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IDENTIFICATION OF SUNFLOWER PATHOGENIC FUNGUS *PLENODOMUS LINDQUISTII* USING PCR WITH SPECIES-SPECIFIC OLIGONUCLEOTIDE PRIMERS

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Plenodomus lindquistii causes Phoma black stem of sunflower which is the most common stem disease of this crop in Russia. The diagnostics of both field specimens and pure cultures of *P. lindquistii* is troublesome. Molecular methods involving the use of the PCR are rapid diagnostic express tests that can precisely identify and detect fungal species. The aim of this study was to develop species-specific oligonucleotide primers for selective amplification of *P. lindquistii* DNA. The primers LepIIF2/LepIIR2 were designed on the basis of ITS region analysis and showed stable amplification of the target fungus DNA with no cross-reaction with other fungal species. The primers are recommended for express detection of the causative agent of Phoma black stem of sunflower. This is the first PCR assay that could be used to rapidly reveal and identify this pathogen.

Keywords: molecular diagnostic, Phoma black stem, sunflower

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Introduction

Plenodomus lindquistii (Frezzi) Gruyter, Aveskamp & Verkley (syn. *Leptosphaeria lindquistii* Frezzi, Revta, *Phoma macdonaldii* Boerema, *Phoma oleraceae* var. *helianthituberosi* Sacc.) causes Phoma black stem of sunflower (*Helianthus annuus* L.). This is the most common stem disease of sunflower in Russia and worldwide (McDonald, 1964; Boerema et al., 1981; Acimovic, 1984; Donald et al., 1987; Maric et al., 1988; Sackston, 1992; Chandreshekar, 1993; Peres, Lefol, 1996; Gulya et al., 1997). In Australia (Chandrashekar, 1993) and China (Wu et al., 2012) the Phoma stem canker causal agent, *P. lindquistii*, is of quarantine significance. In Russia this fungus is widespread in all sunflower producing regions, such as Krasnodar territory (Borodin, Kotlyarova 2006; Saukova et al., 2014), Tambov province (Vypritskaya et al., 2010), Volgograd province (Saukova et al., 2018), Belgorod province, Central Black Earth region, and North Caucasus (Yakutkin, 2001; 2005). Under favorable conditions the fungus can lead to yield losses up to 70%.

The diagnostics of *P. lindquistii* under the field conditions is rather difficult because Phoma black stem can be confused with Phomopsis stem canker (causal agents are *Diaporthe* spp.). Identification of *P. lindquistii* isolates is usually based on morphological criteria of asexual structures: pycnidia

and conidia, but it is often unreliable due to substantial morphological similarity of many related phoma-like species. Correct identification of *P. lindquistii* in pure culture is laborious, time consuming, and requires special conditions and different culture media.

Molecular methods based on PCR are rapid diagnostic express tests that can contribute to detection and precise identification of fungal species in vitro. Nuclear rDNAs particularly in the internal transcribed spacer (ITS) regions are good targets for phylogenetic analysis in fungi (Bruns et al. 1991). It was demonstrated that oligonucleotide specific primers targeting the ITS region selectively detect many agriculturally important fungi including sunflower pathogen *Macrophomina phaseolina* (Babu et al., 2007) and some Phoma-like fungi, e.g. *P. lingam* and *P. biglobosus* (Mahuku et al., 1996).

Currently there are no molecular techniques based on PCR for correct identification of *P. lindquistii* – the causal agent of Phoma black stem of sunflower. The aim of this study was to develop specific oligonucleotide primers and to subsequently evaluate their efficiency and specificity for identification and detection of *P. lindquistii*.

Materials and Methods

Fungal isolates. As a result of the extensive studies of fungal biodiversity on sunflower carried out in 2015–2019 in different geographical locations in Russia 177 *P. lindquistii* isolates were collected by authors from the surface of sterilized stems exhibiting typical symptoms of Phoma black stem. All isolates were stored in the collection of pure cultures of the All-Russian Institute of Plant Protection (VIZR, St. Petersburg).

DNA extraction, PCR and sequencing. Mycelium was obtained from cultures, incubated on potato sugar agar (PSA) and macerated with 0.3 mm glass sand on a MM400 mixer mill (Retsch, Germany). Genomic DNA was then extracted according to a standard CTAB/chloroform method (Doyle, Doyle, 1990).

