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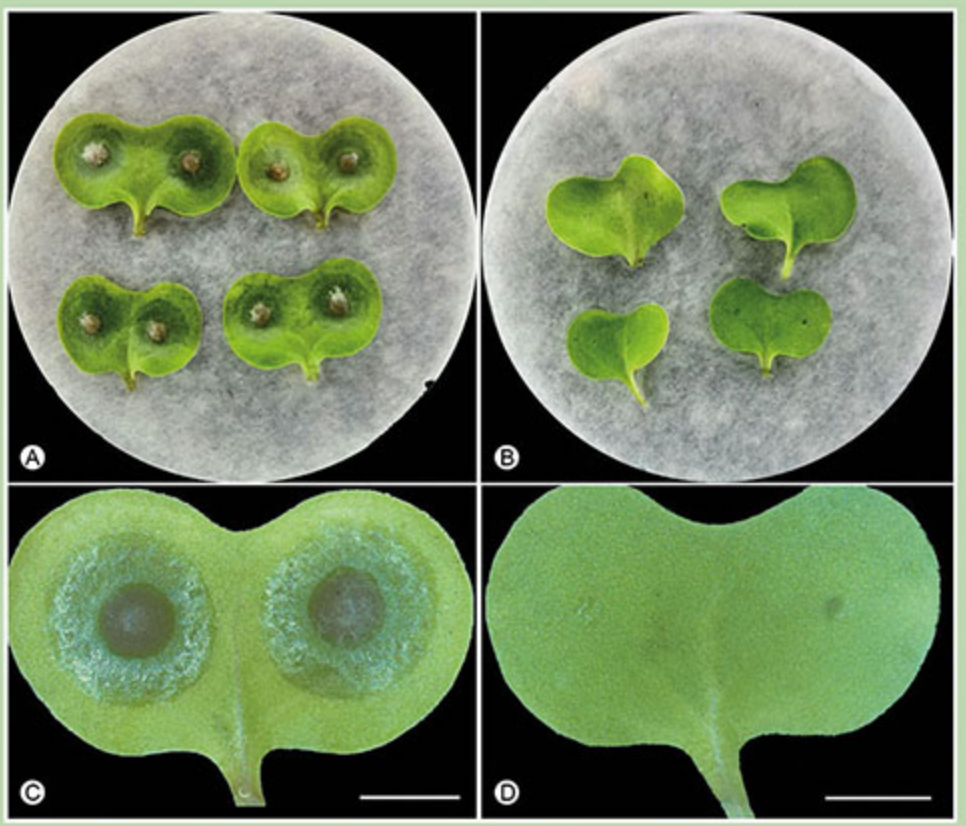

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Full-text article

PLENODOMUS SPECIES INFECTING OILSEED RAPE IN RUSSIA

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The Phoma disease (Phoma stem canker, black leg, Phoma leaf spot) is one of the most harmful diseases of oilseed rape and other *Brassicaceae* in the world, particularly in Russia. The causal agents of this disease are *Plenodomus biglobosus* and *Plenodomus lingam.* Since 2005, a number subclades have been described within *P. biglobosus* and *P. lingam* (2 and 7, respectively). These subclades can be identified by multilocus sequence analysis. So far, biodiversity and geographic distribution of *Plenodomus* spp. infecting oilseed rape in Russia, have not been comprehensively analyzed. For this study, as many as 18 *Plenodomus* spp. isolates were obtained from the samples of stem canker and leaf spot of oilseed rape from four regions of Russia in 2004–2021. The aims of this study were to identify the isolates by phylogenetic analyses inferred from 3 gene sequences: nuclear ribosomal internal transcribed spacer, actin, and β-tubulin, and to assess pathogenicity of the isolates. The phylogenetic reconstructions revealed two well-supported monophyletic clades corresponding to the two species of the genus *Plenodomus*., *P. lingam* 'brassicae' and *P. biglobosus* 'brassicae'.This paper provides robust phylogeny of the *Plenodomus* spp. clade, accompanied with the detailed description of morphological features of both species, and results of pathogenicity tests.

Keywords: *Brassica*, *Leptosphaeria*, molecular phylogeny, pathogenicity, *Phoma*, Phoma leaf spot, Phoma stem canker

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The oilseed rape (*Brassica napus* L.) is a valuable food, fodder and industrial crop. The growing demand for high protein feed for farm animals and biodiesel fuel is the reason for the rising interest in this crop. Expanding the areas of the oilseed rape in crop rotation lead to an increase of known and emergence of novel diseases. In Russia, spring oilseed rape is predominantly cultivated, whereas in the North Caucasus and Kaliningrad Province, winter oilseed rape prevails. In recent years, the area occupied by winter oilseed rape has been expanding also in other regions of Russia.

Phoma disease in *Brassicaceae* (Phoma stem canker, black leg, Phoma leaf spot) is one of the most harmful diseases of the oilseed rape in the world (Fitt et al., 2006). The causal agents of this disease are the two closely related *Phoma*-like fungi, *Plenodomus biglobosus* (Shoemaker & H. Brun) Gruyter, Aveskamp & Verkley (syn. *Leptosphaeria biglobosa* Shoemaker & H. Brun) and *P. lingam* (Tode) Höhn. (syn. *Phoma lingam* (Tode) Desm.). They belong to the family of *Leptosphaeriaceae* (Mendes-Pereira et al., 2003; Liu et al., 2014; Zou et al., 2019). *Plenodomus biglobosus* and *P. lingam* can infect *Brassica oleracea* L. cultivated forms (kale, broccoli, brussels sprouts, cauliflower, Chinese cabbage, mustard, oilseed rape, rutabaga, turnip, and white cabbage), *Eutrema japonicum* (Miq.) Koidz., *Raphanus* (radish and daikon), and *Sinapis* (black and white mustard). Several wild *Brassicaceae* (*Descurainia, Sisymbrium,* and *Thlaspi*) can also be infected with these fungi (West et al., 2001; Fitt et al., 2006; 2008; Zou et al., 2019; King, West, 2022). *Plenodomus biglobosus* and *P. lingam* differ from each other by micromorphological, cultural, physiological, biochemical and molecular phylogenetic features (Mendes-Pereira et al., 2003; Liu et al., 2014; Frac et al., 2022). It was considered that *P. lingam* is more aggressive than *P. biglobosus* and causes higher crop losses (West et al., 2001; Lob et al., 2013; Zou et al., 2019). *Plenodomus lingam* is a fungus of quarantine concern in China (Chen et al, 2010; Zhao et al., 2021).

Phoma-like fungi including the species of the *Leptosphaeriaceae* family display a limited range of morphological features useful for the species identification, and the latter is to be based on multilocus sequencing (de Gruyter et al., 2013). For rapid and accurate identification of *P. biglobosus* and *P. lingam*, PCR with species-specific primers was developed (Xue et al., 1992; Mahuku et al., 1995).

Intraspecific diversity has been described for both of these species. Two subclades have been identified for *P. lingam*, the subclade 'brassicae' (widely distributed) and the subclade 'lepidii' (the sole isolate IBCN84 derived from *Lepidium*, Canada) (Mendes-Pereira et al., 2003; Voigt et al., 2005). *Plenodomus biglobosus* was characterized by a greater genetic diversity. Seven subclades have been identified for *P. biglobosus* within the subclade 'brassicae' occurring mostly in *Brassicaceae* crops (Mendes-Pereira et al., 2003; Zou et al., 2019). The representatives of this subclade was also found in wasabi in the UK (King, West, 2022). In Oregon, USA, the subclade *P. biglobosus* 'americensis' was recorded in oilseed rape (Zou et al., 2019). In Australia, Canada, China, Mexico, the UK and the USA, *P. biglobosus* 'canadensis' was recorded in the *Brassicaceae* species, such as *E. japonicum* and *Thlaspi arvense* L. (Dilmaghani et al., 2009; 2010; Mendes-Pereira et al., 2003; Van de Wouw et al., 2008; Luo et al., 2021; King, West, 2022). In Australia and the USA, *P. biglobosus* 'australensis' was recorded in *B. napus* and *B. juncea* (L.) Czern. (Plummer et al., 1994; Voigt et al., 2005). In Australia, Chile and Georgia, *P. biglobosus* 'occiaustralensis' was

recorded in oilseed rape and wild radish (Vincenot et al., 2008; Dilmaghani et al., 2009) and in Canada, *P. biglobosus* 'thlaspii' and *P. biglobosus* 'erysimii' were recorded in the wild *Brassicaceae* species, *T. arvense* and *Erysimum* sp., respectively (Mendes-Pereira et al., 2003).

