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Review

# Molecular Marker-Based Identification and Genetic Diversity Evaluation of *Fusarium* spp. - A Review

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**Abstract**

The *Fusarium* genus encompasses filamentous fungi of global significance in agriculture, ecology, and the food industry. Certain members of this genus act as plant pathogens, leading to substantial fungal infections in crucial crops, especially cereals. Many *Fusarium* species produce mycotoxins that are species or even strain-specific, contaminating the food chain and posing risks to both animal and human health. Identifying *Fusarium* species remains a formidable challenge due to the dynamic taxonomy within the genus. Traditional identification relies on biological, phylogenetic, and morphological criteria. However, modern genomic analysis techniques have revolutionized species identification, including that of *Fusarium* spp. Established molecular markers like the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA-ITS1), Translation elongation factor 1-α (*TEF1-α*), Intergenic spacer region of rDNA (ISG), and Beta-tubulin (β-tub) facilitate precise genetic identification. The "DNA barcoding" method, recognized in scientific literature, offers a meticulous approach to molecular identification and taxonomic classification of *Fusarium* species. Integrating traditional identification methods with contemporary DNA analysis techniques broadens the possibilities for discerning and establishing phylogenetic relationships among closely related mycotoxigenic species. This integrated approach greatly aids in the development of effective control and management strategies.

**Keywords**: *Fusarium* spp., genetic diversity, molecular markers, identification

**Резюме**

Родът *Fusarium* обхваща филаментозни гъби с глобално значение в селското стопанство, екологията и хранително-вкусовата промишленост. Част от членовете на този род са растителни патогени, причиняващи гъбични инфекции в ключови зърнени култури. Много видове *Fusarium* spp. продуцират микотоксини, които са видово- или дори щамово-специфични, контаминират хранителната верига и представляват риск за здравето на животните и хората. Идентифицирането на видовете от род *Fusarium* остава огромно предизвикателство, поради динамично променящата се таксономия в рамките на рода. Традиционната идентификация се основава на биологични, филогенетични и морфологични критерии. Същевременно развитието на съвременните молекулярни техники за геномен анализ революционизират видовата идентификация, включително и на представителите на род *Fusarium*. Установени са молекулярни маркери като вътрешно транскрибиран спейсърен регион (ITS) кодиращ ядрено рибозомална ДНК (nrDNA-ITS1), фактор на удължаване на транслацията 1-α (*TEF1-α*), междугенна спейсърна област на rDNA (ISG) и бета-тубулин кодиращ ген (β-*tub*), които улесняват прецизната генетична идентификация. В допълнение, използваният метод за „ДНК баркодиране" е признат в научната литература като прецизен подход за молекулярна идентификация и таксономична класификация на видовете от род *Fusarium*. Интегрирането на традиционните методи за идентификация със съвременните техники за ДНК анализ, разширява възможностите за разпознаване и установяване на филогенетични връзки между тясно свързани микотоксикогенни видове.

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Въвеждането на подобен интегриран подход играе значителна роля при разработването на ефективни стратегии за контрол и управление на фузариумните инфекции и образуването на микотоксини.

## Introduction

The *Fusarium* genus, originally described as *Fusisporium* by Link in 1809, derives its name from the Latin word "spindle," owing to the distinctive spindle-shaped macronidia characterizing this genus. Taxonomically, it falls within the phylum *Ascomycota*, class *Ascomycetes*, and order Hypocreales (Ajma et al., 2023). Members of this genus are widely recognized as natural pathogens, endophytes, or saprobes, exhibiting versatility in ecological roles (Okungbowa and Shittu, 2012). Although *Fusarium* species are commonly associated with soil environments and root systems as saprophytes or parasites, they possess mechanisms for spore dispersal into the atmosphere, enabling colonization of aerial plant parts and causing various fungal infections (Abdel-Azeem *et al.,* 2019). The genetic structure of *Fusarium* spp., coupled with sexual stages in some species, contributes to the resilience of its ascospores in extreme conditions, such as high temperatures and high altitudes (Dusengemungu, 2021).