Four isolates, i.e. one from Lipetsk region (MF Ha15-001) and three from Krasnodar territory (MF Ha16-001, MF Ha16-004, and MF Ha16-005), were selected for sequencing of ITS region. The primers ITS1 and ITS4 (White et al., 1990) were used to amplify the ITS region. The amplification reactions had a total reaction volume of 25 µl which was composed of dNTPs (200 µM), each of the forward ITS1 and reverse ITS4 primers (0.5 µM), Taq DNA-polymerase (5 U/µl), 10× PCR buffer with Mg²⁺ and NH₄⁺ ions and total genomic DNA (approx. 1 ng). The PCR conditions were as follows: predenaturation of DNA at 95 °C for 5 min; 35 cycles of denaturation at 92 °C for 50 s, annealing at 55 °C, 40 s, and elongation at 72 °C for 75 s; followed by a final elongation step for 5 min at 72 °C.

Amplicons were purified according to the standard method with a DNA-binding silica matrix (Boyle, Lew, 1995). Visualization and concentration measurements of the purified PCR products were implemented by electrophoresis in 1% agarose gel stained with ethidium bromide and MassRuler 1000 bp as a marker of concentration.

Amplicons were sequenced by Sanger's method (1977) on ABIPrism 3500 (Applied Biosystems – Hitachi, Japan), with the Big Dye Terminator v3.1 Cycle Sequencing Kit (ABI, Foster City, USA), according to the manufacturer's instructions. All sequences were deposited in the GenBank with the following accession numbers: MK495985, MK495986, MK495987, and MK495988.

Development of specific oligonucleotide primers. Four sequences obtained during this study, reference sequence of the ex-type culture of *P. lindquistii* CBS 381.67 and sequences

of other fungi were aligned using the ClustalX 1.8 (Thompson et al., 1997). The regions, which were conserved among the isolates and specific for *P. lindquistii*, were selected to design species specific oligonucleotide primers. Three pairs of primers were designed using Primer3plus online software with default options. The parameters such as percentage of G+C content and absence of self-complementarity were analyzed by Primer3plus. Sequences, annealing temperature and size of product are listed in the Table. The theoretical specificity of the primers set was checked with the sequences from the other fungi in GenBank by the BLASTn analysis.

The PCR conditions were as follows: predenaturation of DNA at 94 °C for 2 min; 30 cycles of denaturation at 92 °C for 50 s, annealing at according temperature (Table) for 30 s, and elongation at 72 °C for 75 s; followed by a final elongation step for 5 min at 72 °C.

Table. New oligonucleotide primers for species-specific amplification of ITS locus in rDNA *Plenodomus lindquistii*

Primer pair	Primer name	Nucleotide sequence, 5'→3'	Annealing temperature, °C	Expected amplicon size, b.p.
1	LepliF	CTGGGTCTTTTGCTCCATGT	60.1	104
	LepliR	TTTTGTCCTATCGGCGGG	61.9	
2	LepliF2	TGCTCCATGTACCAGCTCA	58.9	178
	LepliR2	CGATGCCAGAACCAAGAGAT	60.2	
3	LepliF3	TCCATGTACCAGCTCACCTC	58.7	250
	LepliR3	TGTGCGTTCAAAGATTCGAT	59.3	

Specificity evaluation of oligonucleotide primers was carried out by PCR with DNA of eight *P. lindquistii* isolates (MF Ha16-001 – MF Ha16-008) as positive amplification control. As negative amplification control we used the DNA of the next 16 isolates representing various groups of fungi, including both ascomycetes and basidiomycetes, i.e. *Alternaria atra* (MF 150-011), *Armillaria* sp. (MF A1), *Ascochyta kamchatica* (MF 010-031), *Boeremia exigua* (MF 17-75), *Colletotrichum floriniae* (MF Vm17-043), *Diaporthe gulyae* (MF Ha17-042),

D. eres (MF Vm17-001), *Didymella glomerata* (MF 32.38.1), *D. pomorum* (MF 9.232.1), *Fusarium avenaceum* (MF 60101), *Ganoderma* sp. (MF G), *Plenodomus biglobosus* (MF 4.105), *P. lingam* (MF 4.34), *Paraphoma melnikiae* (MF 9.88), *Neopyrenochaeta acicola* (MF 52.5), *Stagonosporopsis inoxydabilis* (MF 010-020). The most specific primer pair was tested with all 177 *P. lindquistii* isolates from the VIZR pure culture collection.

Results and Discussion

The primers LepliF/LepliR failed to amplify ITS region of eight tested *P. lindquistii* isolates. Whereas primers LepliF2/LepliR2 and LepliF3/LepliR3 yielded single amplified product each of 250 and 180 bp respectively (Fig. 1). However, amplification with the primers LepliF3/LepliR3 generated the target product for six isolates out of eight. Amplification with primers LepliF2/LepliR2 was successful for all DNA samples (Fig. 1).