To reconstruct phylogeny and identify subclades within *P. biglobosus* and *P. lingam*, the nucleotide sequences of the regions of internal transcribed rDNA spacers (ITS-locus) and genes coding for actin (*act*) and β-tubulin (*tub*) have been used (Mendes-Pereira et al., 2003; Voigt et al., 2005; Van de Wouw et al., 2008).

In Russia, occurrence of Phoma disease (black leg and leaf spot) has been reported in the North Caucasus and Krasnodar Area (Serdyuk et al., 2011) since 1933. However, this disease was recorded more recently in Russian Far East (Ussuriysk, Amur Province, and Khabarovsk Area), Siberia, Non-Chernozem and Central Chernozem zones (Bilay et al., 1988; Fedotov et al., 2008). Also, All-Russian Research Institute of Oilseed Rape (Lipetsk, Russia), All-Russian Research Institute of Oil Crops named after V.S. Pustovoit (Krasnodar, Russia), and some other scientific institutions assess oilseed rape genotypes for resistance to these diseases (Bochkareva et al., 2006; Bochkareva, Aliferova, 2009; Artamonov, Gorshkov, 2009; Artamonov, 2012; 2014). Even though it has been known for many years that these diseases of oilseed rape are caused by two *Plenodomus* species, only one of them, namely *Phoma lingam*, is usually mentioned as a causative agent in Russian literature. Moreover, there are only few Russian reports which include species identification of the causative agent of Phoma stem canker (black leg) and Phoma leaf spot.

Plenodomus biglobosus was isolated from winter oilseed rape in the Stavropol Area (Kachlicki et al., 2001), Krasnodar Area, Moscow and Lipetsk Provinces (Dakowska, personal communication, Gasich., 2004), and in spring oilseed rape – in the Lipetsk and Leningrad Provinces (Artamonov, Gorshkov, 2009; Artamonov, 2012). Several *Phoma*-like isolates that failed to produce sirodesmins typical of *P. lingam* were obtained from the seedlings of spring oilseed rape in the Leningrad Province (Kachlicki et al., 2001). When cultured on solid and liquid media, these isolates did not produce yellow-brown pigment, normally synthetized by *P. biglobosus*. Thus, most likely, these isolates belong to other *Phoma*-like species (Jedryczka et al., 2002).

The aim of this study was to unambiguously identify the *Plenodomus* isolates collected from oilseed rape in various locations across Russia according to the species recognition concept currently used for this genus.

Material & Methods

During extensive studies of fungal biodiversity in oilseed rape in Russia and neighboring countries from 1990 to 2021, plants with symptoms of Phoma leaf spot and Phoma stem canker were collected (Fig. 1). Samples of infected plants were deposited in the Mycological Herbarium (LEP) of All-Russian Institute of Plant Protection (VIZR).

To isolate pure cultures of fungus from the plant tissues, fragments of infected material were surface sterilized with 20 ml of 5% NaClO. After surface sterilization, the samples were placed on potato-sucrose agar (PSA) (Samson et al., 2002) containing antibiotics (100 μg/ml ampicillin, streptomycin, penicillin, HyClone, GE Healthcare Life Science, Austria) and 0.4 μl/l Triton X-100 (Panreac, Barcelona, Spain). Petri dishes were incubated at 24 °C in the dark and were examined after 7–10 days (Fig. 2). All isolates were stored in plastic microtubes on PSA at 4 °C in VIZR pure culture collection. From this collection, 18 isolates of local geographical origins were selected for study (Table 1). Isolates from various regions of Russia, collected in different years and represent diversity of *Plenodomus* species.

DNA isolation, PCR and sequencing

The mycelia were obtained from the cultures incubated on PSA and macerated with 0.3 mm glass beads in a MM400 mixer mill (Retsch, Haan, Germany). The genomic DNA was extracted according to the standard CTAB and chloroform protocol (Doyle, Doyle, 1990).

The isolates were identified at the species level using PCR with species-specific primer pairs WV17S, WV5.8C and HV17S, HV26C to ITS locus of *P. biglobosus* and *P. lingam* DNA, respectively (Mahuku et al., 1995). Identification of subclades in *P. biglobosus* and *P. lingam* was implemented by sequencing the taxonomic informative DNA loci, such as ITS region, and partial *act* and *tub* genes. The primers ITSF (PN3) and ITSR (PN10) (Mendes-Pereira et al., 2003), ActinF and ActinR (Van de Wouw et al., 2008), and β tubulinF and β tubulinR (Van de Wouw et al., 2008) were used to amplify the ITS region, and partial *act* and *tub* genes, respectively. The amplification reactions had a total volume of 25 μl, including dNTPs (200 μM), forward and reverse primers (0.5 μM each), *Taq* DNA polymerase (5 U/μL), 10× PCR buffer with Mg^{2+} and NH^{4+}, and 1–10 ng of total genomic DNA. The PCR conditions were: 95 °C for 5 min; followed by 35 cycles of 92 °C for 50 s; 55 °C, 40 s, (HV17S and HV26C), 56 °C (β tubulinF, β tubulinR and ActinF, ActinR), 40 s, 58 °C, 40 s WV17S, WV5.8C and ITSF, ITSR; 72 °C for 75 s; and a final elongation for 5 min at 72 °C.

The PCR amplification products were checked by electrophoresis in 1% agarose gel stained with ethidium bromide. The amplicons were purified according to the standard method (Boyle, Lew, 1995). Single-strand DNA of amplicons was sequenced by Sanger's method (Sanger et al., 1977) on an ABI Prism 3500 analyzer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) with a Bigdye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer's instructions. The obtained nucleotide sequences of the ITS region, as well as partial *act* and *tub* genes, were deposited in the GenBank database (Table 1).

Phylogenetic analysis

The sequences were assembled using Vector NTI advance v. 11.0 (Invitrogen, Thermo Fisher Scientific) and aligned with ClustalX 1.8 (Thompson et al., 1997). The alignments were optimized with Molecular Evolutionary Genetics Analysis 10 (MEGA X; Kumar et al., 2018) and concatenated using Sequence Matrix (Vaydia et al., 2011).

The phylogenetic analysis was based on the alignment of concatenated sequences of the ITS region, and partial *act* and *tub* genes. 54 sequences from local isolates obtained in this study and 84 GenBank sequences of all known genetic subclades of *P. biglobosus* (subclades 'americensis', 'australensis',

