

RESEARCH ARTICLE

First report of the fungus *Diaporthe phaseolorum* on sunflower in Russia

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ABSTRACT

Fungi of the ascomycete genus *Diaporthe* have been identified worldwide. Typically, *Diaporthe* species are saprobes, endophytes, or plant pathogens. The distinction between the species of this genus has historically been based on the combination of the morphological information, cultural characteristics, and host affiliation. The correct identification of the *Diaporthe* species should be carried out based on a combination of molecular genetic traits. A comprehensive analysis of *Diaporthe* species in the Russian Federation using molecular phylogeny methods has never been accomplished.

The goal of this study was the identification of the isolate *Diaporthe* sp. MF 16-010, extracted from stems of *Helianthus annuus* L. that was collected in the Krasnodar region of the Russian Federation. According to the morphology data and DNA sequence analyses of the nuclear ribosomal internal transcribed spacer (ITS) region as well as of the translation elongation factor-1 α (EF-1 α) and β -tubulin genes, the isolate MF 16-010 was identified as *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. To the best of our knowledge, this isolate represents the first report of *Diaporthe phaseolorum* associated with sunflower in the Russian Federation. The development of the stem lesions as a result of the artificial inoculation of MF 16-010 to sunflower proved that this isolate is pathogenic for sunflower.

INTRODUCTION

Fungi of the genus *Diaporthe* Nitschke are widely distributed worldwide. They are known as saprotrophs (opportunistic saprobes), endophytes, and phytopathogens [1], and they cause diseases among a wide range of economically important agricultural crops. In particular, several species of these fungi cause the Phomopsis stem canker of sunflower.

Since the exact identification of fungi to species level is necessary for understanding their epidemiology as well as for choosing the appropriate methods to combat the corresponding diseases, it is quite clear that the study of phytopathogenic species of this genus is of high importance for theory and practice [1]. The association of most *Diaporthe* species with the host plant and their geographical distribution are currently unknown.

Fungi of the genus *Diaporthe* have two stages of development in their life cycle. In the anamorphic (asexual) stage, they are well known as a species of the genus *Phomopsis*. Whereas in the teleomorphic (sexual) stage, these species are commonly called *Diaporthe*. To avoid dual nomenclature, currently the name *Diaporthe* should be considered as the priority generic epithet, since this genus was described earlier than the genus *Phomopsis* [2]. Traditionally, the micromorphological features of the spore-bearing structures and the association with a host plant have been considered as the taxonomically significant

features used to distinguish *Diaporthe* species. However, the micromorphological features are unstable and their range of variation may overlap for members of different species of the genus, and associations to the host plant may not be limited to one species. Therefore, at present, it is recommended to carry out the reliable identification of members of the genus *Diaporthe* at the species level using the methods of molecular phylogeny and a comparison of the nucleotide sequences of phylogenetically informative DNA loci of internal transcribed spacer (ITS) of ribosomal DNA (rDNA) fragment, β -tubulin, calmodulin, and translation elongation factor 1 α (EF-1 α) genes [1, 3].

The revision of the genus, carried out over the past few years within the framework of a polyphasic approach to the systematics of microorganisms and based on a comprehensive analysis of molecular genetics, micromorphological, cultural, physiological, and biochemical characteristics, has radically changed the views on the taxonomic status and species boundaries of the genus *Diaporthe* [1]. It also led to the refining of the geographic ranges of some phytopathogenic species that cause significant losses in agriculture.

The biodiversity and geographic distribution of the *Diaporthe* species in Russia were never studied using methods of molecular phylogeny and according to the

modern systematics of the genus. Data on the biological diversity and distribution of certain *Diaporthe* species across the Russian Federation, especially the species that have a significant economic impact, need to be clarified. To the best of our knowledge, there are only two *Diaporthe* species that were found in the Russian Federation and confirmed by molecular studies: *D. eres* on a *Salsola tragus* [4] and *D. phaseolorum* on a tomato (Gurkina TA, First report of the fungus Phomopsis phaseoli on tomato, International scientific conference of students and young scientists “Lomonosov-2018”, April 2018).

