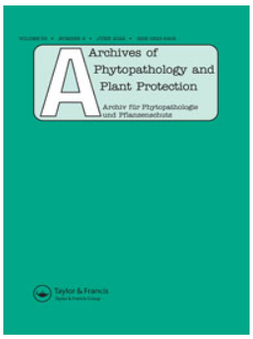

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RESEARCH ARTICLE

Genetic structure and phylogenetic relationships of *Leptosphaeria maculans* and *L. biglobosa* in Northern regions of Iran

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ABSTRACT

Blackleg, caused by *Leptosphaeria maculans,* and *L. biglobosa,* is a major disease that hinders canola (*Brassica napus*) production worldwide. In this study, 51 *Leptosphaeria* isolates, and previously deposited sequences were examined for genetic diversity using *ITS*, *β-tubulin*, *tef* and *act* gene regions. Phylogenetic analyses showed that the isolates fell within two sub-species of *brassicae* and *canadensis*. Out of 51 Iranian isolates, 55%, 37%, and 8% were found as *L. biglobosa canadensis*, *L. biglobosa brassicae*, and *L. maculans*, respectively. Accordingly, the *canadensis* sub-species was found to be the predominant sub-species in Iran. There was no difference between Iranian and foreign *brassicae* sub-species in terms of *ITS* nucleotide sequences. This paper confirms the presence of *brassicae* and *canadensis* sub-species in Iran and describes the distribution of *L. biglobosa* in the country. Moreover, *Leptosphaeria* species were distinguished using a phylogenetic tree based on the *tef* region.

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Oilseed rape; blackleg; phylogenetic tree; *Leptosphaeria maculans*; *Leptosphaeria biglobosa*

Introduction

The Pleosporales is the largest order in the fungal class Dothideomycetes, containing 91 families (Hongsanan et al. 2020; Haridas et al. 2020). The order includes important genera that cause plant diseases, such as *Leptosphaeria*, *Pleospora*, *Alternaria*, *Venturia*, *Cochliobolus,* and *Stagonospora* (Berbee 2001; Schoch et al. 2009). The genus *Leptosphaeria*, considered a monophyletic clade, is one of the most important genera

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of the Leptosphaeriaceae family (Haridas et al. 2020). The genus includes two species *maculans* and *biglobosa*; the causal agents of blackleg (Câmara et al. 2002), a ubiquitous fungal disease threatening Brassica crops, especially canola (*Brassica napus* L.) (Fitt et al. 2006; Hwang et al. 2016). The pathogen can infect canola from germination through to maturity. It progressively damages the crop as the season proceeds by girdling stems, restricting water and nutrient uptake, and eventually leading to lodging and yield loss. The co-existence of both species has been reported in different parts of the world, such as the Czech Republic, Hungary (Szlavik et al. 2003), Poland (Jedryczka et al. 2010), Lithuania (Brazauskiene et al. 2011), Latvia (Bankina et al. 2012), China (West et al. 1999, 2000) and Iran (Fernando et al. 2007; Zamanmirabadi et al. 2008). Although both species of *Leptosphaeria* co-exist in the Brassica field and can infect the plants, *L. maculans* is the most frequent and more aggressive than *L. biglobosa* (Dilmaghani et al. 2010; Paulitz et al. 2017).

Leptosphaeria is a heterothallic ascomycete that requires two compatible partners to produce sexual spores from homothallic ones capable of sexual reproduction from a single organism (Venn 1979). Formations of the fungus's sexual form increase greater genetic diversity and allow the fungus to persist in a protected and dormant form as spores until conditions improve or disperse to a more favorable environment (Sun et al. 2001; Gout et al. 2006). Therefore, in areas such as the northern region of Iran, where the sexual form is present, increased diversity of *Leptosphaeria* species is expected.

The two closely related species, *L. maculans* and *L. biglobosa* can be divided into at least eight sub-clades based on specific host plants, geographic origin, or pathogenicity (Mendes-Pereira et al. 2003; Voigt et al. 2005; Vincenot et al. 2008), including *L. maculans*' *brassicae*' from *Brassica*, *L. maculans*' *lepidii*' from *Lepidium* spp., *L. biglobosa* '*brassicae*', from various *Brassica* species, *L. biglobosa* ' *erysimii*' from *Erysimum* spp., *L. biglobosa* '*thlaspii*' from *Thlaspi arvense*, and *L. biglobosa* '*canadensis*', *L. biglobosa* '*australensis*' and *L. biglobosa* '*occiaustralensis*' from *B. rapa*.

Several molecular techniques have been utilized to study the genetic diversity of *Leptosphaeria* species. For example, (Barrins et al. 2004) used AFLP and RFLP markers and the *MAT1-1* gene to investigate the genetic diversity of Australian isolates of *L. maculans*. In another study, 28 *Leptosphaeria* isolates were examined and compared to 20 other species of the Pleosporales using sequences of the mating-type *MAT1-2*, fragments of *actin* and *β-tubulin* genes for phylogenetic analyses, and seven sub-clades (*L. maculans*, '*brassicae*', *L. maculans*, '*lepidii*', *L. biglobosa*, '*thlaspii*', *L. biglobosa*, '*brassicae*', *L. biglobosa*, '*canadensis*', *L.*

biglobosa, '*australensis*' and *L. biglobosa*, '*erysimii*') were identified for both *Leptosphaeria* species (Voigt et al. 2005). Similarly, Vincenot et al. (2008) have reported that *L. biglobosa* species compose at least seven distinct sub-clades based on biochemical data or *ITS*, mating-type *MAT1-2*, or fragments of *act* or *β-tubulin* genes. The *ITS*, *β-tubulin,* and *act* genes were also used by Dilmaghani et al. (2009) to identify sub-clades of 1,123 *Leptosphaeria* isolates collected from different North and South America. The *ITS* gene was used to identify two sub-clades (*L. biglobosa* '*occiaustralensis*' and *canadensis*') from isolates collected from Mexico (Dilmaghani et al. 2010). The *L. biglobosa* '*brassicae*' sub-clade was also identified in New Zealand using the *ITS* gene (Lob et al. 2013). The *L. biglobosa* '*brassicae*' sub-clade was identified by Hao et al. (2015), who used the *ITS* gene to genotype 84 isolates.

In Iran, *L. maculans* and *L. biglobosa* were first reported in 2007(Fernando et al. 2007) and 2009 (Zamanmirabadi et al. 2009a), respectively. Since then, most of the research on these species in Iran has been focusing on morphology (Zamanmirabadi et al. 2017, 2020), ecophysiology (Zamanmirabadi et al. 2010), and identification of pathogenic races (Zamanmirabadi et al. 2009b, 2009a; Vakili-Zarj et al. 2017) while the genetic structure of the pathogen is rarely investigated (Zamanmirabadi et al. 2021).

Understanding the pathogens' genetic structure and phylogenetic relationships contributes to understanding pathogenesis and breeding program efforts to develop resistant varieties. It also helps develop disease management strategies (Zamanmirabadi et al. 2018). The current study aimed to identify the population structure of *L. maculans* and *L. biglobosa* and determine their status and phylogenetic relationships in Iran to develop a proper management plan in canola fields.

