, Lm5 and Lb3′ were multiplexed that can be used for detection of this two species in a single PCR reaction.

Highly sensitive detection from a very low DNA concentration and detection from crushed pseudothecia indicate that these markers can be used for mass detection without requiring the extraction of template DNA. However, the marker efficacy using more diverse and global collection of *L. maculans* and *L. biglobosa* isolates and the direct detection from infected leaf and infested seed samples remains to be tested. The developed markers, designed from species specific effector proteins will serve as precise, rapid and sensitive detection tools that can be used for mass screening of the pathogenic agents, routine diagnosis and surveillance of the disease and in international quarantine.

4. Materials and Methods

4.1 Fungal Isolates: Collection, Culture and DNA Extraction

Fourteen and seven different isolates belonging to *Leptosphaeria maculans* and *Leptosphaeria biglobosa*, respectively and one isolate belonging to each of the other fungus (such as *Didymella bryoniae*, *Mycosphaerella brassicicola, Podosphaera xanthii* and *Sclerotinia sclerotiorum),* bacteria (such as *Acidovorax citrulli*, *Xanthomonas campestris pv. campestris and Pseudomonas syringae pv. maculicola*) and protist *Plasmodiophora brassicae,* collected from various sources of South Korea, Canada, UK, The Netherlands and New Zealand, were used in this study. These plant pathogenic agents were cultured on various growth media as shown in Table 1 and Figure S3. Fungal cultures were incubated for several weeks and bacterial cultures were generally incubated for 48 hours at 26±2°C in culture room. Fully grown fungal cultures (with mycelia and conidia) and inundated bacterial cultures (by 5 mL of sterile water) were collected by scraping off the colonies gently using a sterile glass slide. The fungal cultures were ground to fine powder using liquid nitrogen. Genomic DNA of both fungal isolates and bacterial strains were extracted using the

QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) following manufacturer's protocols and were stored at -20°C until further use. The DNA quality was tested via electrophoresis in 0.8% agarose gel and the concentrations were measured spectrophotometrically using Nanodrop ND-1000 (NanoDrop,Wilmington, DE, USA).

4.2 Retrieval and alignment of whole genome

The whole genome sequences of three *L. maculans* (JN3, Nz-T4 and WA74) and two *L. biglobosa* (B3.5 and G12-14) isolates were retrieved from the 'Leptolife project' database (http://www.genoscope.cns.fr/externe/leptolife/assemblies.html) and European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) database (https://www.ebi.ac.uk/) (Table 2). The syntenic relationship between these isolates were analyzed using 'SyMap v4.2' and the whole genomic sequences of these isolates were aligned using 'MAUVE v.2.4.0' and 'Geneious free trial version' (https://www.geneious.com/free-trial/) with default parameters.

4.3 Extraction of species specific genomic sequences, effector prediction and primer design

The genomic sequences of *L. maculans* and *L. biglobosa* specific local colonial blocks (LCBs) of ≤3 Kb were extracted and subjected to BLAST Analysis. The sequences corresponding to top blast hits of each LCBs were analyzed for effector prediction using 'EffectorP' (http://effectorp.csiro.au/). Primers were designed from the *L. maculans* and *L. biglobosa* specific sequences that encodes effector proteins using 'Primer3Plus' (Table 3).

4.4 PCR assays

The specificity of the designed *L. maculans* and *L. biglobosa* specific markers were tested in polymerase chain reaction (PCR) assay using the genomic DNA of all plant pathogenic agents used in this study. The final PCR reaction (20 µl) contained 8 µl of Prime Taq Premix, 2X (GeNetBio, Daejon, Korea), 1 µl of forward and reverse primers each, 9 µl of ultra-pure water and 1.0 µl of template DNA (~100 ng/µL). PCR amplification was carried out in a thermocycler (Takara, Shiga, Japan) using initial denaturation at 94°C for 4 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and 72°C for 30 s, and a final elongation at 72°C for 5 min. Electrophoresis was carried out in 1.2% agarose gel stained with blue mango (BioD, Gwangmyeong, Korea) in TAE buffer at 100 V for 30 min and visualized in an ENDURO™ GDS gel documentation system. HIQ 100 bp plus DNA Ladder Mix (Bioneer, Daejeon, Korea) was used as size marker.

