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MOLECULAR GENETIC APPROACHES FOR INVESTIGATION OF TAXONOMY AND SPECIFIC IDENTIFICATION OF TOXIN- PRODUCING *Fusarium* SPECIES: ACHIEVEMENTS AND PROBLEMS (review)

A.A. STAKHEEV¹, L.V. SAMOKHVALOVA¹, D.Yu. RYAZANTSEV¹, S.K. ZAVRIEV^{1, 2}

¹M.M. Shemyakin and Yu.A. Ovchinnikov Institute of Bioorganic Chemistry, Federal Agency of Scientific Organizations, 16/10, ul. Miklukho-Maklaya, Moscow, 117997 Russia, e-mail samokhlar@mail.ru, d.yu.ryazantsev@gmail.com, stakheev.aa@gmail.com;

²All-Russian Research Institute of Phytopathology, Federal Agency of Scientific Organizations, 5, ul. Institute, pos. Bol'shie Vyazemy, Odintsovskii Region, Moscow Province, 143050 Russia, e-mail szavriev@ibch.ru

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Abstract

Ascomycetous fungi of the genus *Fusarium*, which was separated as a single taxonomic group in 1809 by Heinrich Friedrich Link, are distributed worldwide and have different ecological niches. They have been found in soil and plants as endophytes, saprotrophs and parasites. Infections of agricultural plants, caused by *Fusarium* fungi, lead to annual damage, estimated in hundred million dollars. In addition to decrease harvest quality and quantity, *Fusarium* fungi are able to produce toxic metabolites (fusariotoxins). The consumption of fusariotoxin-contaminated winter grain led to deaths of tens of thousands of people in Volga and Ural regions in 1930s-1940s. These factors make necessary investigation of morphology, biochemistry and genetics of those organisms. Nowadays there is no universal taxonomic system of the genus *Fusarium*, so it is not possible to identify an isolate to species level in many cases. The high variability of morphological structures, and, on the other side, their similarity for closely related species, are major problems for researchers which use classical methods for identification and systematization of this group of fungi. Today, the molecular methods, based on the use of specific DNA sequence, are playing an increasingly important role in *Fusarium* systematics. The application of this approach has led to establishment of a number of new species in the genus. T. Yli-Mattila et al. (2009, 2011) used multilocus phylogenetic analysis to describe the novel species *F. sibiricum* and *F. ussurianum* which were found in Siberia and Far East and proved to be morphologically similar to *F. graminearum* and *F. langsethiae*, respectively. The authors of this paper identified the species *F. torulosum* (morphologically similar to *F. avenaceum*) in Russia for the first time based on the use of DNA markers. It is no less important that the analysis of inter- and intraspecific polymorphism makes possible the development of highly-specific assays for molecular detection of *Fusarium* fungi, including ones which infect plants and produce mycotoxins. The use of these assays (for example, ones described by M. Nicolaisen et al., 2009 and A.A. Stakheev et al., 2011) allows not only to detect the contamination of plants by mycotoxin producers, but also to estimate the quantity of their content in a probe. At the same time, a lot of questions are still unanswered: particularly, simultaneous application of different species concepts (morphological, biological and phylogenetic) makes it difficult to develop universal taxonomic system of the genus; also the most informative and reliable marker, which would reflect the evolutionary history of the genus, has not been found. The main achievements and problems of *Fusarium* phylogenetics, and perspectives of the application of molecular approach for studies of this group of organisms are discussed in this review.

Keywords: genus *Fusarium*, taxonomy, DNA marker, mycotoxins, phylogenetic analysis

The genus *Fusarium* was separated as a taxonomic group in 1809 by Heinrich Friedrich Link [1, 2]. Since then, many attempts to create a single universal system of the genus has been made, but they have not been successful to date. For a long time the genus *Fusarium* taxonomy was rather chaotic, and at a certain stage it included more than 1000 species. In many ways, this was due to the mistaken belief that each fungus species affects a single host plant species

[3], so that each new isolate could be classified as a separate species. The number of *Fusarium* species in various taxonomic systems varied widely and ranged from 9 [4] to 78 [5], thereby demonstrating problems in classification of this group of organisms, which are largely due to the high morphological variability that impedes species-specific criteria and, as a result, makes the taxonomic status of many members of the genus *Fusarium* controversial [6]. In addition, the accumulating data further complicate the interpretation. For example, it was found that fusiform to sickle-shaped conidia that has been used by H.F. Link to separate *Fusarium* as a genus, could not be considered strictly typical for this group. Besides, it is revealed that the morphologically similar species can differ significantly on both the genetic and metabolic levels. To date it is apparent that the identification based solely on the comparison of morphological data, often is not quite correct. Last but not least, this is due to the so-called human factor, since not all researchers have the necessary skills and experience to deal with fungi. All these necessitate modern molecular methods to study taxonomy and diversity of *Fusarium* fungi, as well as new approaches for rapid and correct diagnosis of plant infection by these dangerous pathogens [3, 6].