Materials and methods

Sampling and isolation

A total of 51 *Leptosphaeria* isolates (4 *L. maculans* and 47 *L. biglobosa* isolates) were collected from Mazandaran (46 isolates) and Golestan (5 isolates) Provinces (Table 1). The isolates were cultured by plating leaf samples onto potato dextrose agar (PDA) medium. Briefly, leaves showing blackleg disease symptoms were cut into small pieces. The small diseased pieces were surface sterilized by dipping into 10% sodium hypochlorite for 1 min, rinsed in sterile distilled water for 2 min, soaked in 70% ethanol for 30 s, and washed again with sterile distilled water for another 2 min. Disinfected sections were blot dried in sterile filter paper. Finally,

Table 1. Regional information on *Leptosphaeria* isolates.

Code	Province	City/region	isolate(s)	Longitude	Latitude	Host/sample
Fa	Golestan	fazelabad/fazelabad	3	54.73573	36.89424	Canola/leaf
Azad	Golestan	Azadshahr/Azadshahr	1	55.15069	37.07659	Canola/leaf
Go	Golestan	Galikesh/goggle	1	55.40107	37.24618	Canola/leaf
khal	Mazandaran	Kiasar road/khalkhail	4	53.3759	36.32088	Canola/leaf
Amr	Mazandaran	Kiasar road/Amreh	11	53.12524	36.41202	Canola/leaf
Sad	Mazandaran	Kiasar road/Sad	7	53.21195	36.27136	Canola/leaf
Ki	Mazandaran	Miandoroud/Kiapay	1	53.19761	36.55924	Canola/leaf
Ai	Mazandaran	Miandoroud/airport	1	53.18999	36.63627	Canola/leaf
cha	Mazandaran	Kiasar road/ chahardangeh	5	53.19245	36.35004	Canola/leaf
D1	Mazandaran	Simorgh/dehkola1	1	52.82598	36.49325	Canola/leaf
D2	Mazandaran	Simorgh/dehkola2	1	52.82598	36.49325	Canola/leaf
Gy	Mazandaran	Goybar/Goybar	1	52.8388	36.59081	Canola/leaf
Ko	Mazandaran	Behshahr/Kohestan	2	53.46691	36.70248	Canola/leaf
PS	Mazandaran	Miandoroud/Police station	2	53.22377	36.60361	Canola/leaf
Ro	Mazandaran	Behshahr/Rostamkola	1	53.41766	36.67581	Canola/leaf
pk	Mazandaran	Kiasar road/police station	6	53.09186	36.46446	Canola/leaf
AR	Mazandaran	Sari/Arabkhail	2	53.04467	36.69189	Canola/leaf
Alam	Mazandaran	Kiasar road/ Alamdardeh	1	53.25651	36.36606	Canola/leaf

leaf sections were placed on Petri dishes containing PDA and incubated in darkness at 18 ± 2 °C for five days.

Isolation and purification of fungal samples from leaf samples were performed according to (Zamanmirabadi et al. 2010a). The mycelia grown on the PDA medium were harvested using a sterile scalpel, placed in 1.5 mL micro-centrifuge tubes, and kept at −20 °C before DNA extraction and downstream analyses.

DNA extraction

The samples were ground in liquid nitrogen into powder with a mortar and pestle. Total genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method described by Calderon et al. (2002). The concentration of DNA crude extracts was checked using a Qubit® 3.0 Fluorometer kit (Thermo Fisher Scientific, Q33216), following the manufacturer's instructions. DNA quality was checked using a LabChip GX Touch Nucleic Acid Analyzer (PerkinElmer, CLS138162). The samples were stored at −20 °C for further PCR analysis.

PCR amplification and sequencing

PCR amplification of *ITS*, *act*, *tef* and *β-tubulin* regions was performed using primers indicated in Table 2. The PCR amplification was conducted in a total volume of 25 µL, with a reaction mixture containing 12.5 µL Master Mix (Thermo Scientific), 6 µL sterile deionized water, 1.25 µL of

Table 2. Primers used to amplify *ITS*, *act*, *tef* and *β- tubulin* genes of *L. maculans* and *L. biglobosa*.

Region amplified	Primer/ orientation	Sequence (5′-3′)	Reference	Annealing temperature
ITS	V9G/F[a]	TTACGTCCCTGCCCTTTGTA	(De Hoog and van den Ende 1998)	52 °C
	ITS4/R[b]	TCCTCCGCTTATTGATATGC	(White et al. 1990)	
β-tubulin	Btub2/ F	GTBCACCTYCARACCGGYCARTG	(Woudenberg et al. 2009)	64 °C
	Btub4/ R	CCRGAYTGRCCRAARACRAAGTTGTC		
act	ACT-1/F	TGGGACGATATGGAIAAIATCTGGCA	(Voigt et al. 2000)	57 °C
	ACT-5ra/R	TTAGAAGCACTTNCGGTG		
tef	Efa1-983/ F	GCYCCYGGHCAYCGTGAYTTYAT	(Rehner and Buckley 2005)	58 °C
	ef1a-2218/ R	ATGACACCRACRGCRACRGTYTG		

[a]F: Forward.
[b]R: Reverse.

0.5 µM each primer, and 4 µL DNA (25 to 50 ng). The PCR thermocycling conditions were a 2-min first step at 95 °C for initial denaturation, and then 30 cycles of amplification at 95 °C for 30 s, annealing temperature (52° 64° 57° and 58 °C, Table 2) for 30 s and extension at 72 °C for 30 s, and then completed with a final extension step at 72 °C for 5 min. The PCR products were sequenced at the Australian Genome Research Facility (AGRF) and Sinaclon Company. Sequencing was performed in both forward and reverse directions so that each base was read at least twice.

Sequencing data analysis

Post-processing data analysis was performed with CLC Genomics Workbench V. 20.0 (QIAGEN Aarhus A/S). After removing the primer binding sites, the sequences were deposited on NCBI GenBank (Table 3). The NCBI BLAST tool was used to find any possible similarity between the studied isolates and previously deposited species or sub-species (Table 4). The obtained sequences from each isolate for each gene (*ITS*, *β-tubulin*, *tef,* and *act*) were aligned using CLC and then joined to construct the phylogenetic tree.

Phylogenetic analysis

Alternaria and *Leptosphaeria* are relatively closely related since both are ascomycetes belonging to the order Pleosporales. Therefore, four isolates of *Alternaria alternate* were also used to construct a phylogenetic tree (Table 4).

The phylogenetic analysis was carried out using the concatenated *ITS*, *β-tubulin*, *tef,* and *act* sequences. Sequences were aligned with the Classical Sequence Analysis plugin in CLC. Matrices were analyzed using Create Pairwise Comparison. The best substitution model to construct

Table 3. Codes, species, and GenBank accession numbers of registered sequences in this study.