Statistics from the American Phytopathological Society reveal that 81 out of 101 economically significant plants suffer from at least one *Fusarium* disease (Babadoost, 2018). *Fusarium* species produce diverse secondary metabolites, known as mycotoxins, with varying toxigenic potential, categorized based on their chemical structures into different classes, including trichothecenes, fumonisins, zearalenone, eniatins, moniliformin, and bovericin. These mycotoxins adversely affect the quality of feeds and common food staples like wheat, barley, corn, and nuts (Ferrigo *et al*., 2016; Perincherry et al., 2019). Leading mycotoxin producers include *F. graminearum*, *F. culmorum, F. sporotrihioides, F. poae, F. equiseti*, *F. verticillioides*, and *F. proliferatum*, with the latter two being major contributors to fumonisin production (DSM, 2022; Perincherry *et. al.,* 2019). Some *Fusarium* species have the capacity to simultaneously produce multiple mycotoxins, which can accumulate to hazardous levels in grains and food products (Ferrigo *et al*., 2016; Majeed *et al*., 2018). Notably, *F. graminearum*, one of the most aggressive pathogens, generates mycotoxins like nivalenol (NIV), deoxynivalenol (DON), and zearalenone (ZEA). The presence of mycotoxins in food and feed poses significant health risks, including estrogenic, gastrointestinal, and renal disorders, as well as carcinogenic, mutagenic, and immuno suppressive effects (Awuchi *et al*., 2021).

*F. graminearum* stands as a primary causative agent of fusariosis, a disease affecting wheat, maize, and barley, with substantial global economic implications. It is classified by the journal Molecular Plant Pathology in 4th place among the most economically important fungal pathogens (Dean *et al*., 2012). Numerous crops face diseases like vascular wilt and stem rot instigated by members of the *F. oxysporum* species complex. Given the pathogenicity of many *Fusarium* species, accurate identification and study are imperative.

*Fusarium* spp. can infect plants at any growth stage, and the specific pathogen and plant type influence infection dynamics. Due to their mycotoxigenic potential and the varying impacts of misidentification on contamination risk assessment and management, precise identification is indispensable. This review focuses on modern molecular marker-based methods to evaluate genetic diversity and identify *Fusarium* spp.

## Methods for the identification of mold fungi

### *Traditional methods for the identification of mold fungi*

Identification of molds at the species level traditionally relies on the assessment of morphological traits, primarily focusing on structures generated through sexual (meiosis) and asexual (mitosis) reproduction (Raja *et al*., 2017). Notably, several authors, including Nirenberg, (1981); Nelson *et al*. (1983), and Marasas *et al*. (1984), have advocated the microscopic examination of specific morphological characteristics within isolated single-spore cultures cultivated on selective nutrient media for seven days as a means to achieve species-level identification.

*Fusarium* spp., for instance, typically produces both macroconidia and microconidia. Microconidia exhibit characteristics such as being one- or two-celled, hyaline, pyriform, fusiform to ovoid, and straight or curved (Nelson *et al*., 1994). On the other hand, macroconidia are hyaline, with two or more cells, featuring distinct transverse compartments and a species-specific pointed apical cell. The shape of macroconidia, ranging from banana-shaped to spindle-shaped or sickle-shaped, sometimes displays elongated apical and basal cells. When cultivated on Sabouraud agar at 25°C, *Fusar-*

*ium* spp. form colonies that are wavy, cottony, or flat (Cighir *et al.,* 2023). Additionally, *Fusarium* species are characterized by the color of their mycelium, which can vary from white and cream to tan, salmon, cinnamon, yellow, red, violet, pink, or purple. The color on the underside of the mycelium may also range from red and dark purple to brown, tan, or colorless, providing another important distinguishing feature (Okungbowa and Shittu, 2012; Infantino *et al*., 2023).