The aim of this study was the identification of the specimen of *Diaporthe* sp. isolated from sunflower harvested in the Krasnodar region (Russian Federation).

MATERIALS AND METHODS

The specimen isolation

As a result of the phytosanitary monitoring of sunflowers carried out in 2016 in the Krasnodar region, 65 fungal isolates were obtained from sunflower stems, exhibiting the typical symptoms of the Phoma stem canker, which is caused by the fungus *Plenodomus lindquistii* (*Pl. lindquistii*). The majority of them – 64 specimens – were identified as *Pl. lindquistii* according to the morphological characteristics while one – MF 16-010 – was preliminarily identified as *Diaporthe* sp. The isolate MF 16-010 was studied in our project.

To isolate the pure culture of fungus from the sunflower stalks, the fragments of infected material were surface sterilized with 20 ml of 2% sodium hypochlorite (NaClO) solution, then washed for 2 min with 0.1% sodium dodecyl sulfate (SDS), with 5% sodium hypochlorite, and then three times with 20 ml of sterile water. After the surface sterilization, the samples were placed on potato-sucrose agar (PSA) [5] containing antibiotics (100 µg/ml ampicillin, streptomycin, penicillin, HyClone™, GE Healthcare Life Science, Austria) and 0.4 µl/l Triton X-100 (Panreac, Spain) that restricts the growth of fungi. The Petri dishes were incubated at 24°C in the dark and were analyzed on the 7th-10th day of cultivation. Monopycnidial isolate was stored in plastic microtubes on the PSA at + 4°C. Isolate MF 16-010 was deposited in the collection of pure cultures of the Mycology

and Phytopathology Laboratory of the All-Russian Institute of Plant Protection.

DNA extraction and amplification

DNA extraction from pure culture was performed according to a standard protocol using cetyltrimethylammonium bromide (CTAB, Helicon, Russia) and chloroform [6].

ITS locus, β -tubulin and EF-1 α genes of the *Diaporthe* sp. isolate were amplified with the corresponding primer pairs: ITS1F [7]/ITS4 [8]; β tub2Fw/ β tub4Rd [9]; EF1-728F/EF1-986R [10].

Each PCR mixture (25 µl) contained: 0.5 µl of a mixture of deoxynucleoside triphosphates (200 µM), 0.5 µl of each primer (ITS1F/ITS4, β tub2Fw/ β tub4Rd, EF1-728F/EF1-986R) (25 µM), 0.2 µl of Taq polymerase (5 units/µl) (Evrogen, Russia), tenfold buffer for polymerase and 1 µl of DNA solution.

DNA was amplified according to the following protocol: DNA pre-denaturation at 94°C (2 min); denaturation at 92°C (50 s); primer annealing at 55°C (40 s) (ITS1F/ITS4), or at 52°C (40 s) (β tub2Fw/ β tub4Rd), or at 55°C (60 s) (EF1-728F/EF1-986R); elongation at 72°C (75 s); final synthesis (3–5 min) at 72°C; number of cycles: 30. The PCR products were visualized by electrophoresis in 1% agarose gel stained with ethidium bromide.

DNA sequencing and analysis of nucleotide sequences

DNA obtained after PCR was purified according to the standard protocol [11]. The purified DNA fragments were sequenced using the Sanger method [12]. Sequencing was performed on an ABI Prism 3500 instrument according to manufacturer's protocols (Applied Biosystems – Hitachi, Japan) using a reagent kit (BigDye Terminator v3.1 Cycle Sequencing Kit, ABI, USA).

The nucleotide sequences were aligned using the ClustalX 1.8 program [13]. The alignment was adjusted manually where needed. Phylogenetic trees were constructed by method of the Maximum Likelihood (ML) analysis using the RAxML v. 7.2.8 software [14]. The reliability of the phylogram topology was evaluated using 1,000 bootstrap replicates. The sequences of the ITS region of the rDNA, β -tubulin and EF-1 α genes obtained from the GenBank database [15] were used as reference sequences (Table 1).