Both primer pairs, LepliF2/LepliR2 and LepliF3/LepliR3, were found to be specific for *P. lindquistii* as none of the other fungi tested could yield any amplification product under identical conditions of amplification (Fig. 2, 3).

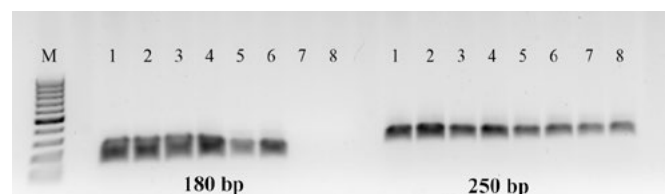


Figure 1. Test for primers LepliF3/LepliR3 (left) and LepliF2/LepliR2 (right) specificity for DNA of eight *Plenodomus lindquistii* isolates. M marks GeneRuler ladder 1000 bp.

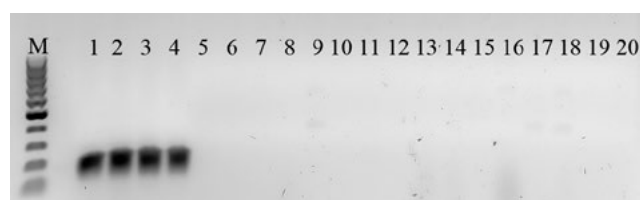


Figure 2. Test for LepliF2/LepliR2 primers specificity for DNA of *Plenodomus lindquistii* isolates (lanes 1–4) and isolates of other fungi (lanes 5–20; fungal species are listed in Material and Methods section.

M marks GeneRuler ladder 1000 bp.

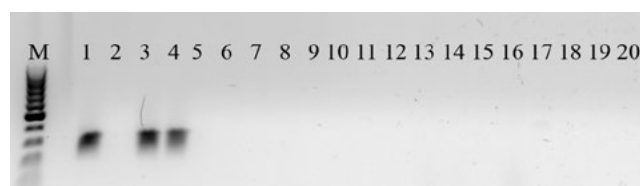


Figure 3. Test for LepliF3/LepliR3 primers specificity for DNA of *Plenodomus lindquistii* isolates (lanes 1–4) and isolates of other fungi (lanes 5–20; fungal species are listed in Material and Methods section.

M marks GeneRuler ladder 1000 bp.

The primer pair LepliF2/LepliR2 was preliminary verified as having the highest specificity for amplification of *P. lindquistii* ITS region. The PCR analysis has resulted in sustainable yield of single products of 250 bp for all 177 *P. lindquistii* isolates, collected from infected sunflower harvested in different years in various geographical locations in Russia

Thus, the use of LepliF2/LepliR2 primers resulted in more specific, reproducible and consistent amplification of rDNA of different *P. lindquistii* isolates than other two primer pairs. This is the first report on development of specific primers for the molecular identification and detection of *P. lindquistii*.

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Краткое сообщение

ИДЕНТИФИКАЦИЯ ПАТОГЕННОГО ДЛЯ ПОДСОЛНЕЧНИКА ГРИБА *PLENODOMUS LINDQUISTII* С ИСПОЛЬЗОВАНИЕМ ПЦР С ВИДОСПЕЦИФИЧНЫМИ ПРАЙМЕРАМИ

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Plenodomus lindquistii – возбудитель фомоза подсолнечника (чёрной стеблевой пятнистости) – заболевания, которое широко распространено в России во всех регионах, возделывающих эту культуру. Диагностика этого заболевания, как в полевых, так и в лабораторных условиях весьма затруднительна. Одним из методов молекулярной

диагностики фитопатогенных грибов является ПЦР с видоспецифичными праймерами. Такой метод позволяет проводить высокоточную детекцию и идентификацию целевых объектов. Цель данной работы заключалась в разработке видоспецифичных олигонуклеотидных праймеров, избирательно амплифицирующих ДНК гриба *P. lindquistii*. Праймеры Lep1F2/Lep1R2, разработанные на основе анализа ITS локуса, показали стабильную амплификацию ДНК целевого гриба при отсутствии кросс-реакции с другими видами грибов. Эти праймеры могут быть рекомендованы для проведения экспресс-диагностики возбудителя фомоза подсолнечника. Данная работа представляет собой первую разработку в области молекулярной экспресс-диагностики этого патогена.

Ключевые слова: молекулярная диагностика, фомоз, чёрная стеблевая пятнистость, подсолнечник

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