While the morphological approach is commonly employed in the field of taxonomy to determine fungal families or species, as well as to elucidate phenotypic traits in the context of evolutionary development (Wang *et al*., 2016), it has certain limitations. This method is time-consuming, demands extensive taxonomic knowledge, and can only be applied to viable mycelium. Furthermore, traditional identification methods may fail to distinguish between species like *F. graminearum*, *F. pseudograminearum*, and *F. crookwellense*. Therefore, to overcome these constraints, it is advisable to combine conventional strategies for the identification of microscopic fungi with DNA-sequencing-based methods (Wang *et al*., 2016).

*Molecular markers for the identification of Fusarium spp.*

Modern methods for molecular identification of filamentous fungi, using DNA marker technologies, have emerged as the new "gold standard" and a fundamental component of microbiological practice (Raza *et al.,* 2016). Various polymerase chain reaction-based (PCR) marker systems have been successfully developed and applied, including amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR), single-nucleotide polymorphisms (SNPs), and real-time PCR. Additionally, contemporary markers such as DNA barcoding, DNA microarrays, and pyrosequencing have been employed (Chandra *et al*., 2011). These methods target specific regions of the *Fusarium* genus genome, providing potential candidate genes for analyzing genetic diversity and phylogeny. This technological advancement represents a pivotal shift in the field, offering precise tools for the identification and classification of *Fusarium* species (van Dam *et al.,* 2018).

**Internal Transcribed Spacer region (nrDNA-ITS).** The utilization of molecular methods for mold species identification emerged following the discovery of the ribosomal operon, a functional unit within DNA housing a cluster of genes controlled by a single regulatory signal (Jacob *et al*., 1960). Primers for this operon were described by White *et al*. (1990). The operon includes three nuclear ribosomal genes: a large ribosomal unit (nrLSU-26S or 28S), a small subunit (nrSSU-18S), and a variable-sized internal transcriptional region acting as a spacer (ITS1, 5.8S, ITS2) (Tekpinar and Kalmer, 2019).

In 2011, the International Consortium of Mycologists in Amsterdam acknowledged the ITS region as the primary fungal barcode marker, chosen among six different regions due to its clear distinction between inter- and intraspecific variation (Schoch *et al*., 2012). While the ITS region lacks coding function, it plays a critical biological role in rRNA and ribosome maturation (Hausner and Wang, 2005). It offers advantages like simple amplification with universal primers complementary to ribosomal RNA gene sequences and high variability among mold species, making it a reliable identification tool (Gardes and Bruns, 1993; Nilsson *et al*., 2009; Blaalid *et al*., 2013).

The choice of ITS1 and ITS2 region length in *Fusarium* spp. depends on the applied primers. For example, using universal primers in some studies resulted in amplicons ranging from 550 to 570 bp (Abd-Elsalam *et al.,* 2003), while taxon-selective primers (ITS-Fu-f and ITS-Fu-r) designed for differentiating closely related fungal species yielded 398 bp products (Abd-Elsalam *et al*., 2003).

Data from 2000-2015 and 2010-2016 show a rising trend in the usage of the nrDNA-ITS region as a mold species identification marker (22% vs. 42%) (Raja *et al*., 2017). The ribosomal ITS region has facilitated comparisons of various species, such as *F. fujikuroi*, *F. napiforme*, *F. proliferatum*, establishing their identity with *F. oxysporum*. Based on ITS-RFLP analysis, Visentin *et al*. (2009) studied a collection of a total of one hundred strains of the genus *Fusarium* isolated from infected maize grains grown in northwestern parts of Italy, with different geographical origins and hosts. In conclusion, the authors suggest that the *Fusarium* ITS region should be identified as taxonomically informative in distinguishing between the morphologically similar maize pathogens *F. verticillioides* and *F. proliferatum*. Distinguishing between morphologically similar species like *F. verticillioides* and *F. proliferatum* has relied on the presence or absence of polyphialides. ITS-RFLP analysis confirmed the taxonomic informativeness of the ITS region for distinguishing between these maize pathogens (Visentin *et al*.,

2009).