To date, morphological, biological and phylogenetic species concepts are three main ones. Morphological concept is based on the description of shape and size of macroconidia, microconidia and chlamydospores, and the type of conidiogenous cells, with pigments and secondary metabolites also taken into consideration. Biological concept is based primarily on crossbreeding ability. As to the genus *Fusarium*, this approach is limited by the fact that a significant number of the representatives do not have a sexual stage in the life cycle and reproduce only asexually [7-9]. It should be noted that the biological concept, when applicable, allows for most accurate defying species boundaries due to unequivocal data on whether the strains can or can not mate with each other and produce fertile offspring [3, 10]. The study of complex species *Gibberella fujikuroi* (*Fusarium fujikuroi* as asexual stage), which included nine species identified is an example of the biological concept [11-13]. The basis of the phylogenetic species concept is a comparative analysis of the DNA nucleotide sequences that allows to find out the relationship of taxa and clarify their evolution [14]. The approaches used to study the genetic polymorphism of different taxonomic groups are based either on arbitrary DNA labeling, such as random amplified polymorphic DNA (RAPD) [15, 16], inter simple sequence repeats (ISSR) [17], and amplification fragments lengths polymorphism (AFLP) [18, 19] methods, or on polymorphism analysis based on sequence characterized amplified regions (SCAR) markers [3, 6, 20]. Among the significant disadvantages of the first approach are low reproducibility of the experimental results (particularly with respect to RAPD), and high DNA purity and concentration necessary to ensure accuracy. SCAR-markers are currently considered as the most reliable tools for genetic marking, which allow, in most cases, for unique identification of organisms by a characteristic sequence of one or more DNA loci. In phylogenetic approach the concept of cryptic species is often used, which is separated as a single taxon solely on the genetic differences [21, 22]. Genealogical concordance phylogenetic species recognition (GCPSR) is used to establish the boundaries of species in a multilocus phylogenetic analysis [23]. The most important task of the phylogenetic approach is to find a universal marker (DNA barcode) which 'would allow to characterize the taxonomic status of the representatives of a broad group of organisms [24]. Gene encoding subunit 1 of mitochondrial cytochrome C-oxidase can be an example of the barcode for animal kingdom (*Animalia* Linnaeus, 1758). At the same time this marker is poorly applicable to the kingdom Fungi [*Fungi* (L., 1753) R.T. Moore, 1980], although it was used successfully for

taxonomic studies of the genus *Penicillium*. Most researchers believe the internal transcribed spacers of ribosomal DNA (*ITS*) most phylogenetically informative marker for fungi [25]. However, some data shows that phylogenetic analysis of these loci does not always objectively reflects the evolution and taxonomic relationships among species for the genus *Fusarium*. One of the reasons is the fact that in many species (e.g., in *Fusarium fujikuroi* complex) the *ITS2* spacer presents in two nonorthologous copies [3, 26]. Thus, the search for highly informative polymorphic markers that would uniquely identify the taxonomic status of the *Fusarium* genus remains an urgent task [27–29].

A significant number of reports on genetic polymorphism in *Fusarium* fungi with analysis of several genes encoding proteins and ribosomal RNA sequences have been published in the last 10–15 years. These are genes for α translation elongation factor (*tef1 α*) [30], β -tubulin (*β -tub*) [31], phosphate permease (*PHO*) [32, 33], sterol 14-demethylase (*CYP51C*) [19], aminoadipate reductase (*lys2*) [20, 21], intergenic spacers (*IGS*) of rRNA genes [22], and a number of genes involved in cascade reactions of chemical synthesis. Due to high inter- and intraspecific polymorphism they can be successfully used in phylogenetic studies of the genus. Currently, the *tef1 α* gene, which is the most similar to the DNA barcode is considered the most informative loci for phylogenetic analysis of the genus *Fusarium*. A database FUSARIUM-ID v. 1.0 [23] composed of nucleotide sequence of the *tef1 α* gene fragments in many cases contributes to faster and easier identification of unknown strains. However, incomplete sequences for some species in the database, and not always correct initial identification of strains lead to a confusion when associating the sequence with a particular species.