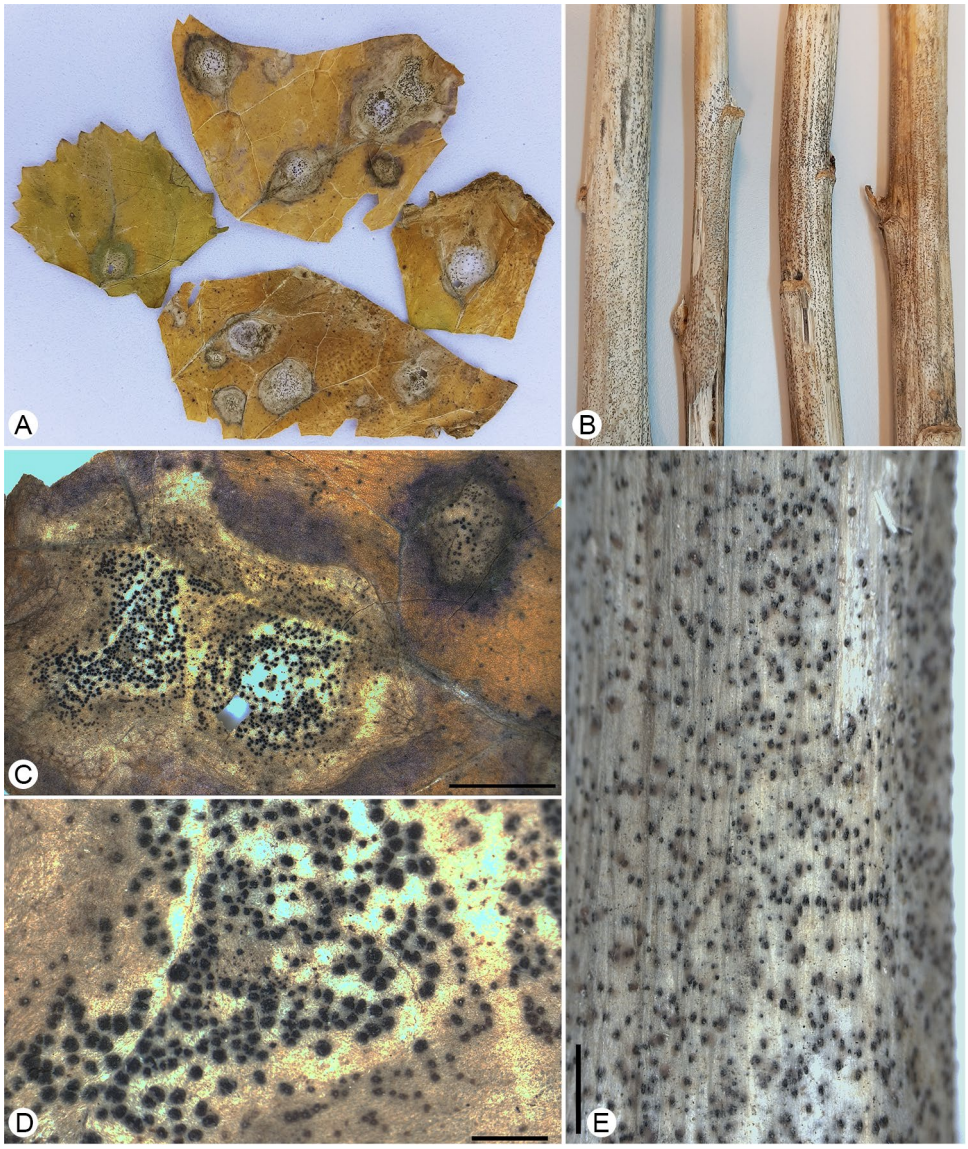

Figure 1. Leaves with symptoms of Phoma leaf spot (A, C, D) from the herbarium specimen LEP 109937. Stems with symptoms of Phoma stem canker (B, E) from the herbarium specimen LEP 92901. Scale bars: C, 5 mm; D, 1 mm; and E, 2 mm

Рисунок 1. Листья (A, C, D), гербарный образец LEP 109937 и стебли (B, E), гербарный образец LEP 92901 рапса с симптомами фомоза. Масштабная линейка: C, 5 мм; D, 1 мм; и E, 2 мм

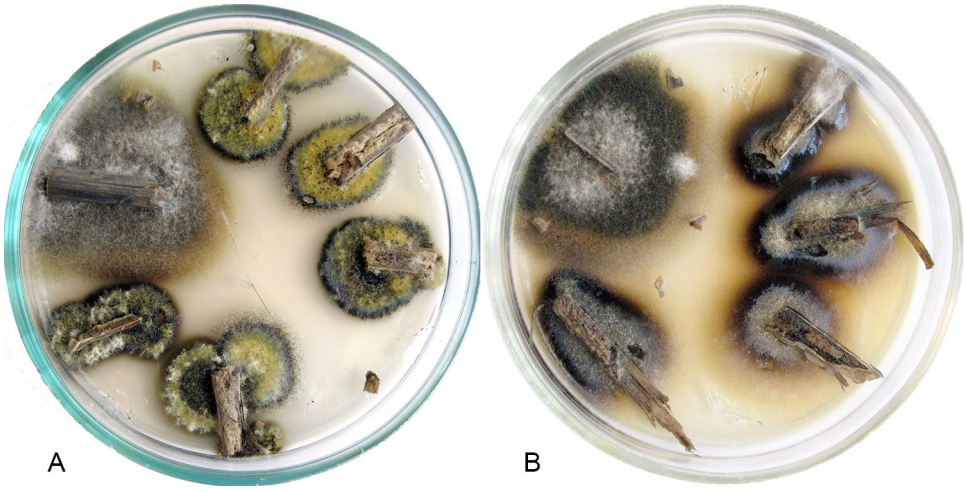

Figure 2. Surface sterilized fragments of stems from the same sample in Petri dishes with PSA after 7 days of incubation. A. *Plenodomus biglobosus* 'brassicae' isolates. B. *Plenodomus lingam* 'brassicae' isolates

Рисунок 2. Поверхностно стерилизованные фрагменты стеблей рапса из одного образца, КСА, 7 суток. А. Изоляты *Plenodomus biglobosus* 'brassicae'. В. Изоляты *Plenodomus lingam* 'brassicae'

Table 1. Isolates of *Plenodomus* spp. used in the study

Plenodomus Species	Subclade	Isolate	Source, cultivar	Location	Collection year	GenBank accsession ITS	*act*	*tub*
biglobosus	'brassicae'	MF-Br17-022	stems	Kaliningrad Province, Gurievsky district	2017	ON730830	ON734111	ON734131
biglobosus	'brassicae'	MF-Br17-023	stems	Kaliningrad Province, Gurievsky district	2017	ON730831	ON734112	ON734132
biglobosus	'brassicae'	MF-R-4.67	stems	Republic of Adygea, Podgorniy	2004	ON730832	ON734113	ON734133
biglobosus	'brassicae'	MF-R-4.92	stems	Republic of Adygea, Podgorniy	2004	ON730833	ON734114	ON734134
biglobosus	'brassicae'	MF-R-4.148	stems, Oniks	Krasnodar Area	2004	ON730834	ON734115	ON734135
biglobosus	'brassicae'	MF-R-4.167	stems	Republic of Adygea, Podgorniy	2004	ON730835	ON734116	ON734136
biglobosus	'brassicae'	MF-R-4.265	roots	Kaliningrad Province, Nesterovskiy district	2019	ON730836	ON734117	ON734137
biglobosus	'brassicae'	MF-R-4.268	leaves	Kaliningrad Province	2019	ON730837	ON734118	ON734138
biglobosus	'brassicae'	MF-R-4.276	leaves	Leningrad Province, Volosovskiy district	2021	ON730838	ON734119	ON734139
biglobosus	'brassicae'	MF-R-4.277	leaves	Leningrad Province, Volosovskiy district	2021	ON730839	ON734120	ON734140
lingam	'brassicae'	MF-Br17-029	stems	Kaliningrad Province, Gurievsky district	2017	ON730840	ON734121	ON734141
lingam	'brassicae'	MF-Br17-031	stems	Kaliningrad Province, Gurievsky district	2017	ON730841	ON734122	ON734142
lingam	'brassicae'	MF-Br17-042	leaves	Kaliningrad Province, Mamonovo	2017	ON730842	ON734123	ON734143
lingam	'brassicae'	MF-Br17-050	leaves	Kaliningrad Province, Mamonovo	2017	ON730843	ON734124	ON734144
lingam	'brassicae'	MF-R-4.266	leaves, Ksenon	Kaliningrad Province, Gurievsky district	2019	ON730844	ON734125	ON734145
lingam	'brassicae'	MF-R-4.274	leaves, Mersedes	Leningrad Province, Volosovskiy district	2021	ON730845	ON734126	ON734146
lingam	'brassicae'	MF-R-4.275	leaves	Leningrad Province, Volosovskiy district	2021	ON730846	ON734127	ON734147
lingam	'brassicae'	MF-R-4.278	leaves	Leningrad Province, Gatchinskiy district	2021	ON730847	ON734128	ON734148

Таблица 1. Исследованные изоляты *Plenodomus* spp.

