Diaporthe species infecting sunflower in Russia

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Abstract. *Diaporthe* is an important group of plant pathogenic fungi revealed all over the world. Early classification and species identification of this genus was mostly based on combination of morphological characteristics, cultural features, and affiliation with a host plant. According to recent investigations, valid distinction between *Diaporthe* species should have combined molecular techniques, morphological and cultural observations, and mating type data. In Russia a comprehensive and extensive analysis of biodiversity and geographic distribution of *Diaporthe* species infecting sunflower has not been performed. There were seven *Diaporthe* sp. strains isolated from this plant maintained in the Laboratory of Mycology and Phytopathology of All-Russian Institute of Plant Protection. In previous study a strain from Krasnodar region, based on combination of molecular and morphological features was identified as *Diaporthe phaseolorum*. The aim of this study was to identify all other strains using primarily molecular phylogenetic approach and traditional morphological analysis. The strains were identified as *Diaporthe gulyae*, *Diaporthe eres*, and *Diaporthe helianthi*. Two species - *D. gulyae* and *D. eres* are found for the first time on sunflower in Russia. Detection of *D. helianthi* is the first report of this fungus in Russia as confirmed by molecular analysis.

1 Introduction

Fungal species belonging to *Diaporthe* Nitschke are the most common and widespread fungi worldwide. This genus is extremely large and species are known as saprotrophs, endophytes, predominately phytopathogens, causing leaf and stem spots in a broad range of economically important agricultural crops, including sunflower [2]. Phomopsis stem canker is ubiquitous in many sunflower-producing regions [3, 4, 5]. This disease is one of the primary limiting factors for sunflower production in Europe, where yield losses reached up to 50% and losses in oil content exceeded 10% [4].

The pathogen causing Phomopsis stem canker on Helianthus annuus was initially described as Diaporthe helianthi Munt.-Cvetk., Mihaljc. & M. Petrov (anamorph synonym – Phomopsis helianthi Munt.-Cvetk., Mihaljc. & M. Petrov) in the former Yugoslavia in 1980. All *Diaporthe* spp. isolated from infected sunflower were assumed to be *D. helianthi*. Although *D. helianthi* was described as the common causal agent, the possibility of multiple species infecting sunflower was raised in the early 1980s [6]. However, there was

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little evidence to support this hypothesis. At that time identification of *Diaporthe* spp. was based on morphological and cultural features and on association with a certain host [7].
Since the description of *D. helianthi,* recent investigations [5, 8-12], employing multi-locus molecular phylogenetic approach analyses of DNA sequence data of the ITS, β-tubulin (*TUB*), translation elongation factor-1α (*TEF*) genes resulted in resolving additional at least 12 Diaporthe species, which together with including D. helianthi could be harmfull for sunflower.
The study of the biodiversity and geography of the Diaporthe species associated with H. annuus in Russia according to actual taxonomy of the genus with introduction of molecular techniques just getting started. To the moment there is a single record, which has been verified by molecular phylogenetic features. This is report about *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. on sunflower in Krasnodar region [1]. The objective of this study was to correctly reidentify isolates of *Diaporthe* sp. from seeds and stems of sunflower originated from the Saint Petersburg, Belgorod and Stavropol regions.

2 Materials and methods

2.1 Isolates

Survey of 50 sunflower fields in 10 regions of Russia (Altay, Krasnodar, Stavropol territories, Belgorod, Lipetsk, Penza, Rostov, Tchelyabinsk, Tula regions and Saint Petersburg) has been conducted. Twenty-two samples of seeds derived from nine regions of Russia (Astrakhan, Orlyol, Belgorod, Tchelyabinsk, Tambov, and Volgograd regions as well as Altay, Krasnodar and Stavropol territories) has been analyzed. Six *Diaporthe* strains were collected from stems and seeds (table 1). To isolate a pure culture of fungus from the sunflower, fragments of material were surface sterilized with 20 ml of 2% sodium hypochlorite (NaClO) solution. After the surface sterilization, the samples were placed on potato sucrose agar (PSA) [13] 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 7-10th day of cultivation. Samples of infected stems were deposited in the Mycological Herbarium (LEP) of All-Russian Institute of Plant Protection (VIZR). All *Diaporthe* isolates were stored in plastic microtubes on PSA at + 4°C in the VIZR pure culture collection.

Table 1. Isolates used in this study.

Diaporthe species	Isolate ID	Isolation source	Origin	Date of collection
Diaporthe gulyae	MF-Ha17-042, MF-Ha17-043	seeds	Russia, Belgorod region (center of European part)	August 2017
Diaporthe eres	MF-Ha18-001, MF-Ha18-002	stems	Russia, Saint Petersburg (North West)	January 2018
Diaporthe helianthi	MF-DS1, MF-DS4	seeds	Russia, Stavropol territory (South of European part)	August 2018