Certain difficulties in multilocus phylogenetic analysis are due to differences in the evolutionary history of different genes. An example is phosphate permease gene (*PHO*), which was used for the taxonomic studies of several groups of *Fusarium* pathogens. Thus, J.B. Scott and S. Chakraborty [40] have successfully used it as a marker to classify *F. pseudograminearum* as a separate species. Later *PHO* gene was used in four-locus phylogeny analysis [33] which allows to distinguish a number of isolates from Siberia and the Far East, originally identified as *F. poae*, as a separate species *F. sibiricum*. At the same time *PHO* gene is polymorphic enough to clearly distinguish *F. culmorum* from the closely related *F. cerealis* [41]. We used *PHO* as a marker for intra- and inter-specific polymorphism study in *F. avenaceum*, *F. tricinctum*, *F. acuminatum*, *F. torulosum*, producing toxins of enniatin group and moniliformin [32]. It has been shown that for this group of species *PHO* has a higher variability than the «classical» markers such as *tef1 α* , *β -tub*, or enniatin synthetase (*Esyn1*) genes. Phylogenetic analysis of *F. avenaceum* strains from different regions of Russia using the *PHO* gene sequences showed four of them to form an independent cluster phylogenetically closer to *F. torulosum*, than to *F. avenaceum*, which is conventionally called *F. avenaceum*-V. Perhaps this group of strains can be treated as a separate phylogenetic species, but this fact has not been confirmed in the analysis of other markers and requires further study. At the same time, we have not found a correlation between the phylogeny of the strains and their division into conventional groups I and II proposed by T. Yli-Mattila et al. [31, 38]. Moreover, these markers did not allow to identify *F. arthrosporioides* strain as a separate taxa other than *F. avenaceum*, thereby confirming the hypothesis that *F. arthrosporioides* is not a separate species. Amino adipate reductase gene (*lys2*) is another example of a different evolutionary history. The *lys2* gene has been shown to be phylogenetically more informative than the number of traditional markers, but the topology of the phylogenetic tree constructed on the basis of comparative

analysis of *lys2* sequences differed significantly from that obtained from other genes [35, 36]. A possible solution is to use a multilocus analysis for several genes to smooth down the possible differences in the evolution and polymorphism of the loci and get a picture as close to the objective as possible [42, 43].

Despite all the difficulties, the use of molecular markers have significantly contributed to biology and taxonomy of *Fusarium* fungi. New taxonomic groups revealed within the genus, such as *F. sibiricum*, *F. pseudograminearum*, and *F. ussurianum* [44], are the examples. Furthermore, DNA-markers allowed distinguishing nine phylogenetic species in *F. graminearum* [45]. Due to *tef1α* gene sequence phylogenetic analysis, northern European and southern European groups have been clustered within *F. equiseti* [46]. Also new taxonomic groups have been found in the *F. fujikuroi* and *F. solani* complexes [3].

Due to study of genetic polymorphism in the Russian population of *Fusarium* pathogens and development of the appropriate markers we have identified the species *F. torulosum*, morphologically similar to *F. avenaceum*, in Russia for the first time [47]. Also species identification was verified for *Fusarium* strains from the Russian National Collections, including All-Russian Collection of Microorganisms (G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms RAS, Pushchino), Collection of All-Russian Institute of Plant Protection (St. Petersburg) and Collection of All-Russian Research Institute of Phytopathology (Moscow Province). Molecular approach made it possible to evaluate the relationship between species, which was non-obvious when comparing morphological features. An example is *F. tricinctum*, traditionally regarded as a closely related to *F. sporotrichioides* and *F. poae* species and attributable to *Sporotrichiella* section [48-50]. However, *F. tricinctum* can produce enniatins and moniliformin, the toxic metabolites, that distinguishes it from *F. sporotrichioides* and *F. poae*, secreting mainly trichothecene mycotoxins. On this criterion *F. tricinctum* is close to *F. avenaceum* and *F. acuminatum*, attributable to *Roseum* and *Gibbosum* sections, respectively [51]. Phylogenetic analysis from multiple genes confirmed the close relationship between *F. tricinctum*, *F. avenaceum* and *F. acuminatum*.