Plenodomus Вид	Субклада	Изолят	Орган, сорт	Происхождение	Год сбора	Номер доступа в ГенБанке ITS	*act*	*tub*
biglobosus	'brassicae'	MF-Br17-022	стебли	Калининградская обл., Гурьевский р-н.	2017	ON730830	ON734111	ON734131
biglobosus	'brassicae'	MF-Br17-023	стебли	Калининградская обл., Гурьевский р-н.	2017	ON730831	ON734112	ON734132
biglobosus	'brassicae'	MF-R-4.67	стебли	Адыгея, Подгорный	2004	ON730832	ON734113	ON734133
biglobosus	'brassicae'	MF-R-4.92	стебли	Адыгея, Подгорный	2004	ON730833	ON734114	ON734134
biglobosus	'brassicae'	MF-R-4.148	стебли, Оникс	Краснодарский край	2004	ON730834	ON734115	ON734135
biglobosus	'brassicae'	MF-R-4.167	стебли	Адыгея, Подгорный	2004	ON730835	ON734116	ON734136
biglobosus	'brassicae'	MF-R-4.265	корни	Калининградская обл., Нестеровский р-н.	2019	ON730836	ON734117	ON734137
biglobosus	'brassicae'	MF-R-4.268	листья	Калининградская обл.,	2019	ON730837	ON734118	ON734138
biglobosus	'brassicae'	MF-R-4.276	листья	Ленинградская обл., Волосовский р-н.	2021	ON730838	ON734119	ON734139
biglobosus	'brassicae'	MF-R-4.277	листья	Ленинградская обл., Волосовский р-н.	2021	ON730839	ON734120	ON734140
lingam	'brassicae'	MF-Br17-029	стебли	Калининградская обл., Гурьевский р-н.	2017	ON730840	ON734121	ON734141
lingam	'brassicae'	MF-Br17-031	стебли	Калининградская обл., Гурьевский р-н.	2017	ON730841	ON734122	ON734142

Продолжение таблицы 1

Plenodomus Вид	*Plenodomus* Субклада	Изолят	Орган, сорт	Происхождение	Год сбора	Номер доступа в ГенБанке ITS	*act*	*tub*
lingam	'brassicae'	MF-Br17-042	листья	Калининградская обл., Мамоново	2017	ON730842	ON734123	ON734143
lingam	'brassicae'	MF-Br17-050	листья	Калининградская обл., Мамоново	2017	ON730843	ON734124	ON734144
lingam	'brassicae'	MF-R-4.266	листья, Ксенон	Калининградская обл., Гурьевский р-н.	2019	ON730844	ON734125	ON734145
lingam	'brassicae'	MF-R-4.274	листья, Мерседес	Ленинградская обл., Волосовский р-н.	2021	ON730845	ON734126	ON734146
lingam	'brassicae'	MF-R-4.275	листья	Ленинградская обл., Волосовский р-н.	2021	ON730846	ON734127	ON734147
lingam	'brassicae'	MF-R-4.278	листья	Ленинградская обл., Гатчинский р-н.	2021	ON730847	ON734128	ON734148

'brassicae', 'canadensis', 'erysimii', 'occiaustralensis' and 'thlaspii' and *P. lingam* (subclades 'brassicae' and'lepidii') were taken in the analyses.*Leptosphaeria doliolum* (Pers.) Ces. & De Not. was an outgroup (Table 2).

Phylogenetic analysis of single locus and combined aligned data consisted of maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference. Both ML and MP analyses were performed with MEGA X. Bayesian inference was performed by MrBayes v. 3.2.1. in ARMADILLO v. 1.1 (Lord et al., 2012) using a Markov chain Monte Carlo (MCMC) sampling method. The general time-reversible model of evolution, including estimation of invariable sites and assuming a gamma distribution with six rate categories was used for Bayesian inference analyses. Four MCMC chains were run simultaneously, starting from random trees for 1000 generations and sampled every tenth generation for a total of 10000 trees.

Morphology

Morphological features were determined for *P. biglobosus* 'brassicae' isolate MF-Br17-023 and *P. lingam* 'brassicae' isolate MF-Br17-050. Pure cultures of these isolates were incubated on PSA and oatmeal agar (OA) (Boerema et al., 2004). These two media were optimal, particularly for cultivation of *Phoma*-like fungi. Petri dishes were placed for a week in darkness and for another week under 12 h near-ultraviolet light/12 h dark to stimulate sporulation. Colony

Table 2. GenBank accession numbers of the reference *Plenodomus* strains, included in the study

Таблица 2. Номера доступа в ГенБанке референсных штаммов *Plenodomus*, включенных в исследование

Plenodomus Species	*Plenodomus* Subclade	Strain designation	GenBank accession numbers ITS	*act*	*tub*
lingam	'brassicae'	Leroy (IBCN 80)	AJ550883	AY748970	AY749018
lingam	'lepidii'	IBCN 84	AJ550890	AY748972	AY749020
biglobosus	'americensis'	Phl002	MG321243	MG282088	MG282089
biglobosus	'australiensis'	IBCN 29	AJ550869	AY748952	AY749000
biglobosus	'australiensis'	IBCN 91 (PHW1268)	AJ550870	AY748953	AY749001
biglobosus	'brassicae'	IBCN 89	AJ550863	AY748949	AY748997
biglobosus	'brassicae'	IBCN 93	AJ550857	AY748951	AY748999
biglobosus	'brassicae'	Roth_LbCN 01	KJ574216	KJ574238	KJ574243
biglobosus	'brassicae'	Roth_LbCN21	KJ574208	KJ574239	KJ574244
biglobosus	'brassicae'	Roth_LbCN57	KJ574215	KJ574234	KJ574255
biglobosus	'brassicae'	Roth_LbCN58	KJ574209	KJ574232	KJ574249
biglobosus	'brassicae'	Roth_LbCN59	KJ574211	KJ574230	KJ574248
biglobosus	'brassicae'	Roth_LbCN60	KJ574213	KJ574233	KJ574245
biglobosus	'brassicae'	Roth_LbPL30	KJ574214	KJ574240	KJ574251
biglobosus	'brassicae'	Roth_LbUK28	KJ574210	KJ574231	KJ574252
biglobosus	'brassicae'	Roth_LbAT01	KJ574212	KJ574235	KJ574246
biglobosus	'brassicae'	Roth_LbAT03	KJ574206	KJ574237	KJ574247
biglobosus	'brassicae'	Roth_LbFR08	KJ574207	KJ574236	KJ574250
biglobosus	'canadensis'	IBCN 63	AJ550868	AY748956	AY749004
biglobosus	'canadensis'	IBCN 82	AJ550866	AY748958	AY749006
biglobosus	'canadensis'	UNITY (IBCN 81)	AJ550867	AY748957	AY749005
biglobosus	'canadensis'	Roth_LbCA02	KJ574220	KJ574225	KJ574253
biglobosus	'canadensis'	Roth_LbCA03	KJ574219	KJ574229	KJ574256
biglobosus	'canadensis'	Roth_LbCA07	KJ574217	KJ574227	KJ574257
biglobosus	'canadensis'	Roth_LbCA08	KJ574218	KJ574228	KJ574254
biglobosus	'erysimii'	IBCN 83	AJ550872	AY748960	AY749008
biglobosus	'thlaspii'	IBCN 65	AJ550891	AY748962	AY749010
biglobosus	'occiaustralensis'	UWA A21-8	AM410082	AM410084	AM410083

diameter was measured after 7 and 14 days, and colony morphology examined after 14 days (Boerema et al., 2004). One thousand conidia for each isolate were observed and measured with Olympus SZX16 stereomicroscope (Olympus, Tokyo, Japan) and an Olympus BX53 microscope. Nomarski differential interference contrast optics was used to examine conidia morphology. Images were captured with a PROKYON camera (Jenoptik, Jena, Germany).

Pathogenicity test

Pathogenicity tests with all studied isolates were performed by inoculating oilseed rape cv. Oredezh 4. Isolates for tests were grown for 4 days in liquid soy media [KH_2PO_4, 2 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4$, 1 g; glucose, 20 g; soy flour, 10 g; water, 1 l; pH 6]; 50 ml media per 250 ml flask on orbital shakers Innova 44R (Eppendorf, Framingham, MA, USA; 5 cm, 100 rpm, 24 °C). Liquid media were inoculated by placement of a 5-mm block cut from 14-d-old colonies cultured on PSA. After 4 days of incubation mycelium was separated from the liquid culture, dried with filter paper at room temperature, ground, adjusted with sterile water to suspension with final concentration 100 mg/ml. Four healthy cotyledons 3-week-old seedlings were placed in the Petri dishes for each isolate. On the upper surface of each cotyledon, two wounds were made symmetrically in the central vein. The drops (10 µl) of mycelial suspension were placed on the wounded area with a micropipette (Purwantara et al., 1998, Van de Wouw et al., 2008, Gasich et al., 2015).

The control plants were wounded and inoculated with a drop of sterile water. The plants were assessed for lesion development (diameter of necrosis) 2–3 days after inoculation. Subsequent re-isolation of pathogens from the inoculated stems and their identification were performed to fulfill Koch's postulates.

Results

Phylogenetic analysis

Initial identification based on PCR with species-specific primers determined that eight local isolates were *P. lingam* and 10 were *P. biglobosus.*

The multilocus phylogenetic analysis inferred the intraspecific relationships within all known subclades in *P. biglobosus* and *P. lingam*. The topology of both single-locus phylogenies inferred from ITS and *tub* revealed no conflicts. The analysis of the single gene phylogeny (data not shown) based on *act* sequences revealed that *P. biglobosus* 'americensis' clustered within *P. biglobosus* 'brassicae' subclade. The individual gene trees (data not shown), as well the combined tree (Fig. 3), demonstrated that the local isolates were grouped in two distinct phylogenetic subclades with high bootstrap support. Parsimony informative characters and the nucleotide models used for analysis are summarized in (Table 3).