Species/isolate name	Species	GenBank accession numbers			
		ITS	*act*	*β-tubulin*	*tef*
Sad31	*L. biglobosa*	MW411245	MW417257	MW428330	MW428377
Khal111	*L. biglobosa*	MW411254	MW417266	MW428339	MW428386
Pk20	*L. biglobosa*	MW411255	MW417267	MW428340	MW428387
Fa16	*L. biglobosa*	MW411256	MW417268	MW428341	MW428388
SAd113	*L. biglobosa*	MW411257	MW417269	MW428342	MW428389
Amr12	*L. biglobosa*	MW411258	MW417270	MW428343	MW428390
D23	*L. biglobosa*	MW411259	MW417271	MW428344	MW428391
d13	*L. biglobosa*	MW411260	MW417272	MW428345	MW428392
Chap12	*L. biglobosa*	MW411261	MW417273	MW428346	MW428393
Pk18	*L. biglobosa*	MW411262	MW417274	MW428347	MW428394
Sad1	*L. biglobosa*	MW411263	MW417275	MW428348	MW428395
Azad4	*L. biglobosa*	MW411246	MW417258	MW428331	MW428378
Khal17	*L. biglobosa*	MW411264	MW417276	MW428349	MW428396
amr16	*L. biglobosa*	MW411265	MW417277	MW428350	MW428397
sad14	*L. biglobosa*	MW411266	MW417278	MW428351	MW428398
gy8	*L. biglobosa*	MW411267	MW417279	MW428352	MW428399
sad5	*L. biglobosa*	MW411268	MW417280	MW428353	MW428400
chap18	*L. biglobosa*	MW411269	MW417281	MW428354	MW428401
pk7	*L. biglobosa*	MW411270	MW417282	MW428355	MW428402
sad9	*L. biglobosa*	MW411271	MW417283	MW428356	MW428403
khal110	*L. biglobosa*	MW411272	MW417284	MW428357	MW428404
cha3	*L. biglobosa*	MW411273	MW417285	MW428358	MW428405
Ro9	*L. biglobosa*	MW411247	MW417259	MW428332	MW428379
amr5	*L. biglobosa*	MW411274	MW417286	MW428359	MW428406
khal24	*L. biglobosa*	MW411275	MW417287	MW428360	MW428407
ps18	*L. biglobosa*	MW411276	MW417288	MW428361	MW428408
amr23	*L. biglobosa*	MW411277	MW417289	MW428362	MW428409
amr3	*L. biglobosa*	MW411278	MW417290	MW428363	MW428410
amr26	*L. biglobosa*	MW411279	MW417291	MW428364	MW428411
ps55	*L. biglobosa*	MW411280	MW417292	MW428365	MW428412
pk8	*L. biglobosa*	MW411281	MW417293	MW428366	MW428413
ko14	*L. biglobosa*	MW411282	MW417294	MW428367	MW428414
amr8	*L. biglobosa*	MW411283	MW417295	MW428368	MW428415
Ki5	*L. biglobosa*	MW411248	MW417260	MW428333	MW428380
chap26	*L. biglobosa*	MW411284	MW417296	MW428369	MW428416
fa5-2	*L. biglobosa*	MW411285	MW417297	MW428370	MW428417
ko8	*L. biglobosa*	MW411286	MW417298	MW428371	MW428418
Amr9	*L. biglobosa*	MW411287	MW417299	MW428372	MW428419
sad40	*L. biglobosa*	MW411288	MW417300	MW428373	MW428420
amr11	*L. biglobosa*	MW411289	MW417301	MW428374	MW428421
Ai3	*L. biglobosa*	MW411290	MW417302	MW428375	MW428422
Go9	*L. biglobosa*	MW411291	MW417303	MW428376	MW428423
Fa7	*L. biglobosa*	MW411249	MW417261	MW428334	MW428381
Amr22	*L. biglobosa*	MW411250	MW417262	MW428335	MW428382
Amr4	*L. biglobosa*	MW411251	MW417263	MW428336	MW428383
Pk5	*L. biglobosa*	MW411252	MW417264	MW428337	MW428384
Cha26	*L. biglobosa*	MW411253	MW417265	MW428338	MW428385
Ar5	*L. maculans*	MW444864	MW463909	OK391244	OK357144
Alam10	*L. maculans*	MW444865	MW463910	OK391245	OK357145
Pk4	*L. maculans*	MW444866	MW463907	OK391242	OK357142
Ar3	*L. maculans*	MW444867	MW463908	OK391243	OK357143

a phylogenetic tree was determined using the default setting of Model Testing. The phylogenetic tree was constructed using the HKY + G + T model for *ITS*, *act,* and *tef* genes and the HKY + T model for the *β-tubulin* gene. Considering the lack of isolates with deposited sequences for *ITS*, *β-tubulin*, *tef,* and *act* genes in the databases, outgroup isolates

Table 4. *Leptosphaeria* isolates for constructing phylogenetic tree based on *ITS* region.

Species	Sub-clade	country	*ITS*	*act*	*β-tubulin*	*tef*
Leptosphaeria biglobosa	*thlaspii*	Canada	AJ550891	AY748962	FO905876	FO905823
Leptosphaeria maculans	*lepidii*	Canada	AJ550890	AY748972	FO906017	na
Leptosphaeria biglobosa	*erysimii*	Canada	AJ550872	AY748960	na	na
Leptosphaeria biglobosa	*australensis*	Australia	AJ550869	AY748952	na	na
Leptosphaeria biglobosa	*canadensis*	Canada	AJ550868	AY748956	na	na
Leptosphaeria biglobosa	*brassicae*	Canada	AJ550863	AY748949	na	na
Leptosphaeria biglobosa	*brassicae*	USA	AJ550857	AY748951	na	na
Leptosphaeria maculans	*brassicae*	Uk	DQ133891	na	na	na
Leptosphaeria maculans	*brassicae*	France	na	AY748970	na	na
Leptosphaeria biglobosa	*brassicae*	Uk	DQ133890	na	na	na
Leptosphaeria maculans	*brassicae*	Australia	GU205260	na	na	na
Leptosphaeria biglobosa	*brassicae*	China	AJ550858	na	na	na
Leptosphaeria biglobosa	*brassicae*	France	AJ550859	na	na	na
Leptosphaeria biglobosa	*brassicae*	Germany	AJ550860	na	na	na
Leptosphaeria biglobosa	*brassicae*	China	AJ550861	na	na	na
Leptosphaeria biglobosa	*brassicae*	France	AJ550864	na	na	na
Leptosphaeria biglobosa	*brassicae*	Poland	AJ550865	na	na	na
Leptosphaeria biglobosa	*australensis*	USA	AJ550870	na	na	na
Leptosphaeria biglobosa	*australensis*	Australia	AJ550871	na	na	na
Leptosphaeria biglobosa	*occiaustralensis*	Australia	AM410082	na	na	na
Leptosphaeria biglobosa	*thlaspii*	Eu	AJ550892	na	na	na
Leptosphaeria biglobosa	*canadensis*	Canada	AJ550867	na	na	na
Leptosphaeria biglobosa	*canadensis*	Australia	FJ172238	na	na	na
Leptosphaeria biglobosa	*canadensis*	China	MK335631	na	na	na
Leptosphaeria biglobosa	*canadensis*	Canada	AJ550866	na	na	na
Leptosphaeria biglobosa	*canadensis*	China	MK335623	na	na	na
Leptosphaeria biglobosa	*thlaspii*	Canada	na	AY748961	na	na
Alternaria alternata *	na	nk	MH329779	MK637426	MN061574	MN078929

no: not known; na: not available.

were used to construct a single-gene phylogenetic tree using the maximum likelihood method with bootstrap calculation using 1,000 replications (Table 4).

Due to the lack of *tef*, *β-tubulin*, and *act* genes in the databases, 9, 2, and 1 accession numbers (from NCBI) and two reference genomes (NJ3 and G12-14) were found to differentiate *tef* and *β-tubulin* gene regions. Also, due to the lack of samples in classifying some sub-species of *tef* and *B-tubulin* gene regions, the nomenclature was based on their location in the *ITS* and *act* gene regions.

