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FIRST DETECTION OF FUNGUS *Fusarium coffeatum* IN THE TERRITORY OF THE RUSSIAN FEDERATION

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Abstract

According to the data of Rosstat, cereals and pulses took 17.2-20.2 % of agricultural production in Russia in 2014-2018, and their gross harvest in the same period was from 105.2 to 135.5 million tons. At the same time, the problem of contamination of grain and grain products by plant pathogens of different nature, including toxigenic Fusarium fungi, is still actual. Estimation of composition of species, affecting agricultural crops in different regions, is one on the key measures against *Fusarium*-induced infections spread. A complex investigation, which includes both traditional microbiological procedures and analysis of nucleotide sequences of marker genes followed by their comparison with reference ones from GenBank database, has been widely used for species-specific identification. This work is devoted to the description of the fungus of the genus *Fusarium* strain ION-3/4, isolated from wheat grain in Tula region of the Russian Federation (2014). DNA extraction and purification were performed by DNAeasy® Plant Pro Kit (Qiagen, Germany). Sanger sequencing of marker fragments of translation elongation factor 1 alpha (*TEF1α*, fragment size 587 b.p.) and RNA polymerase II subunit gene (*RPB2*, fragment size 689 b.p.) was carried out on an automated sequencer ABI PRISM 3730 (Applied Biosystems, USA). To analyze *TEF1α* and *RPB2* sequences, BLAST algorithm was used (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis and phylogenetic tree constructions were performed by MEGA-X software (https://www.megasoftware.net/) using maximum likelihood method and Kimura two-parameter model. Macromorphological characteristics of the isolate were studied on several culture media, micromorphology was studied using an Olympus CX33 microscope (Olympus Corporation, Japan). Complex of the results made it possible to identify the isolate — strain ION-3/4 as recently described *Fusarium coffeatum* species, belonging to the *F. incarnatum-equiseti* species complex. Phylogenetically the isolate (strain ION-3/4) formed a separate group with ex-type *F. coffeatum* strain 635.76. Key morphological characters (mycelium type, conidia shape and size, structure of mono- and polyphialides) also corresponded to the typical features of this species. As *F. coffeatum* is a member of *F. incarnatum-equiseti* species complex, which includes plant pathogens and mycotoxin producers, this species also can be considered as a potential cause of plant diseases and needs serious attention and further investigations.

Keywords: *Fusarium coffeatum*, *Fusarium incarnatum-equiseti* species complex, phylogenetic analysis, DNA-markers, morphology

Species identification of fungi of the genus *Fusarium*, especially in the case of closely related species, is often difficult due to the high similarity of key morphological structures. This can be confirmed by the fact that during the history of the study of the genus *Fusarium*, up to 10 taxonomic systems were proposed based on the analysis of morphological traits, in which the number of species ranged from 9 to 75 [1-4]. Molecular genetic methods based primarily on the sequencing of short marker DNA segments ("barcodes") [5, 6], phylogenetic analysis, and PCR identification are currently of great importance in resolving these problems.

Phylogenetic species recognition based on genealogical concordance (Genealogical Concordance Phylogenetic Species Recognition, GCPSR) are considered the most reliable method of molecular taxonomy [7-10]. This method is used to establish species boundaries and is based on multilocus phylogenetic analysis. The genes *TEF1α* (translation elongation factor 1 alpha gene) [11] and *RPB2* (RNA polymerase II subunit gene) [12] are mostly used as phylogenetically informative DNA markers for fungi of the genus *Fusarium*. Importantly, the molecular genetic methods cannot replace classical microbiological procedures, but should be a reasonable tool which significantly expands the arsenal of analytical capabilities of a researcher. Examples of a successful combination of microbiological and molecular approaches are works devoted to the detection of previously unidentified species *F. torulosum* [13] and *F. globosum* [14] in Russia.

Since the late 1990s, the use of multilocus analysis has allowed for deeper species differentiation, as a result of which many morphologically similar isolates (strains) of *Fusarium*, previously considered as representatives of the same species, were re-classified and assigned to a group or complex of species (species complex) [15]. One of the largest and most intensively studied is the *Fusarium incarnatum-equiseti* (FIESC) species complex [16] which currently includes more than 30 phylogenetic species of two clades, *F. equiseti* and *F. incarnatum* [17-20]. Researchers are interested in FIESC representatives due to their high genetic variability, ecological plasticity, and ability to synthesize a wide range of secondary metabolites, including mycotoxins [21].