Eight isolates were placed in the *P. lingam* 'brassicae' subclade with the representative culture of this species (IBCN 80). This subclade had high statistical support (MLBS 100%, MPBS 100% and BPP 0.81). Nucleotide sequences of *act* and *tub* of all local *P. lingam* isolates were identical to that

Table 3. Alignment properties and nucleotide substitution models used for phylogenetic analyses

Character status summary	Loci			
	ITS	*act*	*tub*	Combined loci
Total length	462	394	398	1254
Invariable characters	346	240	77	663
Informative charactrers (%)	53	42	88	183
Uninformative characters	63	112	233	408
Nucleotide substitution models	K2+G	K2+G	T92	K2+I
Tree length (TL)	149	196	415	774
Consistency index (CI)	0.7865	0.7564	0.8594	0.7735
Homoplasy index (HI)	0.2135	0.2436	0.1406	0.2265
Retention index (RI)	0.9597	0.9456	0.9475	0.9399
Rescaled consistency index (RC)	0.7549	0.7152	0.8143	0.7269

K2, Kimura 2-parameter; T92, Tamura 3-parameter.

Таблица 3. Данные об общей длине матриц для каждого изолята, длине каждого локуса в отдельности, числе вариабельных сайтов и оптимальных моделях нуклеотидных замен, использованных для филогенетического анализа

Параметр	Локус ДНК			
	ITS	*act*	*tub*	ITS+*act*+*tub*
Общая длина	462	394	398	1254
Невариабельные значения	346	240	77	663
Информативные значения (%)	53	42	88	183
Неинформативные значения	63	112	233	408
Оптимальная модель нуклеотидных замен	K2+G	K2+G	T92	K2+I
Длина MP дерева	149	196	415	774
Индекс согласованности	0.7865	0.7564	0.8594	0.7735
Индекс гомоплазии	0.2135	0.2436	0.1406	0.2265
Индекс удержания	0.9597	0.9456	0.9475	0.9399
Перемасштабированный индекс согласованности	0.7549	0.7152	0.8143	0.7269

K2, Kimura 2-parameter; T92, Tamura 3-parameter.

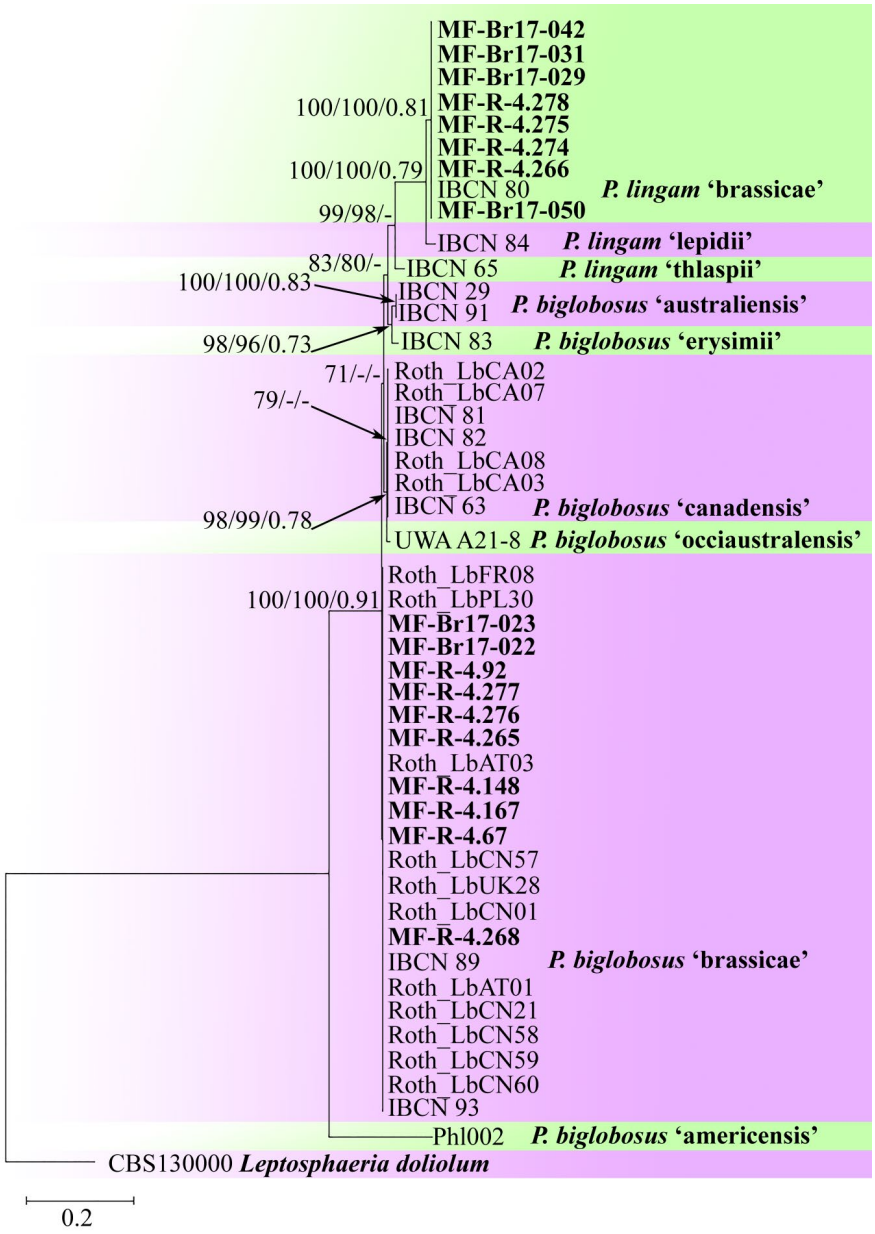

Figure 3. Phylogenetic tree of *Plenodomus s*pp. inferred from a maximum likelihood analysis based on a concatenated alignment of ITS region, and partial *act* and *tub*. The maximum likelihood bootstrap support values (MLBS ≥ 70 %), maximum parsimony bootstrap support values (MPBS ≥ 70 %), and Bayesian posterior probabilities (BPP ≥ 0.70) are given at the nodes (MLBS/MPBS/BPP). Studied isolates given in bold

Рисунок 3. Комбинированное филогенетическое древо видов и субклад *Plenodomus*, построенное методом ML, основанное на нуклеотидных последовательностях ITS, *act* и *tub*. Числовые значения бутстреп-поддержки, полученные методами ML (≥70), MP (≥70) и Байесовский статистики (≥0.7), приведены в узлах ветвей дендрограммы, соответственно. Номера исследованных изолятов выделены полужирным

of reference strain *P. lingam* 'brassicae' IBCN 80. Nucleotide sequences of ITS of local isolates were identical but differed from the reference by transversion (A instead of C) in the position 135.

Ten isolates were placed in the *P. biglobosus* 'brassicae' subclade with the representative culture of this species (IBCN 89). This subclade had high statistical support (MLBS 100 %, MPBS 100 % and BPP 0.91). Nucleotide sequences of ITS and *act* of all local *P. biglobosus* isolates were identical to those of the reference *P. biglobosus* 'brassicae' IBCN 89. Nucleotide sequences of *tub* of single isolate MF-R-4.268 were identical to IBCN 89. Sequences of other isolates differed from each other and from the reference. Sequences of three isolates, MF-R-4.67, MF-R-4.148 and MF-R-4.167, differed from the reference by transition (T instead of C) in the position 8. Sequences of six isolates, MR-Br17-022, MF-Br17-023, MF-R-4.92, MF-R-4.265, MF-R-4.276 and MF-R-4.277, were identical with non-representative *P. biglobosus* 'brassicae' strains, Roth_LbPL30 and Roth_FR08, originating from Poland and France, respectively. These six strains differed from the reference by a transversion (G instead of C) in the position 127.