Table 1. The accession numbers for nucleotide sequences (GenBank) of the reference species and strains of *Diaporthe*.

<i>Diaporthe</i> species	Strain number	Loci and the corresponding GenBank accession numbers for nucleotide sequences		
		ITS	β -tubulin	TEF
<i>D. ambigua</i>	CBS 114015	KC343010.1	KC343978.1	KC343736.1
<i>D. chamaeropsis</i>	CBS 454.81	KC343048.1	KC344016.1	KC343774.1
<i>D. endophytica</i>	CBS 133811	KC343065.1	KC344033.1	KC343791.1
<i>D. infecunda</i>	CBS 133812	KC343126.1	KC344094.1	KC343852.1
<i>D. manihotia</i>	CBS 505.76	KC343138.1	KC344106.1	KC343864.1
<i>D. phaseolorum</i>	CBS 116019	KC343175.1	KC344143.1	KC343901.1
<i>Diaporthe</i> sp. 1	CBS 119639	KC343202.1	KC344170.1	KC343928.1

Morphology

To evaluate the morphological features, isolates were grown on PSA in the dark at 20–22°C for 7 days. The next 7 days Petri dishes were incubated under a 13/11 h day/night regime with ultraviolet light (LE-30 lamps with emission maximum 310–320 nm) [16]. Morphological features were examined on the 14th day of colony growth.

The taxonomically important characteristics of pycnidia and conidia obtained on PSA were determined using an Olympus BX53 microscope and an Olympus SZX16 stereomicroscope (Olympus, Japan).

Pathogenicity

The assessment of the pathogenicity of the isolate was performed using the Tunka sunflower hybrid (selection of Limagren, France) according to the standard method described earlier [17, 18, 19]. Inoculation of intact plants was carried out at the stage of development of R1–R2 (6–8 pairs of leaves, the beginning of budding) in triplicates [18]. Agar blocks 5 mm in size carved from a 10-day pure culture grown on PSA were used as the inoculum. Agar blocks were placed on preliminary wounded leaves and stems. Plants inoculated with blocks cut from a pure agar media were used as a negative control. The observation of the size of necrosis was performed on the 4th–5th day after inoculation. Subsequently, a pathogen was isolated from infected plants and identified in order to confirm the Koch's postulates.

RESULTS

Molecular Phylogeny

The resulting amplified products of the isolate *Diaporthe* sp. MF 16-010 had the following sizes: ITS fragment – about 600 base pairs (bp), β -tubulin gene – 550 bp, EF-1 α gene – 350 bp. The data matrices for subsequent phylogenetic analysis were obtained after the compilation of the composition data. The length of the aligned sequences of the ITS fragment was 555 bp, of the β -tubulin gene – 549 bp, and EF-1 α gene – 313 bp. The GenBank accession numbers of the corresponding nucleotide sequences of the studied isolate are MH732990, MH734197, and MH768350, respectively.

As a result of molecular phylogenetic analysis, four phylograms were constructed: three corresponding to each locus and one combined for all three loci. The isolate MF 16-010 formed a common clade with the reference isolate *Diaporthe phaseolorum* CBS 116019 with a high value of bootstrap support (96–100%) (Fig. 1).

Morphology

Isolate MF 16-010 formed on PSA fast growing colonies with an abundant light aerial mycelium and with numerous pycnidia (Fig. 2). Pycnidia that had the dimension of 370–480 × 340–370 μ m contained three types of conidia: α , β , and γ (Fig. 3). Type α conidia – bean-shaped – had the dimension 6.5–7.25 × 2.25–2.75 μ m. Conidia of type