In 2014, as part of the annual monitoring of the contamination of grain raw materials with mycotoxins and the study of their producers, carried out at the Federal Research Center for Nutrition and Biotechnology, fungus isolate was obtained from a wheat plant grown in the Tula region. The isolate was given the working name ION-3/4. A preliminary analysis of morphological features did not allow unambiguous determination of its species, which initiated subsequent studies using molecular genetic tools.

The purpose of this work is to determine the species status of the ION-3/4 isolate based on an integrated approach which includes the analysis of nucleotide sequences of marker genes and an extended study of cultural, macro- and micro-morphological properties.

Materials and methods. The *Fusarium* strain ION-3/4 was isolated during the grain mycological survey from the mycelium grown directly from the caryopsis. After successive passages, a monospore isolate was obtained. At present, the strain ION-3/4 is stored in the collection of microorganisms in the Laboratory of Biosafety and Nutrimicrobiome Analysis (Federal Research Center for Nutrition and Biosafety).

For molecular genetic studies and phylogenetic analysis, in addition to strain ION-3/4, we used strains of other morphologically similar species of the genus *Fusarium*: *F. equiseti* 64803 and *F. equiseti* 97001 (collection of the All-Russian Institute of Plant Protection, St. Petersburg—Pushkin), *F. incarnatum* F-2681 (collection of the Pushchino Scientific Center for Biological Research RAS) and *F. graminearum* F-892 (collection of the National Research Center Kurchatov Institute—GosNIIgenetika, Moscow), the species identity of which was confirmed in a previous study [22].

DNA from monospore cultures of fungi was isolated using a DNAeasy® Plant Pro Kit (Qiagen, Germany) according to the manufacturer's protocol. The DNA concentrations and purification from protein and low molecular weight impurities were determined (a NanoVue spectrophotometer, GE HealthCare, USA).

The design of universal primers for sequencing marker regions was carried out by aligning the nucleotide sequences of the *TEF1α* and *RPB2* genes of the

genus *Fusarium* fungi deposited in the database of the National Center for Biotechnology Information (GenBank, http://www.ncbi.nlm.nih.gov/GenBank). Algorithm ClustalW [23] was used for alignment. Calculation of the annealing temperature and evaluation of the physicochemical properties of oligonucleotides were performed using the Oligo.6.71 program (https://www.oligo.net/). As a result, of the following primers were designed: TEF30F — 5′-CGTCGTCATCGGCCA-CGT-3′, TEF650R — 5′-ACCAATGACRGTGACATAGTAGC-3′; RPB2F — 5'-ATGRTCMRCMGAGGYATGGAAGT-3', RPB2R — 5'- TTGTGATCG-GGGAADGGA-3′.

PCR was performed in a Tertsik amplifier (DNA-technology, Russia). For a pair of TEF30F-650R, the following amplification program was used: 93 °C, 90 s (1 cycle); 93 °C, 20 s, 64°C, 5 s, 67 °C, 5 s (5 cycles); 93 °C, 1 s, 64 °C, 5 s, 67 °C, 5 s (40 cycles); for a pair of RPB2F-2R, the program was as follows: 93 °C, 90 s (1 cycle); 93 °C, 5 s, 60 °C, 10 s, 72 °C, 5 s (45 cycles). A set of reagents for PCR was used, including Taq polymerase UP (5 units/μl, OOO DNA Technology, Russia). The composition of the PCR buffer and the concentrations of the components of the reaction mixture have been described previously [24].

PCR products were cloned into the pAL2-T plasmid vector using the Quick-TA kit (ZAO Eurogen, Russia) according to the manufacturer's protocol. Sequencing was performed by a modified Sanger method using a fluorescent label (an ABI PRISM 3730 automatic sequencer, Applied Biosystems, USA; sequencing was performed at ZAO Eurogen). The BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to analyze the marker sequences of the genes *TEF1*α and RPB2 and to compare these sequences with the NCBI database (https://www.ncbi.nlm.nih.gov/genome/).