Best performing *L. maculans* and *L. biglobosa* specific primers were multiplexed in same PCR condition in a final reaction volume of 25 µl (Taq Premix = 9.5 µl, ultra-pure water = 10 µl, *L. maculans* and *L. biglobosa* specific forward and reverse primers = 1 µl each and genomic DNA = 1.5 µl). Besides, selected primers were tested for direct amplification of *L. maculans* and *L. biglobosa* (without requiring DNA extraction) where instead of genomic DNA and ultra-pure water, 10 µl of suspension (finely powdered fungal pseudothecia and ascospores suspended in 25 µl of ultra-pure water) were used as template DNA.

4.5 Determination of PCR sensitivity

The minimum genomic DNA of *L. maculans* (12CC 13-03) and *L. biglobosa* (16-21-19) isolates required for detecting these isolates by the developed primers in PCR assay was determined using a dilution series (starting from 250 ng/µL). One microliter of each dilution was used directly as the template DNA with PCR conditions as described in previous section.

Supplementary Materials: The following are available online.

Table S1. Effector prediction of the top blast hits of *Leptosphaeria maculans* and *Leptosphaeria biglobosa* specific LCBs using 'EffectorP' program.

Figure S1. Nucleotide sequences of *Leptosphaeria maculans* (a) and *Leptosphaeria biglobosa* (b) specific 'local colonial blocks (LCBs)'.

Figure S2. Amplification of pathogenic isolates by the designed *Leptosphaeria maculans* and *Leptosphaeria biglobosa* specific primers (A) and by previously published multiplex primers (B) in PCR assay.

Figure S3: Culture plates of *Leptosphaeria maculans* (*Lm*) and *Leptosphaeria biglobosa* (*Lb*) isolates on V8 and PDA media, respectively.

Author Contributions: ISN, JIP, MRH and HTK conceptualized, acquired the fund and supervised the work; DMEJ and MRH conducted the bioinformatics analysis; MJF cultured the fungus and performed all wet lab experiments; HJJ; MJF and MRH analyzed the data, interpreted the results and wrote the manuscript. All authors read the article and approved the manuscript.

Funding: This study was supported by the Golden Seed Project (Grant Number: 213007-05-4-CG100), Center for Horticultural Seed Development, Ministry of Agriculture, Food and Rural Affairs in the Republic of Korea (MAFRA). The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Acknowledgments: We thank Hossein Borhan and Nicholas J. Larkan of Agriculture and Agri-Food Canada (AAFC), Saskatoon and Professor Dilantha Fernando of University of Manitoba for providing the *Leptosphaeria* isolates.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Fitt, B.D.L.; Brun, H.; Barbetti, M.J.; Rimmer, S.R. World-wide importance of phoma stem canker (Leptosphaeria maculans and L . biglobosa) on oilseed rape (Brassica napus). *Eur. J. Plant Pathol.* **2006**, *114*, 3–15.
2. Mendes-Pereira, E.; Balesdent, M.-H. ne; Brun, H.; Rouxel, T. Molecular phylogeny of the Leptosphaeria maculans – L . biglobosa species complex. *Mycol. Res.* **2003**, *107*, 1287–1304.
3. Rouxel, T.; Gall, C.; Balesdent, M.H. Du polymorphisme au complexe d'espèces: combien d'agents pathogènes sont impliqués dans la nécrose du collet du colza? *Agronomie* **1994**, *14*, 413–432.
4. Grandaubert, J.; Lowe, R.G.T.; Soyer, J.L.; Schoch, C.L.; Van De Wouw, A.P.; Fudal, I.; Robbertse, B.; Lapalu, N.; Links, M.G.; Ollivier, B.; et al. Transposable element-assisted evolution and adaptation to host plant within the Leptosphaeria maculans-Leptosphaeria biglobosa species complex of fungal pathogens. *BMC Genomics* **2014**, *15*, 1–27.
5. Zhou, Y.; Fitt, B.D.L.; Welham, S.J.; Gladders, P.; Sansford, C.E.; West, J.S. Effects of severity and timing of stem canker (Leptosphaeria maculans) symptoms on yield of winter oilseed rape (Brassica napus) in the UK. *Eur. J. Plant Pathol.* **1999**, *105*, 715–728.
6. West, J.S.; Kharbanda, P.D.; Barbetti, M.J.; Fitt, B.D.L. Epidemiology and management of Leptosphaeria maculans (phoma stem canker) on oilseed rape in Australia, Canada and Europe. *Plant Pathol.* **2001**, *50*, 10–27.
7. Dilmaghani, A.; Balesdent, M.H.; Didier, J.P.; Wu, C.; Davey, J.; Barbetti, M.J.; Li, H.; Moreno-Rico, O.; Phillips, D.; Despeghel, J.P.; et al. The Leptosphaeria maculans - Leptosphaeria biglobosa species complex in the American continent. *Plant Pathol.* **2009**, *58*, 1044–1058.
8. West, J.S.; Balesdent, M.; Rouxel, T.; Narcy, J.P.; Huang, Y.; Roux, J.; Steed, J.M.; Fitt, B.D.L.; Schmit, J.