Results

In this study, 51 *Leptosphaeria* isolates; four *L. maculans* and 47 *L. biglobosa* isolates (46 isolates from Mazandaran and five isolates from Golestan Provinces) were characterized based on morphological characteristics (Zamanmirabadi et al. 2010) and molecular data obtained from *ITS*, *β-tubulin*, *tef* and *act* gene regions sequencing (Table 1). The amplified fragments of *ITS*, *β-tubulin*, *tef,* and *act* for *L. maculans* were 679, 345, 985, and 233 bp, respectively, whereas for *L. biglobosa* they were 705, 232, 985, and 337, respectively.

The phylogenetic tree was constructed based on *the ITS* gene region using 25 accession numbers of *L. maculans* and *L. biglobosa* extracted from the NCBI (Table 4). The results showed that the Iranian isolates were identified as *L. maculans* sub-species *brassicae, L. biglobosa* sub-species *brassicae*, and *L. biglobosa* sub-species *canadensis* (Figure 1). Out of 51 Iranian isolates, 55%, 37%, and 8% were found as *L. biglobosa canadensis*, *L. biglobosa brassicae*, and *L. maculans*, respectively. The phylogenetic tree constructed based on the *ITS* gene separated *L.*

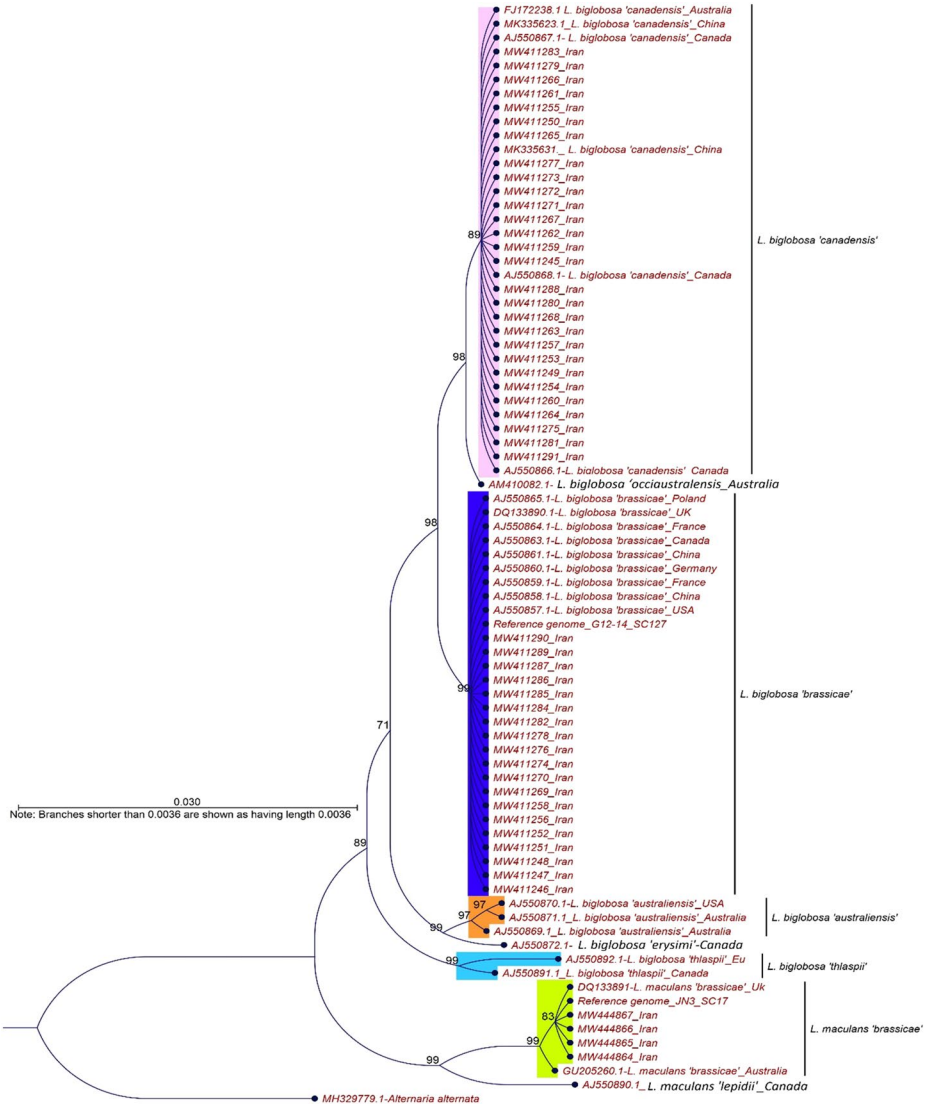

Figure 1. Phylogenetic relationship of *Leptosphaeria* isolates using Maximum likelihood and based on *ITS* sequence. The tree is rooted using *Alternaria alternata* (MH329779). The numbers at the branch node indicate the confidence value obtained from bootstrap analysis using 1000 replications.

maculans and *L. biglobosa* species. Also, phylogenetic analysis showed that *L. biglobosa* sub-species *thlaspii*, *L. biglobosa* sub-species australiensis, *L. biglobosa* sub-species *occiaustralensis*, and *L. biglobosa* sub-species *erysimii* were separated into distinct groups (Figure 1). The australiensis and *erysimii* sub-species were placed in the same group with a 97% similarity; however, they had 14 and 15 SNPs, respectively. Also, 21 and 23 SNPs were observed in the *canadensis* and *brassicae* sub-species (accession number of australiensis), respectively (Figure S1.

L. biglobosa sub-species *occiaustralensis* and *L. biglobosa* sub-species *canadensis* were placed in the same group. The *thlaspii* sub-group was more distant from other *L. biglobosa* species but was still distinct from *L. maculans*. Based on the phylogenetic tree constructed based on the *ITS* region, both *L. maculans* sub-groups, *brassicae* and *lepidii*, were placed in the same monophyletic group (Figures 1 and S1). The phylogenetic trees constructed based on the *act* and *tef* regions (Figures 2 and 3) used 9 and 1 accession numbers, respectively (Table 4). The placement of the isolates in this tree was quite similar to the phylogenetic tree drawn based on the *ITS* region.

Based on this, a total of seven sub-species, including two sub-species *of L. maculans* and five sub-species belonging to *L. biglobosa,* were separated from each other (Figure 2).

Two accession numbers were used to construct a phylogenetic tree based on *β-tubulin* (Figure 4 and Table 4). In the phylogenetic tree constructed based on *β-tubulin,* the maximum diversity was related to *canadensis* sub-species (Figure 4). In the tree constructed based on the *β-tubulin* region, several isolates (MW428340, MW428346, MW428376, MW428353, MW428365, MW428330, MW428358, MW428339, MW428360, MW428348) were in a clade with *canadensis* sub-species (Figure 4). These ten isolates were detected in *L. biglobosa* sub-species *canadensis* based on *ITS*, act, and *tef* regions. Generally, few accession numbers have been deposited on the NCBI website for *β-tubulin* and *tef* compared with *ITS* and *act* to separate sub-species. Thus, sequencing of these regions would result in phylogenetic trees with higher resolution.

The order of Iranian isolates in the phylogenetic tree constructed based on four gene regions (Figure 5) was similar to the phylogenetic trees constructed based on the *ITS*, *act*, *tef*, and *β-tubulin* regions, individually. The results showed that none of the Iranian isolates was classified as australiensis and *occiaustralensis*. The *canadensis* sub-species was found in the western and southern parts of Mazandaran, whitest *canadensis* and *brassicae* sub-species were observed in the central and western parts (Figure 6).