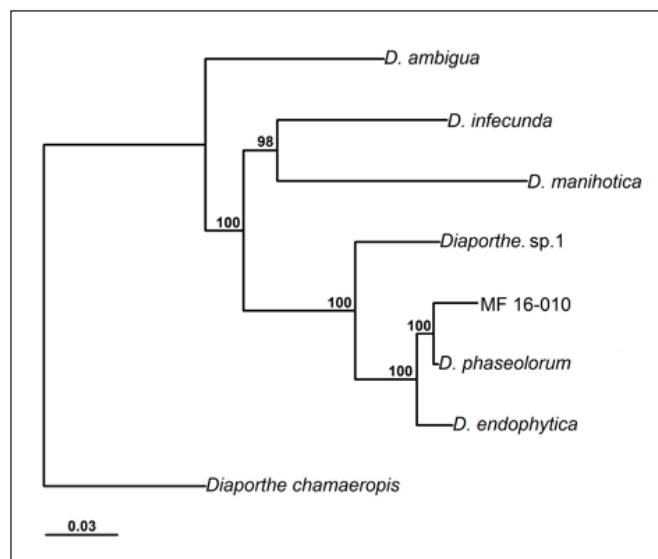


Fig. 1. Combined phylogenetic tree for species and strains of *Diaporthe*, inferred from the combined analysis of the ITS locus of rDNA and the β -tubulin and EF-1 α genes.

β – elongated filiform – had the dimension 15–20 × 0.75–1 μ m. Conidia of type γ had intermediate size between conidia of α and β types.

Pathogenicity

Isolate MF 16-010 caused necrosis in 100% of cases after artificial inoculation of preliminary wounded stems and leaves of sunflower while the inoculation of non-injured stems and leaves of plants did not cause necrosis. The average size of necrosis was 4.00 ± 4.90 mm on the leaves and 2.33 ± 1.47 mm on the stems, 7 days post inoculation.

To confirm the Koch's postulates, the *Diaporthe phaseolorum* isolate was obtained from the necrosis that was formed on sunflower. This isolate had morphological characteristics that were identical to that of the isolate MF 16-010.

DISCUSSION

For a long time, it was considered that the only species of the genus *Diaporthe* – *D. helianthi* – was affecting sunflowers. It was believed that this species can be found everywhere the sunflower is cultivated. In Russia, the species *D. helianthi* is included in The list of quarantine objects (pests, phytopathogens, and weeds) that have limited distribution across the territory of the Russian Federation [20]. However, all these data on the distribution of this species are based on the identification of the pathogen solely according to the symptoms found on the plant or by the morphological characteristics of the isolates in pure culture.

At present, it is known that the reliable identification of *Diaporthe* species can be accomplished only by using molecular phylogenetic methods. As a result of studies on the revision and reidentification of herbarium specimens

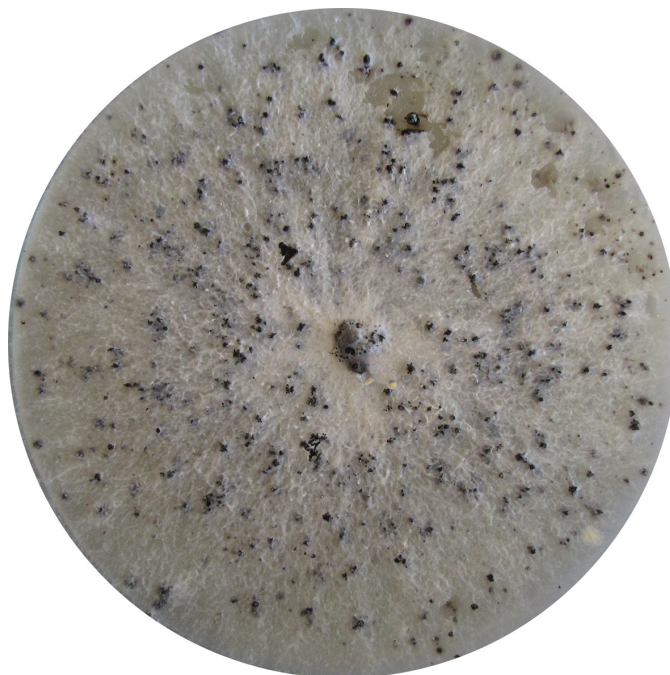


Fig. 2. *Diaporthe phaseolorum* MF 16-010 isolate on PSA on the 14th day of growth.