Colonization of winter oilseed rape tissues by A/Tox+ and B/Tox0Leptosphaeria maculans (phoma stem canker) in France and England. *Plant Pathol.* **2002**, *51*, 311–321.

9. Sprague, S.J.; Balesdent, M.-H.; Brun, H.; Hayden, H.L.; Marcroft, S.J.; Pinochet, X.; Rouxel, T.; Howlett, B.J. Major gene resistance in Brassica napus (oilseed rape) is overcome by changes in virulence of populations of Leptosphaeria maculans in France and Australia. In *Sustainable strategies for managing Brassica napus (oilseed rape) resistance to Leptosphaeria*; Fitt, B.D.L., Evans, N., Howlett, B.J., Cooke, B.M., Eds.; Springer Netherlands: Dordrecht, 2006; pp. 33–40 ISBN 978-1-4020-4525-7.
10. Vincenot, L.; Balesdent, M.H.; Li, H.; Barbetti, M.J.; Sivasithamparam, K.; Gout, L.; Rouxel, T. Occurrence of a new subclade of Leptosphaeria biglobosa in Western Australia. *Phytopathology* **2008**, *98*, 321–329.
11. Balesdent, M.H.; Barbetti, M.J.; Li, H.; Sivasithamparam, K.; Gout, L.; Rouxel, T. Analysis of Leptosphaeria maculans race structure in a worldwide collection of isolates. *Phytopathology* **2005**, *95*, 1061–1071.
12. Liban, S.H.; Cross, D.J.; Kutcher, H.R.; Peng, G.; Fernando, W.G.D. Race structure and frequency of avirulence genes in the western Canadian Leptosphaeria maculans pathogen population, the causal agent of blackleg in brassica species. *Plant Pathol.* **2016**, *65*, 1161–1169.
13. Parlange, F.; Daverdin, G.; Fudal, I.; Kuhn, M.; Balesdent, M.; Blaise, F.; Grezes-besset, B.; Rouxel, T. Leptosphaeria maculans avirulence gene AvrLm4-7 confers a dual recognition specificity by the Rlm4 and Rlm7 resistance genes of oilseed rape , and circumvents Rlm4-mediated recognition through a single amino acid change. *Mol. Microbiol.* **2009**, *71*, 851–863.
14. Hong, S.-K.; Kim, W.-G.; Shin, D.-B.; Choi, H.-W.; Lee, Y.-K.; Lee, S.-Y. Occurrence of stem canker on rape caused by Leptosphaeria biglobosa in Korea. *Plant Pathol. J.* **2009**, *25*, 294–298.
15. Hao, L.; Song, P.; Huangfu, H.; Li, Z. Genetic diversity and differentiation of Leptosphaeria biglobosa on oilseed rape in China. *Phytoparasitica* **2015**, *43*, 253–263.
16. Liu, Z.; Latunde-Dada, A.O.; Hall, A.M.; Fitt, B.D.L. Phoma stem canker disease on oilseed rape (Brassica napus) in China is caused by Leptosphaeria biglobosa 'brassicae.' *Eur. J. Plant Pathol.* **2014**, *140*, 841–857.
17. Zhang, X.; White, R.P.; Demir, E.; Jedryczka, M.; Lange, R.M.; Islam, M.; Li, Z.Q.; Huang, Y.J.; Hall, A.M.; Zhou, G. Leptosphaeria spp., phoma stem canker and potential spread of L. maculans on oilseed rape crops in C hina. *Plant Pathol.* **2014**, *63*, 598–612.
18. Liu, Z. Strategies for managing Leptosphaeria maculans and Leptosphaeria biglobosa to decrease severity of phoma stem canker epidemics on winter oilseed rape 2008.
19. Fitt, B.D.L.; Hu, B.C.; Li, Z.Q.; Liu, S.Y.; Lange, R.M.; Kharbanda, P.D.; Butterworth, M.H.; White, R.P. Strategies to prevent spread of Leptosphaeria maculans (phoma stem canker) onto oilseed rape crops in China; costs and benefits. *Plant Pathol.* **2008**, *57*, 652–664.
20. van den Burg, H.A.; Tsitsigiannis, D.I.; Rowland, O.; Lo, J.; Rallapalli, G.; Maclean, D.; Takken, F.L.W.; Jones, J.D.G. The F-box protein ACRE189/ACIF1 regulates cell death and defense responses activated during pathogen recognition in tobacco and tomato. *Plant Cell* **2008**, *20*, 697–719.
21. Zhang, X.; Fernando, W.G.D. Insights into fighting against blackleg disease of Brassica napus in Canada. *Crop Pasture Sci.* **2018**, *69*, 40–47.
22. Badawy, H.M.A.; Hoppe, H.; Koch, E. Differential reactions between the genus Brassica and