The pairwise comparison of the isolates base *ITS* sequence alignment in terms of similarity percentage (Percent identity) and nucleotides

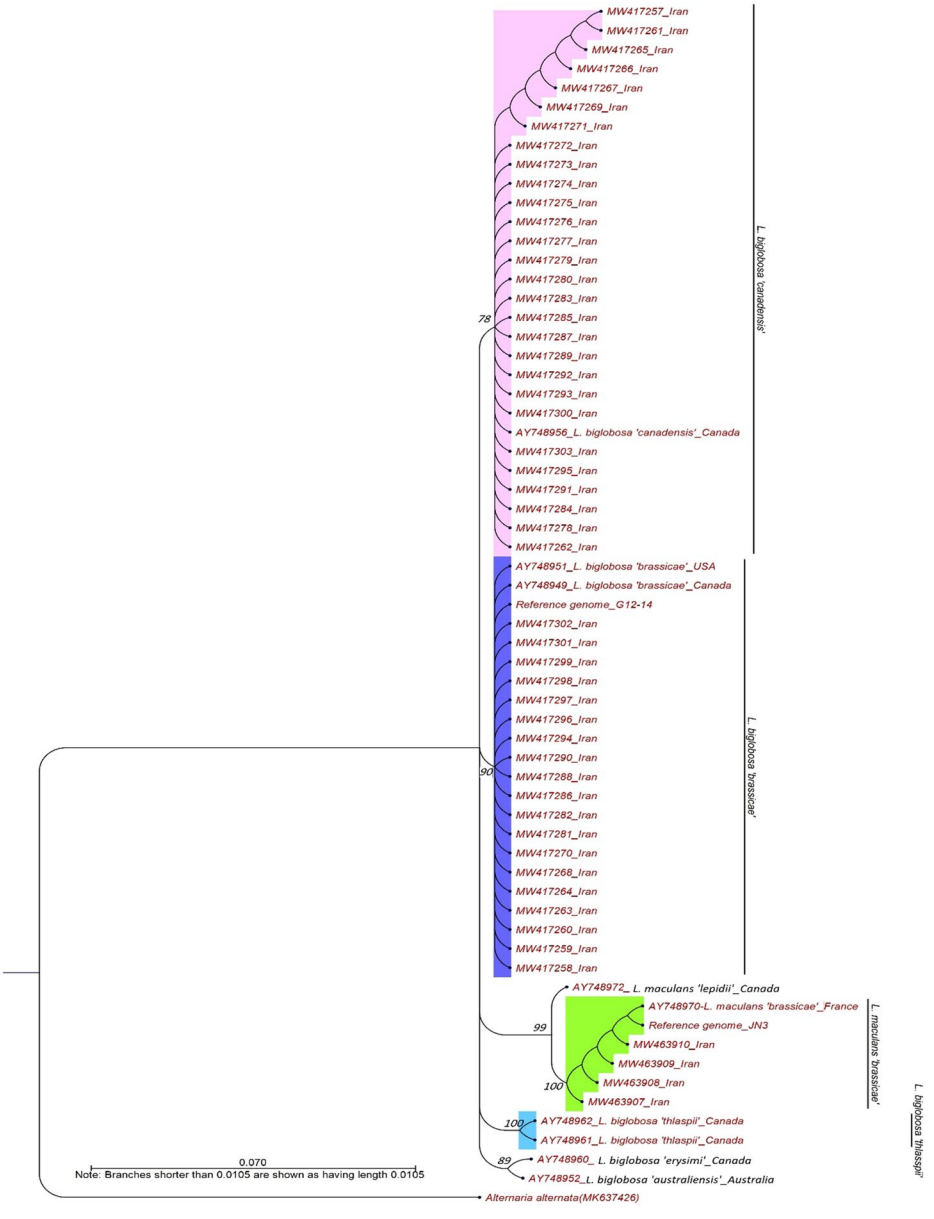

Figure 2. Phylogenetic relationship of *Leptosphaeria* isolates using Maximum likelihood and based on *act* sequence. The tree is rooted using *Alternaria alternata* (MK637426). The numbers at the branch node indicate the confidence value obtained from bootstrap analysis using 1000 replications.

differences is shown in Figure 7. Accordingly, the variation of similarity percentage for all sub-species was 82–100%, and the number of nucleotides difference ranged between 0 and 86 bp. The difference in the number of nucleotides of the *maculans* and *biglobosa* sub-species for the *ITS* region was 83 to 84 nucleotides.