Morphology

Plenodomus biglobosus 'brassicae' (Fig. 4). Colonies on PSA, 35–39 mm in diameter after 7 days (Fig. 4 E, F) and 75–79 mm after 14 days (Fig. 4 M, N). Margin regular,

covered with abundant aerial mycelium gray neutral in the center, then dark gray, and dull gray in the marginal zone, velvety to felty. Abundant pycnidia are composed in concentric rings at the margin of colony. Agar media dark yellow-orange due to pigment production. Reverse deep brown in the center, deep black in the periphery. Colonies on OA, 51–53 mm in diameter after 7 days (Fig. 4 G, H) and 90 mm after 14 days (Fig. 4 O, P). Margin regular, covered with abundant aerial mycelium white aluminum in the center, then signal gray, silver gray in the marginal zone and floccose. Reverse blackish green in the center and natural umber in the periphery. Agar media dark yellow-orange due to pigment production. Conidiomata pycnidial, on PSA and OA formed concentric rings at the edge of colony, on PSA mostly superficial, rare immersed or semi-immersed in agar media, on OA mostly immersed or semi-immersed in agar media, rare superficial (Fig. 4 S, T). Solitary or confluent, globose, (sub)globose, glabrous, often papillate, conidia exuding from the pycnidia in pearl light gray drops (Fig. 4 T, W), 151–548 (average 375±39) × 128–416 (296±28) μm. Pycnidial wall composed of isodiametric cells, outer layers pigmented. Conidiophores formed from the inner cells of the pycnidial wall, straight or slightly curved, subhyaline, non-septate, 8.6–12.4 (9.7±0.6) × 2.9–3.5 (3.1±0.1) μm. Conidia (Fig. 4 X) subcylindrical, occasionally pyriform, straight, biguttulate, hyaline, 3.1–9 (4.7±0.1) × 1.4–4.4 (2) μm. Morphological features of conidia in vitro the same as in vivo.

Plenodomus lingam 'brassicae' (Fig. 4). Colonies on PSA, 32–34 mm in diameter after 7 days (Fig. 4 A, B) and 52–54 mm after 14 days (Fig. 4 I, J). Margin regular, slightly covered with aerial mycelium grayish green in the center, then colorhouse metal, and light gray in the marginal zone, floccose. Abundant pycnidia are composed in concentric rings at the margin of colony. Reverse grayish green in the center, stone gray in the periphery. Colonies on OA, 46–53 mm in diameter after 7 days (Fig. 4 C, D) and 85–87 mm after 14 days (Fig. 4 K, L). Margin irregular, covered with abundant aerial mycelium dark gray olive in the center, then dark greenish yellow, pearly in the marginal zone, floccose, with almost black pycnidial conglomerates. Reverse gray blue in the center, beige-brown in the periphery. No pigmentation of colony observed.

Conidiomata pycnidial, on PSA and OA scattered, on PSA mostly superficial, rare immersed or semi-immersed in agar media, composed in conglomerates on OA mostly immersed or semi-immersed in agar media, rare superficial (Fig. 4 O, R). Solitary or confluent, globose, (sub)globose, glabrous, often papillate, conidia exuding from the pycnidia in mouse gray drops, 120–355 (206±23) × 118–362 (209±24) μm. (Fig. 4 R, U) Pycnidial wall composed of isodiametric cells, outer layers pigmented. Conidiophores formed from the inner cells of the pycnidial wall, reduced to conidiogenous cells, hyaline, flaskform, non-septate, 7.3–10.7 (8.4±0.7) × 4.6–5.7 (5.2±0.2) μm. Conidia (Fig. 4 V) subcylindrical, cyllindrical, straight, biguttulate, hyaline, 3–5 (4) × 1.2–2.3 (1.8) μm. Conidia as in vivo.

Pathogenicity

All studied isolates were pathogenic to oilseed rape cv. Oredezh 4 cotyledons to varies degree; the necrosis diameters for *P. biglobosus* 'brassicae' isolates varied from 1.7 to 8.7 mm after 2 days, and 2.3 to 10.7 mm after 3 days. Isolates MF-Br17-023 from the Kaliningrad Province and MF-R-4.167 from the Republic of Adygea showed the highest pathogenicity. The necrosis diameters for *P. lingam* 'brassicae' isolates varied from 0 to 6.4 mm after 2 days and 1.5 to 7.3 mm after 3 days. Isolate MF-Br17-029 from the Kaliningrad Province showed the least pathogenicity (Table 4).

Table 4. Pathogenicity of *Plenodomus* spp. isolates for *Brassica napus*

Plenodomus species	Subclade	Isolate	Diameter of the necrosis, mm	
			two days	three days
biglobosus	'brassicae'	MF-Br17-022	3.5±0.2	6.1±0.6
biglobosus	'brassicae'	MF-Br17-023	6.4±0.2	9.3±0.4
biglobosus	'brassicae'	MF-R-4.67	2.2±0.6	5.2±0.9
biglobosus	'brassicae'	MF-R-4.92	3.9±0.5	5.6±0.5
biglobosus	'brassicae'	MF-R-4.148	1.7±0.4	2.3±0.5
biglobosus	'brassicae'	MF-R-4.167	8.7±0.2	10.7±0.3
biglobosus	'brassicae'	MF-R-4.265	4.9±0.4	7.6±0.4
biglobosus	'brassicae'	MF-R-4.268	5.7±0.2	7.8±0.3
biglobosus	'brassicae'	MF-R-4.276	4.5±0.2	6.6±0.3
biglobosus	'brassicae'	MF-R-4.277	2.6±0.2	4.5±0.2
lingam	'brassicae'	MF-Br17-029	0	1.5±0.2
lingam	'brassicae'	MF-Br17-031	2.0±0.6	4.1±1.0
lingam	'brassicae'	MF-Br17-042	1.9±0.3	2.8±0.4
lingam	'brassicae'	MF-Br17-050	2.0±0.1	2.0±0.2
lingam	'brassicae'	MF-R-4.266	6.4±0.1	7.3±0.1
lingam	'brassicae'	MF-R-4.274	3.3±0.4	7.2±0.2
lingam	'brassicae'	MF-R-4.275	2.0±0.03	4.6±0.2
lingam	'brassicae'	MF-R-4.278	2.1±0.6	3.2±1.0

Таблица 4. Результаты оценки патогенности изолятов *Plenodomus* spp. в отношении рапса

Вид *Plenodomus*	Субклада	Изолят	Диаметр некроза, мм	
			2 суток	3 суток
biglobosus	'brassicae'	MF-Br17-022	3.5±0.2	6.1±0.6
biglobosus	'brassicae'	MF-Br17-023	6.4±0.2	9.3±0.4
biglobosus	'brassicae'	MF-R-4.67	2.2±0.6	5.2±0.9
biglobosus	'brassicae'	MF-R-4.92	3.9±0.5	5.6±0.5
biglobosus	'brassicae'	MF-R-4.148	1.7±0.4	2.3±0.5
biglobosus	'brassicae'	MF-R-4.167	8.7±0.2	10.7±0.3
biglobosus	'brassicae'	MF-R-4.265	4.9±0.4	7.6±0.4
biglobosus	'brassicae'	MF-R-4.268	5.7±0.2	7.8±0.3
biglobosus	'brassicae'	MF-R-4.276	4.5±0.2	6.6±0.3
biglobosus	'brassicae'	MF-R-4.277	2.6±0.2	4.5±0.2
lingam	'brassicae'	MF-Br17-029	0	1.5±0.2
lingam	'brassicae'	MF-Br17-031	2.0±0.6	4.1±1.0
lingam	'brassicae'	MF-Br17-042	1.9±0.3	2.8±0.4
lingam	'brassicae'	MF-Br17-050	2.0±0.1	2.0±0.2
lingam	'brassicae'	MF-R-4.266	6.4±0.1	7.3±0.1
lingam	'brassicae'	MF-R-4.274	3.3±0.4	7.2±0.2
lingam	'brassicae'	MF-R-4.275	2.0±0.03	4.6±0.2
lingam	'brassicae'	MF-R-4.278	2.1±0.6	3.2±1.0