Phylogenetic analysis was performed using the MEGA-X program [25] using the maximum likelihood (ML) method and Kimura's two-parameter model [26]. The reliability of the topologies of phylogenetic trees was confirmed by bootstrap analysis with 1000 repetitions. The analysis included the sequences of the *TEF1*α and *RPB2* genes of fungi of the genus *Fusarium* deposited in the NCBI database: *F. equiseti* NRRL 264 919 (GQ505599, GQ505777), *F. scirpi* NRRL 36478 (GQ505654, GQ505832), *F. compactum* NRRL 36323 (GQ505648, GQ505826), *F. lacertarum* NRRL 20434 (GQ505593, GQ505771), *F. incarnatum* CBS 132.73 (MN170476, MN170409). The sequences of the type strain *F. coffeatum* CBS 635.76 (MN120736, MN120755) were also used.

The macro- and micromorphological characteristics of the ION-3/4 monospore isolate were studied on several types of nutrient media: potato sucrose agar (PSA) [1], potato dextrose agar (PDA) (Himedia Laboratories Pvt Ltd., India; Neogen Corporation, USA), Czapek-Dox agar (CDA) (Himedia Laboratories Pvt Ltd., India), oatmeal agar (OA) (Difco Laboratories, Fisher Scientific, USA), carnation-leaf agar (CLA) [1] and Nirenberg medium (SNA) [3]. Macromorphological features (mycelium structure and pigmentation) were assessed on PSA, PDA, OA and CDA media on day 7 of growth, in darkness and variable lighting (16 h day/8 h night) at 5 °C, pigmentation changes were observed up to 3 weeks The horizontal growth rate was studied in culture on PDA, CDA, OA, and SNA media in Petri dishes at 20, 25, and 30 °C. The diameter of colonies on days 3, 5, and 7 was measured in two transverse directions for three inoculations (n = 3). Mean values (M) were calculated, the standard deviation for all measurements was 5.3%, calculations were performed using Microsoft Office Excel software. Micromorphological characteristics (shape and size of conidia, types of phialides) were studied on CLA medium at 25 °C on days 7-14 using an Olympus CX33 microscope (Olympus Corporation, Japan). Conidia sizes are presented as mean and smallest

to largest size ranges (min-max) in longitudinal and transverse directions obtained from at least 10 measurements made using ToupView software http://touptek.com/product/ showproduct.php?lang=en&id=103.

The morphological features of strain ION-3/4 were compared with descriptions of fungi of the genus *Fusarium* in guides [2, 3] and in publications [20, 27].

Results. Nucleotide sequences of two marker genes showed 100% (*TEF1*α) and 99.45% (*RPB2*) similarity of the analyzed fragments with the corresponding sequences of the type strain *F. coffeatum* CBS 635.76. A study of the topology of the phylogenetic trees presented in Figure 1 showed that strains ION-3/4 and CBS 635.76 form a separate cluster with bootstrap support for the *TEF1*α and *RPB2* genes of 100% and 99%, respectively. Importantly, the *F. coffeatum* cluster belonged to the phylogenetic clade *F. incarnatum*, which is consistent with the data published earlier [20, 27].