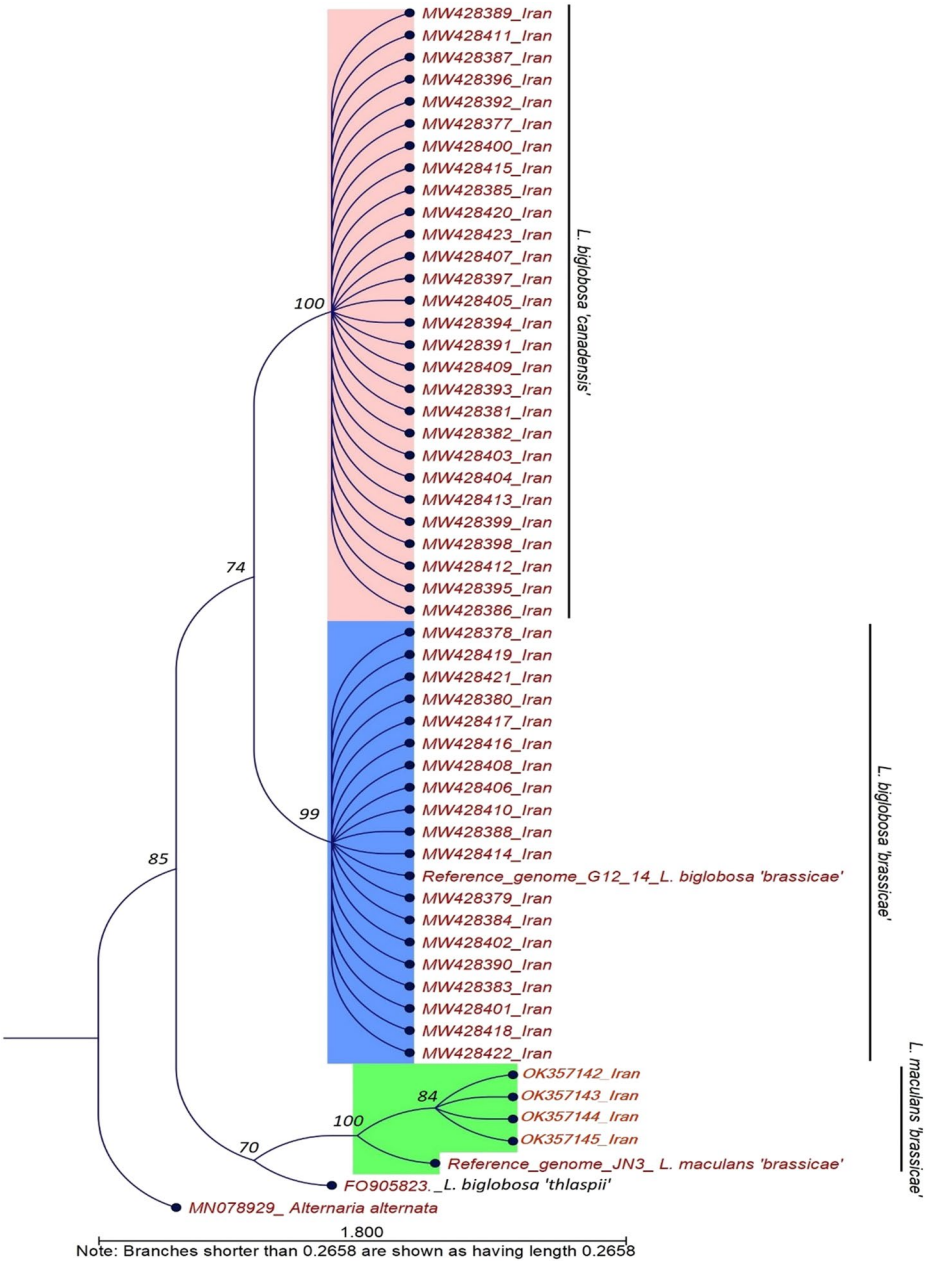

Figure 3. Phylogenetic relationship of *Leptosphaeria* isolates using Maximum likelihood and based on *tef* sequence. The tree is rooted using *Alternaria alternata* (MN078929). The numbers at the branch node indicate the confidence value obtained from bootstrap analysis using 1000 replications.

This difference was one nucleotide for *ITS* and *tef* regions of Iranian isolates and the reference genome of *maculans* species. No difference was observed for *β-tubulin* and *act* regions. In *maculans* species, the nucleotide sequences of the *ITS* region in MW44864 and MW444865

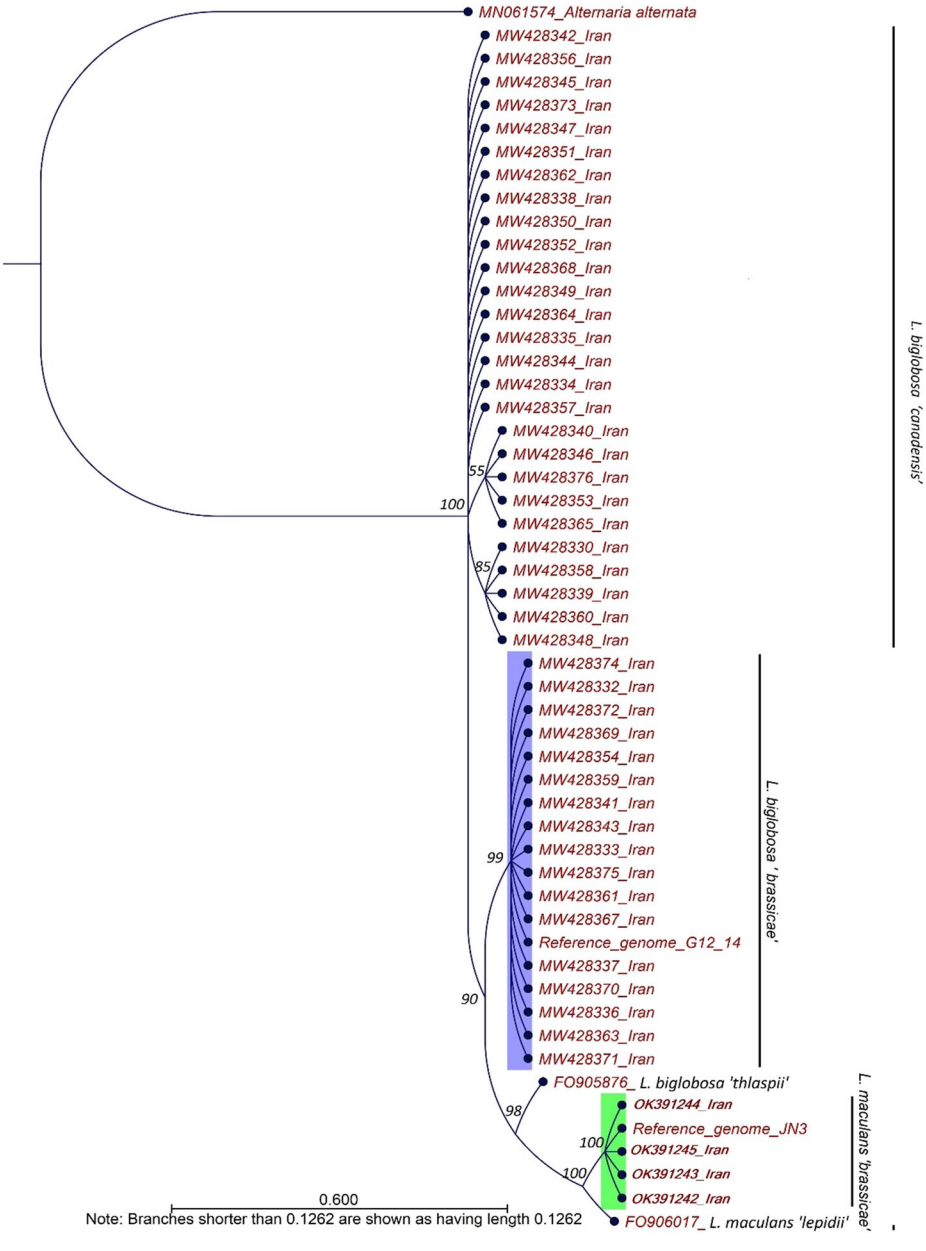

Figure 4. Phylogenetic relationship for 55 *Leptosphaeria* isolates using Maximum likelihood and based on the *β-tubulin* sequence. The tree is rooted using *Alternaria alternata* (MN061574). The numbers at the branch node indicate the confidence value obtained from bootstrap analysis using 1000 replications.

isolates were quite similar to the reference genome. At the same time, there was one SNP difference between the *ITS* region of the reference genome and MW444867 or MW444866 isolates (Figure S3).

Sequences of Iranian *brassicae* and *canadensis* sub-species and foreign samples differed with high similarity in 10 nucleotide loci (Figure 8).