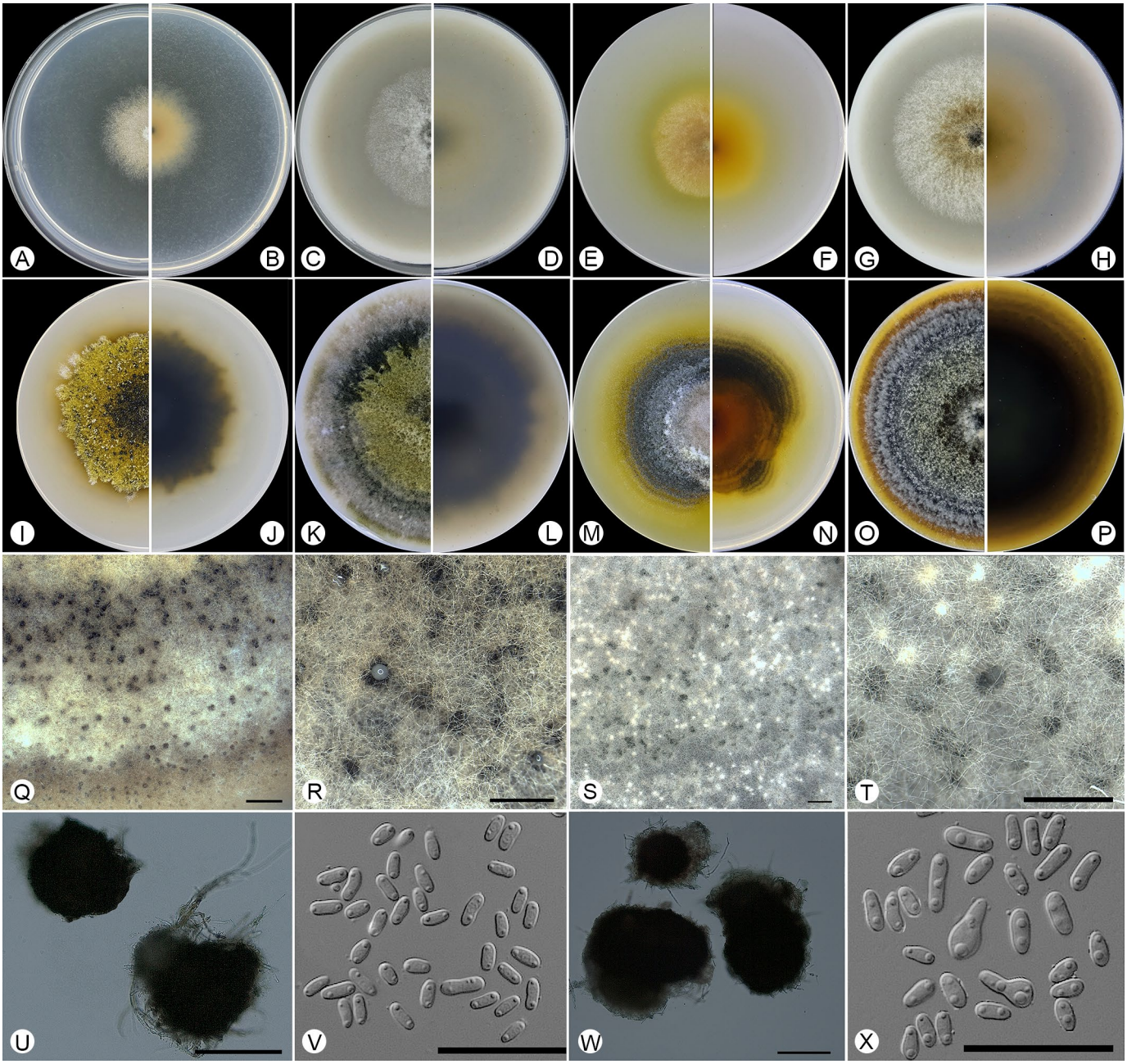

Figure 4. *Plenodomus lingam* 'brassicae' (Plb MF-Br17-050) and *Plenodomus biglobosus* 'brassicae' (Pbb MF-Br17-023). A. Plb cultures on PSA after 7 d of growth, front. B. Plb cultures on PSA after 7 d of growth, reverse. C. Plb cultures on OA after 7 d of growth, front. D. Plb cultures on OA after 7 d of growth, reverse. E. Pbb cultures on PSA after 7 d of growth, front. F. Pbb cultures on PSA after 7 d of growth, reverse. G. Pbb cultures on OA after 7 d of growth, front. H. Pbb cultures on OA after 7 d of growth, reverse. I. Plb cultures on PSA after 14 d of growth, front. J. Plb cultures on PSA after 14 d of growth, reverse. K. Plb cultures on OA after 14 d of growth, front. L. Plb cultures on OA after 14 d of growth, reverse. M. Pbb cultures on PSA after 14 d of growth, front. N. Pbb cultures on PSA after 14 d of growth, reverse. O. Pbb cultures on OA after 14 d of growth, front. P. Pbb cultures on OA after 14 d of growth, reverse. Q, R, U. Pycnidia of Plb on OA. S, T, W. Pycnidia of Pbb on OA. V. Conidia of Plb on OA. X. Conidia of Pbb on OA. **Scale bars:** Q and S, 2 mm; R and T, 1 mm; U and W, 200 µm; and V and X, 20 µm.

Рисунок 4. *Plenodomus lingam* 'brassicae' (Plb MF-Br17-050) и *Plenodomus biglobosus* 'brassicae' (Pbb MF-Br17-023). A. Культура Plb на КСА, 7 суток, верхняя сторона. B. Культура Plb на КСА, 7 суток, реверс. C. Культура Plb на ОА, 7 суток, верхняя сторона. D. Культура Plb cultures на ОА, 7 суток, реверс. E. Культура Pbb на КСА, 7 суток, верхняя сторона. F. Культура Pbb на КСА, 7 суток, реверс. G. Культура Pbb на ОА, 7 суток, верхняя сторона. H. Культура Pbb на ОА, 7 суток, реверс. I. Культура Plb на КСА, 14 суток, верхняя сторона. J. Культура Plb на КСА, 14 суток, реверс. K. Культура Plb на ОА, 14 суток, верхняя сторона. L. Культура Plb на ОА, 14 суток, реверс. M. Культура Pbb на КСА, 14 суток, верхняя сторона. N. Культура Pbb на КСА, 14 суток, реверс. O. Культура Pbb на ОА, 14 суток, верхняя сторона. P. Культура Pbb на ОА, 14 суток, реверс. Q, R, U. Пикниды Plb на ОА. S, T, W. Пикниды Pbb на ОА. V. Конидии Plb на ОА. X. Конидии Pbb на ОА. Масштабная линейка: Q и S, 2 мм; R и T, 1 мм; U и W, 200 µм; и V и X, 20 µм.