aggressive single spore isolates of Leptosphaeria maculans. *J. Phytopathol.* **1991**, *131*, 109–119.

23. Koch, E.; Badawy, H.M.A.; Hoppe, H.H. Differences between aggressive and non-aggressive single spore lines of Leptosphaeria maculans in cultural characteristics and phytotoxin production. *J. Phytopathol.* **1989**, *124*, 52–62.
24. Piliponytė-Dzikienė, A.; Andriūnaitė, E.; Petraitienė, E.; Brazauskienė, I.; Statkevičiūtė, G.; Brazauskas, G. Genetic diversity and occurrence of Leptosphaeria spp. on Brassica oleracea and B. napus in Lithuania. *J. Plant Pathol.* **2015**, *97*.
25. Ashfield, T.; Egan, A.N.; Pfeil, B.E.; Chen, N.W.G.; Podicheti, R.; Ratnaparkhe, M.B.; Ameline-Torregrosa, C.; Denny, R.; Cannon, S.; Doyle, J.J.; et al. Evolution of a complex disease resistance gene cluster in diploid Phaseolus and tetraploid Glycine. *Plant Physiol.* **2012**, *159*, 336–354.
26. Bailey, T.L.; Boden, M.; Buske, F.A.; Frith, M.; Grant, C.E.; Clementi, L.; Ren, J.; Li, W.W.; Noble, W.S. MEME Suite: tools for motif discovery and searching. *Nucleic Acids Res.* **2009**, *37*, W202–W208.
27. Mahuku GS, Hall R, G.P. Co-infection and induction of systemic acquired resistance by weakly and highly virulent isolates of Leptosphaeria maculans in oilseed rape. *Physiol. Mol. Plant Pathol.* **1996**, *49*, 61–72.
28. Dutreux, F.; Da Silva, C.; D'Agata, L.; Couloux, A.; Gay, E.J.; Istace, B.; Lapalu, N.; Lemainque, A.; Linglin, J.; Noel, B.; et al. De novo assembly and annotation of three Leptosphaeria genomes using Oxford Nanopore MinION sequencing. *Sci. Data* **2018**, *5*, 180235.
29. Rouxel, T.; Grandaubert, J.; Hane, J.K.; Hoede, C.; Wouw, A.P. Van De; Couloux, A.; Dominguez, V.; Anthouard, V.; Bally, P.; Bourras, S.; et al. Effector diversification within compartments of the Leptosphaeria maculans genome affected by Repeat-Induced Point mutations. *Nat. Commun.* **2011**, *2*, 202.
30. Petrie, G.A.; Lewis, P.A. Sexual compatibility of isolates of the rapeseed blackleg fungus Leptosphaeria maculans from Canada, Australia, and England. *Can. J. Plant Pathol.* **1985**, *7*, 253–255.
31. Balesdent, M.-H.; Gall, C.; Robin, P.; Rouxel, T. Intraspecific variation in soluble mycelial protein and esterase patterns of Leptosphaeria maculans French isolates. *Mycol. Res.* **1992**, *96*, 677–684.
32. Gall, C.; Balesdent, M.-H.; Desthieux, I.; Robin, P.; Rouxel, T. Polymorphism of Tox0Leptosphaeria maculans isolates as revealed by soluble protein and isozyme electrophoresis. *Mycol. Res.* **1995**, *99*, 221–229.
33. Balesdent, M.H.; Jedryczka, M.; Jain, L.; Mendes-Pereira, E.; Bertrandy, J.; Rouxel, T. Conidia as a Substrate for Internal Transcribed Spacer-Based PCR Identification of Members of the Leptosphaeria maculans Species Complex. *Phytopathology* **1998**, *88*, 1210–1217.
34. Morales, V.M.; Pelcher, L.E.; Taylor, J.L. Comparison of the 5.8s rDNA and internal transcribed spacer sequences of isolates of Leptosphaeria maculans from different pathogenicity groups. *Curr. Genet.* **1993**, *23*, 490–495.
35. Rouxel, T.; Balesdent, M.H. The stem canker (blackleg) fungus, Leptosphaeria maculans, enters the genomic era. *Mol. Plant Pathol.* **2005**, *6*, 225–241.
36. Gudelj, I.; Fitt, B.D.L.; Van den Bosch, F. Evolution of sibling fungal plant pathogens in relation to host specialization. *Phytopathology* **2004**, *94*, 789–795.
37. Jedryczka, M.; Rouxel, T.; Balesdent, M.H. Rep-PCR based genomic fingerprinting of isolates of Leptosphaeria maculans from Poland. *Eur. J. Plant Pathol.* **1999**, *105*, 813–823.