Bulgarian wheat samples yielded *Fusarium* spp. identification via direct sequencing, demonstrating the potential of rDNA-ITS as an early diagnostic marker for *Fusarium* spp. infections (Gencheva and Beev, 2021). Predominant isolates included *F. tricinctum* and *F. poae*, with *F. proliferatum*, *F. graminearum*, and *F. equiseti* also detected, revealing low genetic distances (0.004) among *F. tricinctum* isolates (Gencheva and Beev, 2021).

While the ITS region may not consistently identify specific species from *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, and *Trichoderma* genera due to narrow or absent barcode gaps (Lindner *et al*., 2013), its recognition as an official DNA barcode marker for fungal identification marks a significant advancement, benefiting the research community. Over the years, the number of ITS sequences in GenBank has grown, increasing the method's reliability and data availability.

**Intergenic spacer region of rDNA (ISG).** The intergenic spacer region that separates the repeated sections of rDNA is known for its rapid evolutionary rate, surpassing that of other regions within rDNA. Consequently, closely related *Fusarium* species often exhibit substantial divergence in this region, manifesting as variations in length and nucleotide sequence, along with the occurrence of restriction site variations in some molds (Appel and Gordon, 1995; Konstantinova and Yli-Mattila, 2004). These characteristics make it conducive to utilizing PCR-based technology for research on genetic diversity in *Fusarium* species.

For instance, a comprehensive technique for identifying mycotoxigenic *Fusarium* species, including *F. proliferatum*, *F. verticillioides*, and various members of the *Giberella fujikuroi* complex in crops like maize, effectively employed a PCR-based strategy based on IGS sequences (Jurado et al., 2006). This protocol involved expedited DNA extraction from maize samples (grain and germ), followed by genus-specific PCR, *G. fujikuroi* identification, and trichothecene-producing PCR. Additionally, a PCR assay based on the ISG region, known for its multiple copies in the genome, has been developed and refined for identifying *F. proliferatum*. This assay exhibits heightened sensitivity compared to PCR assays based on single-copy sequences and has been successfully applied to a wide range of fumonisin-contaminated maize samples (Jurado *et al*., 2006).

To explore the genetic diversity of *F. oxysporum* from strawberries (*Rosaceae*) cultivated under varying conditions in different regions of Korea, PCR-RFLP analysis was employed (Kim *et al*., 2017). The study revealed a direct correlation between the phenotypic variations of isolated pathogens and their respective growth regions. Sequencing the *IGS* gene uncovered a plethora of variants, including insertions, deletions, single nucleotide transitions, base substitutions, and duplicated repeats, within several *F. oxysporum* types isolated from rice (Mbofung *et al.,* 2007). In light of the considerable amplicon length, specific internal primers were developed by the authors. Nevertheless, this method is not universally applicable. For instance, the IGS region is unsuitable for identifying species in section Liseola and also *F. avenaceum*, *F. artrosproioides*/*F. tricinctum*, *F. sporotrichioides*/*F. langsethiae* (Chandra *et al.,* 2011). Similarly, the *Gibberella clade* possesses non-orthologous copies of ITS2, leading to unreliable results (Geiser *et al*., 2004).

**Translation elongation factor 1-*α* *(TEF1-α)*.** In the realm of *Fusarium* species identification, the choice of marker genes plays a crucial role. Although the *ITS* region is commonly employed as a standard marker for fungi, its utility in distinguishing closely related *Fusarium* species is limited (Schoch *et al*., 2012). Instead, *TEF1-α* has emerged as a preferred marker due to several advantageous features. Notably, *TEF1-α* is a single-copy gene within the *Fusarium* genus and boasts a substantial proportion of intronic regions, approximately 50% (Torres-Cruz *et al*., 2022).