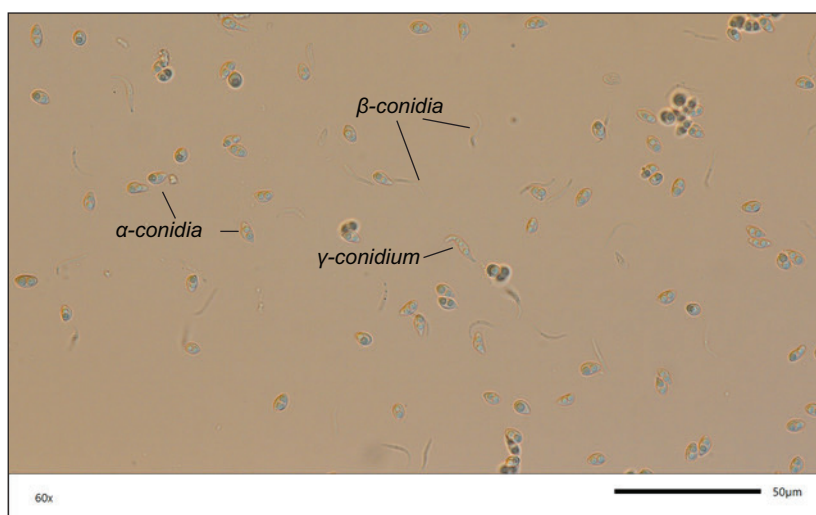


Fig. 3. Conidia of the isolate *Diaporthe phaseolorum* MF 16-010 on PSA on the 14th day of growth.

of infected sunflower and *Diaporthe* strains isolated from sunflower, it was shown that at least 14 *Diaporthe* species – not only one species as it was believed earlier – can affect this plant: *D. ambigua* [21], *D. goulteri* [19], *D. guilyae* [19, 22, 23, 24, 25, 26], *D. helianthi* [19, 22, 23, 24, 25, 26, 27], *D. kochmanii* [19], *D. kongii* [19, 26], *D. masirevicii* [26], *D. miriciae* [26], *D. novem* [28], *D. sackstonii* [26], *D. serafinae* [26], *D. sojae* [22], *D. stewartii* [23, 29], and *D. phaseolorum* [1].

The isolate of *Diaporthe* sp. MF 16-010 obtained from an infected sunflower stem collected in the Krasnodar region of the Russian Federation was identified as a *Diaporthe phaseolorum* according to molecular phylogenetic data. This conclusion is based on the fact that according to all four phylograms – for the ITS locus, β-tubulin gene, and EF-1α gene as well as the combined

phylogram – *Diaporthe* sp. isolate MF 16-010 formed one clade with the reference strain *D. phaseolorum* with the maximum value of bootstrap support. In the pure culture on PSA, this isolate formed only asexual reproduction structures – pycnidia, containing three types of conidia: α, β, and γ, that fit the taxonomical characteristic for this genus. After the artificial inoculation of sunflower with this isolate, it was shown that MF 16-010 is pathogenic for this plant.

According to the published data, the species *D. phaseolorum* was also found on plants of the following families *Fabaceae*: *Glycine max* [27], *Phaseolus vulgaris* [22], *Euphorbiaceae*: *Caperonia palustris* [27], *Cactaceae*: *Hylocerus undatus* [8], *Solanaceae*: *Lycopersicon esculentum* (Gurkina TA, First report of the fungus *Phomopsis phaseoli* on tomato, International scientific conference of students

and young scientists “Lomonosov-2018”, April, 2018), *Asteraceae: Helianthus annuus* [1], *Olearia cf. rani*, *Aster exilis* [23]. In the case of epiphytotic development, this phytopathogen can cause significant crop losses, for example for soybeans – up to 70-100% [29]. It is known that this species can also cause mycoses of immunocompromised people [30].

Therefore, to the best of our knowledge, this is the first report of *Diaporthe phaseolorum*, associated with sunflower in Russia that was confirmed by molecular phylogenetic data.

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CONFLICT OF INTERESTS

The authors declare no commercial or financial conflict of interest.

CITATION

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