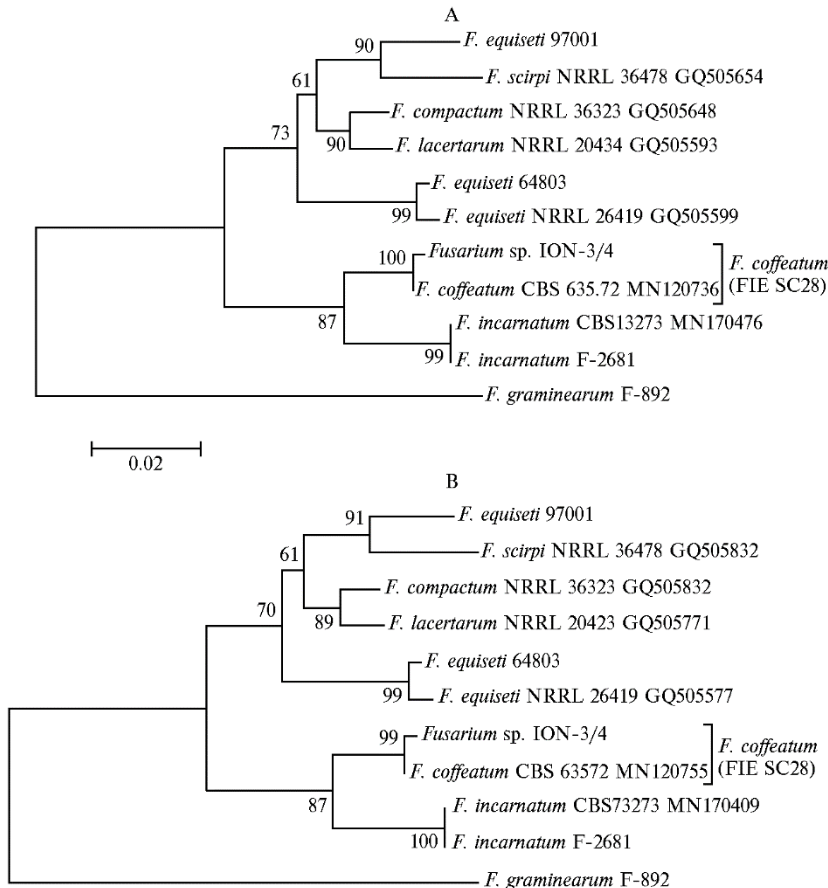

Fig. 1. Phylogenetic trees constructed based on the marker sequences of the *TEF1*α gene (A) and *RPB2* gene (B) of the ION-3/4 isolate and reference strains of seven species of fungi of the genus *Fusarium* using the maximum likelihood method (ML) and Kimura's two-parameter model [26] in program MEGA-X [25]. Only bootstrap values greater than 50% for 1000 replicates are shown. *F. graminearum* F-892 strain sequences are used as an "outgroup".

The macromorphological characteristics of strain ION-3/4 were studied in cultures on PSA, PDA, OA, and CDA nutrient media. On PSA and PDA, there was an abundant dense low aerial mycelium of milky white color with a slight creamy tint was noted, the reverse had a more saturated creamy peach color. As the culture aged (at more than 2 weeks of age), a more pronounced yellow color appeared, which then turned into light brown ("coffee with milk") (Fig. 2, A).

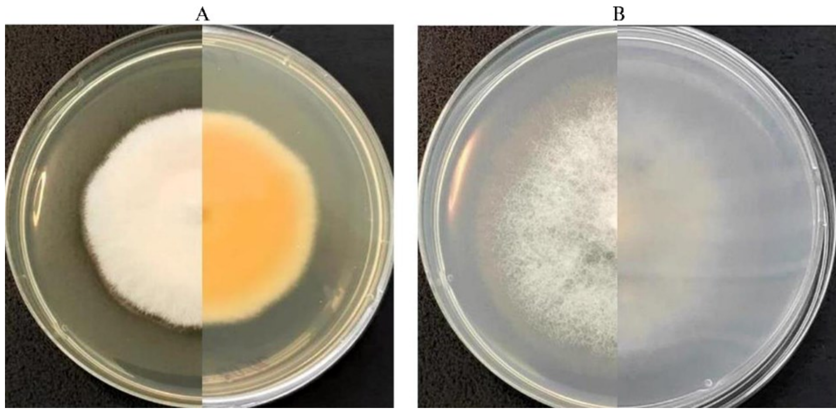

Fig. 2. ***Fusarium coffeatum*** **(strain ION-3/4), day 4 of culture, 25 °C. potato-sucrose agar** (PSA) (A) **and Czapek-Dox agar (**CDA) ***(B).***