2.2 DNA isolation, amplification and sequencing

Mycelium was obtained from cultures incubated on PSA and macerated with 0.3 mm glass sand on MM400 mixer mill (Retsch, Germany). Genomic DNA was extracted according to standard CTAB/chlorophorm method [14].
Strains were screened for three loci ITS region of rDNA, TUB and TEF. The ITS region was amplified with the primers pair ITS1F [15] and ITS4 [16], the partial TUB gene with βtub2Fw/βtub4Rd [17] and T1/T2 [18], and TEF with primers EF1-728F/EF1-986R [19], respectively. Amplicons were purified according to standard method [20]. The visualization and concentration measurement of purified PCR products were implemented by electrophoresis in 1% agarose gel stained with ethidium bromide and MassRuler 100 bp as a marker of concentration.
The resulting fragments were sequenced using the PCR primers together with Big Dye Terminator v3.1 Cycle Sequencing Kit (ABI, Foster City, USA) by Sanger's method (1977) [21] on ABIPrism 3500 (Applied Biosystems – Hitachi, Japan), according to the manufacturer's instructions.
Sequences were assembled using Vector NTI advance v. 11.0 (Invitrogen). Alignments of all consensus sequences, as well the reference sequences were generated with ClustalX 1.8 [22] and were improved manually when necessary. Sequences of reference *Diaporthe* strains and type species with *Diaporthe chamaeropis* (Cooke) R.R. Gomes, C. Glienke & Crous as an outgroup were obtained from GenBank, NCBI. The phylogenetic trees were conducted in RAxML (randomized accelerated maximum likelihood) software (v. 7.2.8) [23] by Maximum likelihood (ML) method including 1000 bootstrap replicates.

3 Results

In all phylogenetic trees inferred from ITS, *TUB*, and *TEF*, as well in combined trees, six studied *Diaporthe* sp. isolates clustered in order Diaporthales, family *Diaporthaceae* with the highest value of bootstrap support (Fig. 1). Two strains (MF-Ha18-001, MF-Ha18-002) in a combined tree, inferred from all three loci, formed one clade with reference strains of *D. eres* Nitschke species group, but they were outside the subclade, which contain the type culture of D. eres. Whereas in the phylogram, based on TUB and TEF genes these two strains joined together in one clade, closely related to that with type D. eres.
Two strains (MF-Ha17-042, MF-Ha17-043) in the one-gene phylogenetic trees, inferred from both ITS and TEF, as well in combined tree, based on these two loci with high values of bootstrap support (100%) clustered together with the reference strains of D. gulyae R.G. Shivas, S.M. Thompson & A.J. Young and D. stewartii A.L. Harrison. Both these Diaporthe sp. strains in the trees, inferred from TUB genes and three gene-based phylogenetic tree clustered in the clade with the reference strain of D. gulyae. Two strains (MF-DS1, MF-DS2) in all phylogenetic trees based on single loci as well in combined trees formed the same clade with type strain of *D. helianthi.*

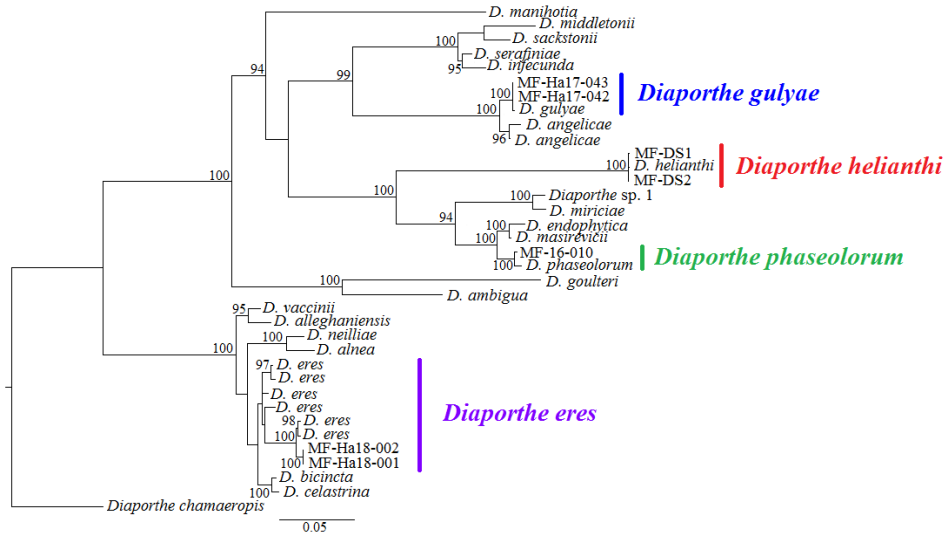

Fig.1. Maximum likelihood phylogenetic tree inferred from *TUB* and *TEF*.

4 Discussion

Currently it is widely known, that species identification in *Diaporthe* should be implemented using a polyphasic approach (Consolidated Species Concepts (CSC)) and based on phylogenetic, morphological, and biological characteristics. A multi-locus phylogenetical analysis should be based on combined DNA data matrix of ITS and partial sequences from TUB and TEF genes.

Six Russian Diaporthe strains isolated from sunflower based on molecular phylogenetic data were identified as D. gulyae, D. eres and D. helianthi. Diaporthe gulyae was found on sunflower seed originated from Belgorod region. Diaporthe eres was detected on sunflower stems from Saint Petersburg. And finally *D. helianthi*, that was previously treated as the only *Diaporthe* species ordinary infected sunflower in Russia, was found only in Stavropol territory. Findings of two species, *D. gulyae* and *D. eres*, are the first reports of these fungi on sunflower in Russia. Detection of *D. helianthi* confirmed by molecular phylogenetic analysis is the first valid identification of this species in the country. Previous reports of *D. helianthi* have been based only on morphological features or symptoms on sunflower stems and leaves.

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