The figure shows a dendrogram based on the phylogeny analysis of *tef1α* gene sequenced and described by the authors of this article for 14 *Fusarium* species from the Russian National Collection, and those deposited in GenBank NCBI (accession numbers listed in the Table). Noteworthy, the species are clustered in strict accordance with their mycotoxin patterns, i.e. A trichothecenes producers (*F. sporotrichioides*, *F. langsethiae*), B trichothecenes producers (*F. graminearum*, *F. culmorum*, *F. cerealis*, and those predominantly synthesizing B trichothecenes, such as *F. poae*, *F. venenatum*, *F. sambucinum*), and enniatins and moniliformin producers (*F. avenaceum*, *F. tricinctum*, *F. acuminatum*, *F. torulosum*).

***Fusarium* strains from the Russian National Collection used in phylogenetic studies**

Strain	Species	Origin	Host plant
96800 ¹	<i>F. acuminatum</i>	North Ossetia	Wheat
CM-9866 ²	<i>F. avenaceum</i>	Smolensk Province	Wheat
64722 ¹	<i>F. cerealis</i>	Хабаровский край	Wheat
74007 ¹	<i>F. culmorum</i>	Arkhangelsk Province	Potato
97001 ¹	<i>F. equiseti</i>	North Ossetia	Wheat
58033 ¹	<i>F. graminearum</i>	Leningradskaya Province	Barley
82901 ¹	<i>F. langsethiae</i>	Orel Province	Oats
61701 ¹	<i>F. poae</i>	Saratov Province	Wheat
F-842 ³	<i>F. sambucinum</i>	Bekaya Tserkov', Ukraine	Potato
HTiMi ²	<i>F. solani</i>	Unknown	Unknown
BR-04-60 ²	<i>F. sporotrichioides</i>	Bryansk Province	Wheat
90604 ¹	<i>F. torulosum</i>	Leningradskaya Province	Barley
30141 ¹	<i>F. tricinctum</i>	Finland	Barley
58242 ¹	<i>F. venenatum</i>	Germany	Unknown

Note. 1, 2 and 3 — collections of All-Russian Institute of Plant Protection (St. Petersburg), of All-Russian Research Institute of Phytopathology (Moscow Province) and All-Russian Collection of Microorganisms (G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms RAS, Pushchino), respectively.

Dendrogram based on *tefla* sequences for 14 Russian *Fusarium* strains from the Russian National Collection and those deposited in GenBank (NCBI). Neighbor joining algorithm (MEGA5) applied, with bootstrap support over 50 %. For deposited sequences the accession numbers are indicated. One, two and three asterisks mark strains from Collection of All-Russian Institute of Plant Protection (St. Petersburg), Collection of All-Russian Research Institute of Phytopathology (Moscow Province) and All-Russian Collection of Microorganisms (G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms RAS, Pushchino), respectively.

Data on interspecies polymorphism of specific DNA loci facilitates the development highly specific detection system for *Fusarium* pathogens based on classical PCR or its modification [52-61]. Routine application of these systems provides rapid and accurate identification of a pathogen at different levels (species, subspecies or species groups depending on the task), and hence allows to predict the potentially accumulated mycotoxins. RT-PCR [62] allows to determine the pathogen DNA amounts, thereby tracking the infection development and the fungicide effectiveness, and also to carry out regular monitoring of the *Fusarium* infestation (e.g., in grain and by-products). L. Niessen et al. [63] suggested a good alternative to PCR. They used a loop-mediated isothermal amplification (LAMP) method to detect *F. tricinctum* in pure cultures and grain [64]. Its sensitivity is comparable to that of the classical PCR (0.95 pg of target DNA per reaction), but cross-reactions were observed with the DNA of *F. acuminatum* strains, that is, this method was not completely specific. Nevertheless, it may be promising compared to the PCR as does not require sophisticated and expensive equipment.

So, despite challenges, molecular genetic approaches have good pro-

spects and potential for solving problems in the taxonomy of the genus *Fusarium*. Nevertheless, these techniques should not be considered «means of escape» from the difficulties in *Fusarium* classification. Phylogenetic methods used without studying morphology, strain crossbreeding and metabolism can only complicate the problem. Only relevant combination of several approaches will provide valid and reliable data on the basis of which a relatively universal taxonomic system can be created to satisfy professionals in one way or another involved in studying this group of plant pathogens.

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