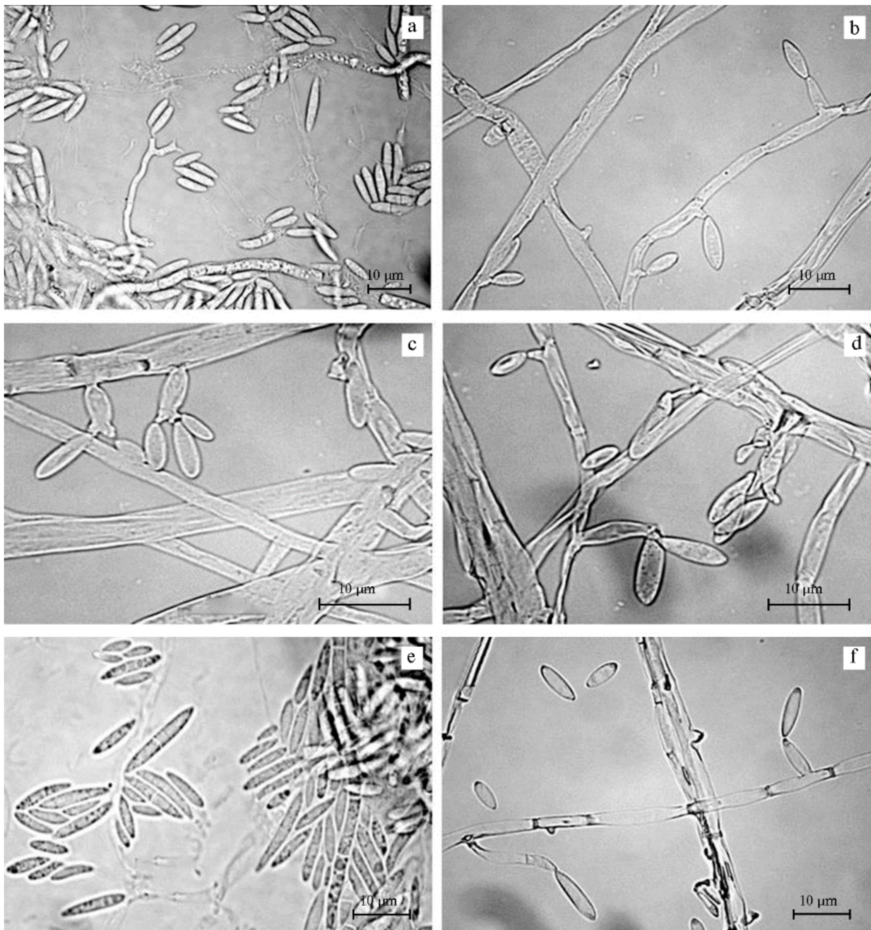

Fig. 3. ***Fusarium coffeatum*** **(strain ION-3/4) micro- and mesoconidia formed in aerial mycelium on mono- and polyphialides on carnation-leaf agar** (CLA)**: a, c — microconidia on polyphialides; b, f — microconidia on monophialides; d — microconidia on mono- and polyphialides; e — micro- and meso-conidia** (Olympus CX33 microscope, Olympus Corporation, Japan).

On potato extract-based nutrient media from different manufacturers, the color saturation of the reverse varied. A lighter reverse was observed during growth on PSA prepared under laboratory conditions from potato broth according to recipe [2], the most intense bright yellow-orange color of the colony was on PDA

medium manufactured by Himedia Laboratories Pvt Ltd. Growing under conditions of variable illumination (16 h day/8 h night) on PSA and PDA media led to the formation of a more saturated colors of the reverse, in contrast to conditions without light. The aerial mycelium always remained milky white regardless of the light conditions. When grown on OA medium, aerial mycelium was less dense, and on CDA it had a loose, flaky structure, less developed than on PDA. The color of aerial mycelium and reverse on OA and CDA remained milky white (see Fig. 2, B).

Micromorphological structures were evaluated on CLA at 25 °C on days 7-14. In aerial mycelium, the culture formed abundant micro- and mesoconidia (Fig. 3), macroconidia were rare. The sizes of conidia are shown in Table 1. The formation of sporodochia was not observed.

1. Size (μm) **of *Fusarium coffeatum* (strain ION-3/4) conidia on carnation-leaf agar** (CLA) (n = 10)

Type	Length (L), average (min-max)	Width (W), average (min-max)	L/W
Microconidia	6.7 (6.0-7.4)	1.6 (1.0-2.1)	4.2
Mesoconidia :			
without a septum	15.7 (8.6-16.0)	2.7 (2.3-3.1)	5.8
with one septum	15.2 (9.4-21.0)	2.4 (2.0-2.7)	6.3
Macroconidia (with two septa)	24.5 (24.0-25.0)	3.8 (3.5-4.0)	7.3

Macroconidia formed on CLA, mostly with two septa, almost straight, with a slight curve on the dorsal side, the terminal cells had a weakly expressed shape (papilla-shaped in the apical part, foot-shaped in the basal part).

In the aerial mycelium, there was an abundance of spindle-shaped mesoconidia without a septum or with one septum (see Fig. 3, e), as well as oval or obovate microconidia (see Fig. 3, a-d, f) formed in young cultures on monophialides (see Fig. 3, b, f), and as it grows, also on polyphialides (see Fig. 3, a, c, d). Polyphialids had two or more loci for the formation of conidia. The size of the phialides varied from 6 to 60 μm (see Fig. 3, a-d, f). Chlamydospores were not found.