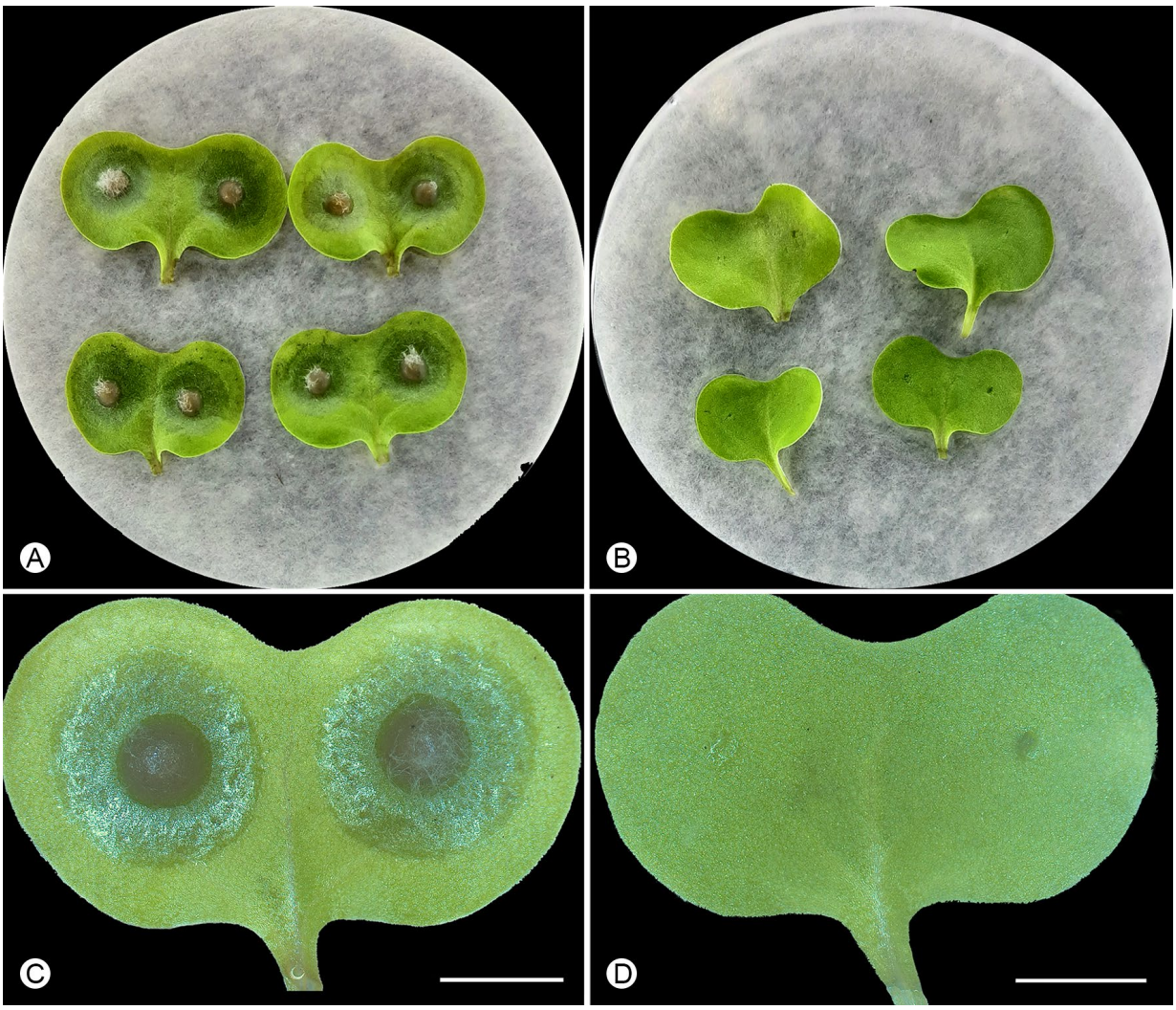

Figure 5. Pathogenicity test of *Plenodomus biglobosus* 'brassicae' MF-R-167 on cotyledons of oilseed rape Oredezh 4. A, C. Tested cotyledons, two days after inoculation with mycelial suspension B, D. Control cotyledons, two days after inoculation with sterile water. Scale bar: C and D, 5 mm.

Рисунок 5. Результаты оценки патогенности изолята *Plenodomus biglobosus* 'brassicae' MF-R-167 на семядолях рапса Оредеж 4. A, C. Инокулированные мицелиальной суспензией семядоли, 2 суток после инокуляции. B, D. Инокулированные стерильной водой семядоли, 2 суток после инокуляции. Масштабная линейка: C и D, 5 мм.

Discussion

This work is the first attempt to determine biodiversity of *Plenodomus* spp. infecting oilseed in Russia confirmed by reliable molecular phylogenetic data. It demonstrated that both *P. biglobosus* and *P. lingam* occur in this host in Leningrad and Kaliningrad Provinces (North West of European part of Russia), and that only *P. biglobosus* occurs in Adygeya and Krasnodar Area (South of European Russia). Both species belong to the subclade 'brassicae'. Other subclades were not found.

It is important to note that it is difficult to diagnose the causal agents of Phoma black stem and Phoma leaf spot of oilseed rape basing on the symptoms alone. For example, *Plenodomus biglobosus* 'brassicae' isolates MF-Br17-022 and MF- Br17-022, and *P. lingam* 'brassicae' isolates MF-Br17-029 and MF-Br17-031 were obtained from symptomatic stems of the same sample. Also, there Plenodomus infection is not associated with a particular organ of the host plant: both species were isolated from stems and leaves. Besides, other morphologically similar *Phoma*-like species were isolated from the symptomatic tissue (data not shown).

The polyphasic approach for reliable species recognition is widely used by mycologists and plant pathologists to distinguish plant pathogenic species, including *Plenodomus,* and relies on the consolidated species concept (CSC) implying incorporating morphological, biological and phylogenetic characters. Data on phylogenetic characters should be obtained according to the genealogical concordance phylogenetic species recognition (GCPSR) method using multilocus phylogenetic approaches for recognizing fungal species. When applying a polyphasic approach for species recognition, it should be noted that GCPSR substantial outweighs the ecological or morphological data combined in the CSC (Crous et al., 2015).

PCR with species-specific primers has been proved to be an effective tool to identify isolates as *P. biglobosus* or *P. lingam* (Mahuku et al., 1995). Our analyses demonstrated that in order to achieve accurate and reliable subclades definition, the

multilocus phylogeny based on combined data matrix of ITS sequences, and partial *act* and *tub* genes might be successfully used.

Although the *P. biglobosus* pure culture on PSA differed from *P. lingam* in production of diffusible dark yellow-orange pigment, this feature alone would not be sufficient for reliable species identification, because it is known that only some *P. biglobosus* isolates produce this pigment (King, West, 2022). There is little difference between these species in micromorphological features of spore-bearing structures. Pycnidia and conidia of *P. lingam* isolates (206 × 207 and 4 × 1.8 μm) were slightly smaller than of *P. biglobosus* (375 × 296 and 4.7 × 2 μm). The data on conidia dimensions corresponded to morphological description of these species made by Boerema (Boerema et al., 2004). Pycnidia of *P. lingam* were subglobose-conical with broad base and usually slightly papillate pore, 150–250 (up to 400) μm diam. Conidia were oblong-ellipsoidal 3.5–5 × 1–2 μm, and usually have 2 small guttules. Pycnidia of *P. biglobosus* were relatively large, globose-papillate up to 330–400 μm diam., or smaller, 150–250 μm diam. Conidia subcylindrical, straight, biguttulate, and mostly 4–5 × 1.5–2 μm. It is clear that the range of variation of the morphological features overlaps for these species, so it can not be used as the sole basis for the species identification. In addition, several morphological features, such scleroplectenchymatous nature of pycnidial wall, in both species are difficult to assess. The pseudoparenchymatous or scleroplectenchymatous wall structure of pycnidia should be studied by staining thin and thick sections with Lugol's iodine. The walls of scleroplectenchyma become red by adsorption of iodine, but pseudoparenchymatous wall structure do not demonstrate such staining pattern (Boerema et al., 2004). No red staining was not observed in our observations of pycnidial walls in both species (data not shown). Thus, for the species identification, morphological, ecological and other features can be used only as additional information.

It has been noted that *P. lingam* isolates are more aggressive than *P. biglobosus* (West et al., 2001; Lob et al., 2013; Zou et al., 2019). However, our pathogenicity results for both species did not confirm this observation.

Phytosanitary measures do not require identification of *Plenodomus* spp. to subclades or even to species. However, we believe that accurate identification at least at the genus level is required given that the other genera of *Phoma*-like fungi may develop on *Brassicaceae*. The genus identification should be based on examination and observation of morphological characters after growing the purified fungal culture on two agar media, PSA and OA.

The results of this study highlight the need to review the biodiversity of causal agents of Phoma stem canker and Phoma leaf spot through theoretical and fundamental studies, not only in oilseed rape but also in other *Brassicaceae* crops cultivated in Russia. This would require regular phytosanitary monitoring of *Brassicaceae* fields in different areas of Russia and identification of species and subclades of the genus *Plenodomus*.

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Полнотекстовая статья

ВИДЫ *PLENODOMUS*, ПОРАЖАЮЩИЕ РАПС В РОССИИ

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Фомоз – одно из самых распространенных заболеваний рапса. Возбудителями заболевания являются грибы *Plenodomus lingam* и *P. biglobosus*. Эти виды грибов могут вызывать такие симптомы как рак стебля,сухая гниль или листовую пятнистость. Внутри этих видов выделяют соответственно две и семь филогенетических линий (субклад). Корректная идентификация этих субклад возможна только в результате мультлокусного филогенетического анализа нуклеотидных последовательностей ITS локуса и участков генов, ответственных за синтез актина и β-тубулина. Комплексный анализ биоразнообразия и географического распространения видов *Plenodomus,* поражающих рапс в России, не проводился. В данной работе было исследовано 18 изолятов *Plenodomus* spp., выделенных из рапса, собранного на территории четырех регионов России в 2004–2021 годах. Целью работы была идентификация этих изолятов с помощью мультилокусного филогенетического анализа и оценка их патогенности. На всех полученных филограммах исследованные изоляты формировали две монофилетические клады, соответствующие двум видам – *P. lingam* (8 изолятов) и *P. biglobosus* (10). Все изоляты каждого вида относились к одной субкладе: *P. lingam* ‘brassicae’ и *P. biglobosus* ‘brassicae’. Помимо детальных филогенетических данных, рукопись сопровождается подробным описанием культуральных и микроморфологических признаков обоих видов, а также оценкой патогенных свойств.

Ключевые слова: *Brassica*, *Leptosphaeria*, *Phoma*, молекулярная филогения, патогенность, субклады, фомоз

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