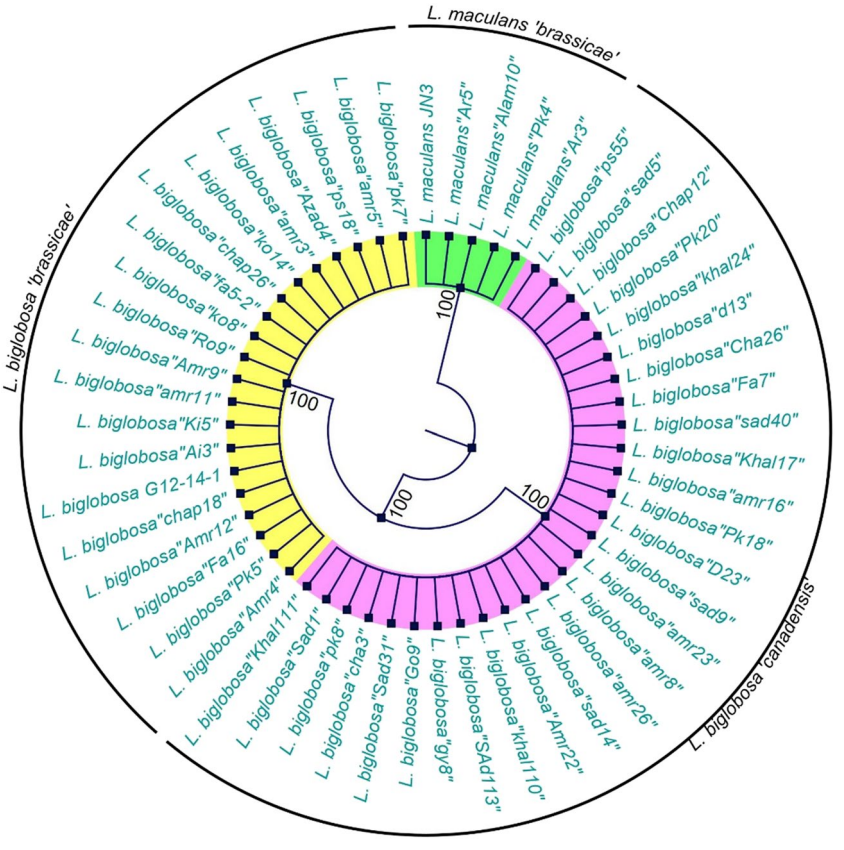

Figure 5. Phylogenetic relationship of *Leptosphaeria* isolates using Maximum likelihood and based on *ITS*, *tef*, *act,* and *β-tubulin* sequence.

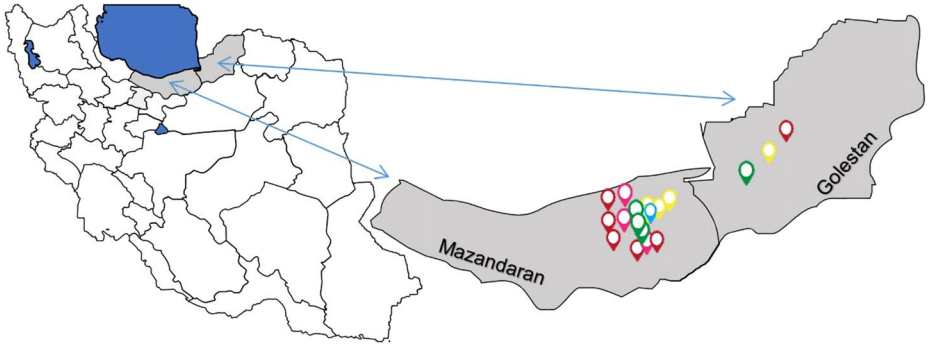

Figure 6. Geographical distribution of *Leptosphaeria maculans* and *Leptosphaeria biglobosa* species and its sub-groups in Mazandaran and Golestan Provinces. Each color indicates the presence of a specific species and sub-group. **brown**: *L. biglobosa canadensis* sub-group, **yellow**: *L. biglobosa brassicae*, **pink**: *L. maculans brassicae*, **green**: the presence of both *L. biglobosa brassicae* and *L. biglobosa canadensis*, **blue**: the precence each of three *L. biglobosa brassicae*, *L. biglobosa canadensis* and *L. maculans brassicae*.

		1	2	3	4	5	6	7	8	9	10	11	12	13
MW411251_Iran_L. biglobosa brassicae	1		100.00	97.98	83.37	83.17	83.37	97.98	98.59	100.00	95.77	94.39	93.00	84.46
Reference genome_G12-14_L. biglobosa brassicae	2	0		97.98	83.37	83.17	83.37	97.98	98.59	100.00	95.77	94.39	93.00	84.46
MW411250_Iran_L. biglobosa canadensis	3	10	10		83.37	83.17	83.37	100.00	99.40	97.98	95.37	93.99	92.60	84.26
Reference genome_JN3_L. maculans brassicae	4	83	83	83		99.79	99.79	83.37	83.17	83.37	84.00	83.07	83.00	90.33
MW444867_Iran_L. maculans brassicae	5	84	84	84	1		99.57	83.17	82.97	83.17	83.80	82.87	82.80	90.12
DQ133891-L. maculans 'brassicae'_Uk	6	83	83	83	1	2		83.37	83.17	83.37	84.00	83.07	83.00	90.33
AJ550867.1- L. biglobosa 'canadensis'_Canada	7	10	10	0	83	84	83		99.40	97.98	95.37	93.99	92.60	84.26
AM410082.1- L. biglobosa 'occiaustralensis'_Australia	8	7	7	3	84	85	84	3		98.59	95.77	94.39	93.20	84.46
AJ550859.1-L. biglobosa 'brassicae'_France	9	0	0	10	83	84	83	10	7		95.77	94.39	93.00	84.46
AJ550869.1_L. biglobosa 'australiensis'_Australia	10	21	21	23	80	81	80	23	21	21		97.19	94.20	85.29
AJ550872.1- L. biglobosa 'erysimi'_Canada	11	28	28	30	85	86	85	30	28	28	14		93.01	83.76
AJ550891.1_L. biglobosa 'thlaspii'_Canada	12	35	35	37	85	86	85	37	34	35	29	35		84.69
AJ550890.1_L. maculans 'lepidii'_Canada	13	78	78	79	47	48	47	79	78	78	74	82	77	

Figure 7. The results of identity percent (upper-left triangle) and difference (lower-left triangle) pairwise comparisons of Iranian and the other representative of *Leptosphaeria maculans* and *Leptosphaeria biglobosa* sub-species based on *ITS* sequence alignment.