When compared to the morphologically similar species *F. chlamydosporum* Wollenweber & Reinking [3], the *F. coffeatum* isolate (strain ION-3/4) shows similarities in the reduction or absence of formation of sporodochia and macroconidia in the form of conidiogenic cells (mono- and polyphialids), in the abundance and shape of microconidia, the formation of white aerial mycelium, however, ION-3/4 cultures lack the pink and burgundy color of the reverse.

In terms of the abundance of straight spindle-shaped mesoconidia in the aerial mycelium on mono- and polyphialides, the isolate of *F. coffeatum* (strain ION-3/4) is similar to *F. semitectum* Berkeley & Ravenel (syn. *F. incarnatum*) [3], the *F. coffeatum* (strain ION-3/4) and *F. semitectum* also show similarity in a reduced formation or the absence of chlamydospores, which, like the formation of sporodochia, is a strain-specific trait. However, unlike *F. semitectum*, the isolate *F. coffeatum* (strain ION-3/4) produces abundant microconidia in young cultures, while in *F. semitectum* in old cultures [3], and beige or brown color of the reverse appears only in the old culture.

The obtained characteristics for the studied isolate (strain ION-3/4) are consistent with the description of a typical strain of *F. coffeatum* (CBS 635.76) given earlier [27]. This concerns, first of all, micromorphological characteristics (shape and size of conidia formed in aerial mycelium, structure of mono- and polyphialids, and absence of sporodochia on CLA). The strain *F. coffeatum* (CBS 635.76) lost the ability to produce pigments (from beige to coffee-brown) on PDA and OA media, and there were no sporodochia on CLA, which, according to the

authors, is a consequence of its degeneration. In the isolate of *F. coffeatum* obtained by us (strain ION-3/4), as described above, the pigment is produced on PDA media, but the intensity of pigmentation varies on media from different manufacturers. There is currently no more detailed description of *F. coffeatum* in scientific publications.

The growth rate of the ION-3/4 strain culture was studied for 7 days when cultured in Petri dishes on four types of nutrient media at 20, 25, and 30 °C (Table 2).

2. Radial growth of *Fusarium coffeatum* (strain ION-3/4) colonies (mm) depending on various nutrient media at different temperatures (n = 3, M±5.3 %)

Days	PDA			CDA			OA			SNA		
	20 °C	25 °C	30°C	20 °C	20 °C	30 °C	20 °C	25 °C	30 °C	20 °C	25 °C	30 °C
3	22	35	23	30	40	35	18	32	23	23	30	17
5	50	65	52	60	77	72	45	72	60	46	53	40
7	75	86	75	80	86	86	67	86	70	64	80	60

N o t e. PDA — potato dextrose agar (Himedia Laboratories Pvt Ltd., India), CDA — Czapek-Dox agar (Himedia Laboratories Pvt Ltd., India), OA — oatmeal agar (Difco Laboratories, Fisher Scientific, USA), and Nirenberg medium (SNA) [3].

The highest growth rate occurred at 25 °C. On day 7, the colonies reached their maximum size (86 mm on PDA, CDA, OA, 80 mm on SNA). At 20 °C and 30 °C, the culture grew much more slowly on PDA, OA, and SNA, while on CDA, the colony size at 30 °C was the same as at 25 °C, and at 20 °C it was somewhat smaller. Thus, it was found that the temperature of 25 °C is the most favorable for the growth of the *F. coffeatum* isolate (strain ION-3/4) on all types of nutrient media used.

Thus, the main result of this study is the first detection of a *Fusarium coffeatum* fungus strain in Russia and a detailed description of its phenotypic characteristics. According to GenBank (NCBI), so far monospore strains of *F. coffeatum* have been isolarted in Australia (FIESC28_10703), South Africa (type strain CBS 635.76), and Romania (CBS 430.81). Based on the data obtained, it can be noted that this species is cosmopolitan and has a wide ecological plasticity, which allows it to exist in various climatic conditions. The discovery of *F. coffeatum* expands the understanding of the species diversity of fungi of the genus *Fusarium* in the Russian Federation and adds new knowledge about the area of this species. However, it is not yet clear whether *F. coffeatum* is capable of infecting crops and synthesizing mycotoxins. The study of these aspects may be the goal of future research.

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