38. Liu, S.; Liu, Y.; Yang, X.; Tong, C.; Edwards, D.; Parkin, I.A.P.; Zhao, M.; Ma, J.; Yu, J.; Huang, S.; et al. The Brassica oleracea genome reveals the asymmetrical evolution of polyploid genomes. *Nat. Commun.* **2014**, *5*, 1–11.
39. Taylor, J.L.; Borgmann, I.E. An unusual repetitive element from highly virulent isolates of Leptosphaeria maculans and evidence of its transfer to a weakly virulent isolate. *Mol. plant-microbe Interact. MPMI* **1994**, *7*, 181–188.
40. Xue, B.; Goodwin, P.H.; Annis, S.L. Pathotype identification of Leptosphaeria maculans with PCR and oligonucleotide primers from ribosomal internal transcribed spacer sequences. *Physiol. Mol. Plant Pathol.* **1992**, *41*, 179–188.
41. Liu, S.Y.; Liu, Z.; Fitt, B.D.L.; Evans, N.; Foster, S.J.; Huang, Y.J.; Latunde-Dada, A.O.; Lucas, J.A. Resistance to Leptosphaeria maculans (phoma stem canker) in Brassica napus (oilseed rape) induced by L. biglobosa and chemical defence activators in field and controlled environments. *Plant Pathol.* **2006**, *55*, 401–412.
42. Zou, Z.; Zhang, X.; Parks, P.; Toit, L.J.; Wouw, A.P. Van De; Fernando, W.G.D. A New Subclade of Leptosphaeria biglobosa Identified from Brassica rapa. *Int. J. Mol. Sci.* **2019**, *20*, 1668.
43. Goodwin, P.H.; Annis, S.L. Rapid identification of genetic variation and pathotype of Leptosphaeria maculans by random amplified polymorphic DNA assay. *Appl. Environ. Microbiol.* **1991**, *57*, 2482–2486.
44. Cozijnsen, A.J.; Popa, K.M.; Purwantara, A.; Rolls, B.D.; Howlett, B.J. Genome analysis of the plant pathogenic ascomycete Leptosphaeria maculans; mapping mating type and host specificity loci. *Mol. Plant Pathol.* **2000**, *1*, 293–302.
45. Berbee, M.L.; Payne, B.P.; Zhang, G.; Roberts, R.G.; Turgeon, B.G. Shared ITS DNA substitutions in isolates of opposite mating type reveal a recombining history for three presumed asexual species in the filamentous ascomycete genus Alternaria. *Mycol. Res.* **2003**, *107*, 169–182.
46. ROUXEL, T.; BALESDENT, M.H. The stem canker (blackleg) fungus , Leptosphaeria maculans , enters the genomic era. *Mol. Plant Pathol.* **2005**, *6*, 225–241.
47. Petit-Houdenot, Y.; Degrave, A.; Meyer, M.; Blaise, F.; Ollivier, B.; Marais, C.L.; Jauneau, A.; Audran, C.; Rivas, S.; Veneault-Fourrey, C.; et al. A two genes – for – one gene interaction between Leptosphaeria maculans and Brassica napus. *New Phytol.* **2019**, *223*, 397–411.
48. van de Wouw, A.P.; Cozijnsen, A.J.; Hane, J.K.; Brunner, P.C.; McDonald, B.A.; Oliver, R.P.; Howlett, B.J. Evolution of linked avirulence effectors in Leptosphaeria maculans is affected by genomic environment and exposure to resistance genes in host plants. *PLoS Pathog.* **2010**, *6*.
49. Sonah, H.; Zhang, X.; Deshmukh, R.K.; Hossein Borhan, M.; Dilantha Fernando, W.G.; Bélanger, R.R. Comparative transcriptomic analysis of virulence factors in leptosphaeria maculans during compatible and incompatible interactions with canola. *Front. Plant Sci.* **2016**, *7*.