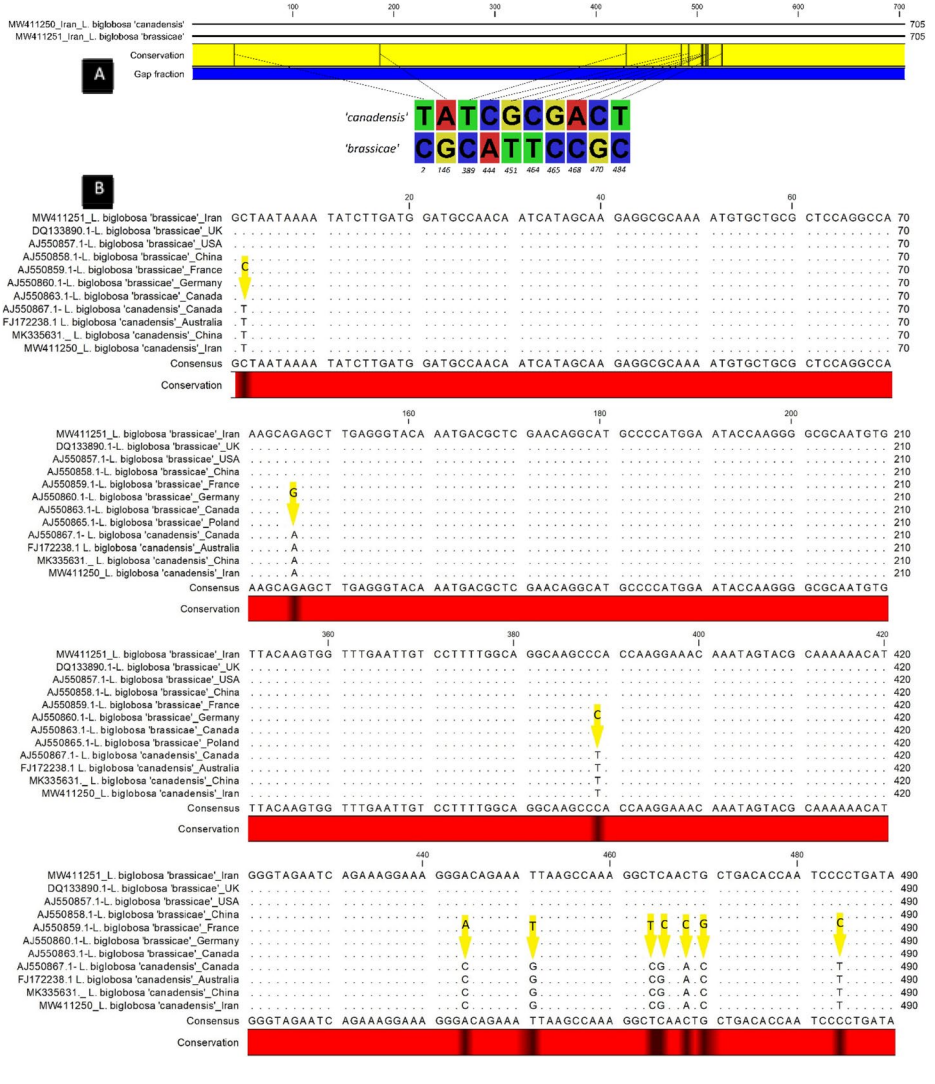

Figure 8. Alignment of *brassicae* and *canadensis* isolates (A) between two samples of Iranian isolates and (B) comparing Iranian and foreign isolates. Ten different positions between sub-species have been identified.

There was no difference between Iranian and foreign *brassicae* sub-species, however out of 28 Iranian *canadensis* sub-species, eight isolates (MW411259, MW411262, MW411272, MW411245, MW411277, MW411267, MW411271, and MW411273) at one common nucleotide site and one isolate (MW411265) at one nucleotide site were different from other Iranian and foreign *canadensis* isolates.

The areas included in this study have a temperature and precipitation difference of up to 20 °C and 300 mm, respectively. Therefore, considering the presence of all three sub-species, *L. maculans brassicae*, *L. biglobosa brassicae*, and *L. biglobosa canadensis*, it does not seem that temperature and humidity changes play a role in the presence or absence of these three sub-species.

Discussion

Many single-gene sequences, including the *ITS*, *LSU*, *SSU*, and *COX1* regions, have been used to identify the genetic diversity of fungi (Schoch et al. 2009). Ribosomal sequences are widely used for species identification and phylogenetic analyzes between and within fungal species, especially for basidiomycetes and ascomycetes (O'Brien et al. 2005). However, to differentiate isolates at the sub-species level, it is necessary to have additional information and use genes encoding proteins such as *RPB1*, *RPB2*, *β-tubulin*, *TEF1-α, γ-actin* (Tekpinar and Kalmer 2019). Many studies show that nucleotide sequences from genes encoding proteins and conserved regions are the best way to differentiate and study sub-species. Still, it seems that the contribution of the *ITS* region is more considerable due to appropriate changes to differentiate species (Voigt et al. 2005).

This study identified three sub-groups using *ITS*, *act*, *tef*, and *β -tubulin* gene regions; however, more sub-groups may be introduced if *MAT1-2* is used. Although sub-species of *Leptosphaeria maculans* and *L. biglobosa* have been previously identified (Mendes-Pereira et al. 2003), this is the first report on the population structure of the *Leptosphaeria* genus using the *ITS*, *act*, *tef*, and *β-tubulin* gene regions in Iran. Like previous studies (Mendes-Pereira et al. 2003; Vincenot et al. 2008), in this study, *L. biglobosa* was more diverse than *L. maculans*. The *L. biglobosa* in the west of Mazandaran Province belonged to the *canadensis* sub-species. Canola cultivation is less developed in those areas than in the central and eastern regions; hence, blackleg disease damage is not that threatening. This is mainly due to the rotation of paddy fields and possibly due to the *canadensis* sub-species being less harmful than the *brassicae* sub-species (Vincenot et al. 2008).

Considering the negligible damage of *L. biglobosa* in Iran, not enough information on the distribution and population of this species was

available. It was thought that *L. maculans* is the only species responsible for yield loss in canola fields. However, this study showed that a significant percentage of the *Leptosphaeria* population in Iran's Northern regions belongs to *L. biglobosa*. This finding will play an essential role in managing invasive *L. maculans* isolates in canola fields. Non-invasive *L. biglobosa* isolates can play a critical role in reducing *L. maculans* populations by inducing systemic acquired resistance (SAR) (Mahuku et al. 1996; Chen and Fernando 2006). Therefore, considering the significant presence of *L. biglobosa* and its role in reducing the pathogenicity of *L. maculans*, one of the reasons for the lack of development and epidemic of canola in the Northern regions of Iran may be the lack of development and epidemic of canola in the presence of many non-invasive isolates.

Most spring-type canola cultivars used in Iran have been supplied from different countries, such as France, Canada, Australia, and Germany, without any quarantine regulation on the pathogen's possible transmission via seeds (West et al. 2001). Considering the 100% nucleotide similarity between *brassicae* and *canadensis* sub-species and Iranian samples (Figure 8), it is not surprising that some of these isolates have been transferred to Iran.

The sequence of *L. biglobosa* isolates used in this study was related to Canada, Australia, the USA, Germany, France, China, Poland, and EU countries. So far, four sub-groups, including *canadensis*, *brassicae*, australiensis, and *occiaustralensis* have been identified for canola. Except for the last two species reported in Australia and the United States, *canadensis* and *brassicae* have been reported from most parts of the world (Dilmaghani et al. 2009a). Non-invasive Iranian isolates were also divided, which indicates the global distribution of these two species.

In recent years, Iran's canola cultivation area has reached its maximum value (Zamanmirabadi 2020). The cultivation area of spring-type canola, except for Mazandaran and Golestan Provinces, which showed the maximum area in the last two decades, has significantly increased (>70,000 ha) in other Provinces such as Khuzestan located in the south of Iran. Therefore, considering the geographical position of this study, it is essential to identify the sub-species of the pathogen in the southern and western Provinces.

According to the findings of this study, the population of the *canadensis* sub-species in Iran, like in Canada, was more significant than the population of the other two sub-species. (Dilmaghani et al. 2009a). However, considering that there is no previous history or report on the prevalence of possible sub-species in Iran, it is not possible to accurately identify any of these or other possible species on Brassicas.

While *B. napus* is the only commercial species in Iran, *B. juncea*, *B. rapa,* and *B. carinata* are only grown for research purposes; hence, the findings of this study are limited to *B. napus;* nevertheless, it is essential to identify possible sub-species of *Leptosphaeria* on other *Brassica* species.

There are few *Leptosphaeria* recorded sequences for *β-tubulin*, *act,* and *tef* regions on the NCBI website; therefore, it was impossible to accurately compare the isolates in this study with other global isolates. More nucleotide and protein differences can be acquired by sequencing these regions and comparing the isolates to global isolates.

In this study, *ITS* sequences related to Iranian and foreign *brassicae* sub-species were not different, while in the *canadensis* sub-species, some isolates observed this difference. However, further studies are needed to pinpoint the exact location of these isolates to accurately identify their sub-species, although based on the nucleotide differences, these isolates differed from each other in only one nucleotide.

The identification of *Leptosphaeria* spp. sub-species based on the gene regions are presently being investigated. This study reports the molecular and phylogenetic characterization of *Leptosphaeria* in Iran for the first time. Although molecular research on this pathogen is relatively scant in Iran, it is predicted that more studies will be conducted due to the spread of this disease.

Disclosure statement

No potential conflict of interest was reported by the authors.

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