The *TEF1-α* gene spans around 700 base pairs and harbors three introns, constituting more than half of the amplicon length in all *Fusarium* genus members (Ignjatov *et al*., 2017). Its utility as a phylogenetic marker was initially demonstrated in the study of various strains within the *F. oxysporum* complex (Geiser *et al*., 2004). This gene exists in a single copy in the *Fusarium* spp. genome and exhibits a high degree of polymorphism among closely related species, surpassing some other protein-coding region-rich markers like calmodulin, beta-tubulin, and histone H3 in terms of effectiveness and informativeness regarding species diversity (Geiser *et al.,* 2004; Dubey *et al.,* 2013). Furthermore, as a coding gene, *TEF1-α* has proven to be more effective in distinguishing *Fusarium* spp. than non-coding genes (Barik and Tayung, 2012).

Different PCR primers for the *TEF1-α* were used for 30 *Fusarium* species and 23 non-*Fusarium* species. Primers EF1-F2 and EF1-R3 were chosen as the most specific for *Fusarium* and for their abil-

ity to produce a distinct single band after agarose gel electrophoresis. These primers amplified a 640 bp segment of the *TEF1-α* gene in *Fusarium* species, demonstrating significant interspecific polymorphism. This segment was successfully amplified using DNA from all 30 *Fusarium* species tested (Boutigny *et al.,* 2019).

The use of *TEF1-α* partial sequences has enabled the identification of saffron isolates belonging to species *F. nirenbergiae*, *F. commune*, and *F. annulatum* (Mirghasempour *et al.,* 2022). This versatile gene has also found successful application in the identification of *Fusarium* spp. infecting economically important crops such as cotton (*Gossypium* spp.), beans (*Phaseolus vulgaris* L.), and wheat (*Triticum aestivum* L.) (Mbofung *et al.,* 2007; Silva *et al*., 2014; Minati 2020).

Another notable advantage is that widely used *TEF1-α* primers (EF1 and EF2) are suitable for both PCR amplification and Sanger sequencing (Geiser *et al*., 2004). Sequences of *TEF1-α* for precise identification of *Fusarium* spp. are accessible in publicly available databases like GenBank or the *Fusarium* ID database (Geiser *et al.,* 2004; Park, 2011). The ~680-bp 5' part of the *TEF1-α* gene has been recognized as the original barcoding locus for the *Fusarium* genus (Torres-Cruz *et al*., 2022). Strain identification using *FUSARIUM*-ID entails generating sequences from *TEF1-α* and/or other markers for an unknown isolate, conducting a BLAST search to find identical and/or highly similar sequences, and subsequently performing a species-level taxonomic assessment based on the results. The extensive use of *TEF1-α* for species identification has led to the accumulation of more than 30,000 *Fusarium TEF1-α* sequences in GenBank (Torres-Cruz *et al*., 2022).

**Beta-tubulin *(β-tub)*.** Tubulins play a pivotal role as integral components of eukaryotic microtubules, contributing significantly to processes such as cell division and intracellular transport. Eukaryotic organisms possess a variety of tubulin isoforms, including α-, β-, γ-, δ-, ε-, and η-tubulin. Remarkably, fungal β-tubulins serve as molecular targets for fungicides, proving effective in combatting plant diseases induced by ascomycete fungi (Zhao *et al*., 2014). These fungicides, which target β-tubulins, exert their inhibitory effects on hyphal growth. Methyl benzimidazole carbamate (MBC), for instance, is one such fungicide that specifically targets β-tubulin proteins in molds (Zhou *et al.,* 2016).

While many ascomycetes possess only a single β-tubulin gene, *F. graminearum* stands out with its two distinct genes - *TUB1* and *TUB2*. These genes exhibit varying functions during vegetative growth and sexual reproduction but share common roles in hyphal growth (Qiu *et al.,* 2012).

Recent research has made significant strides in this field. The complete sequencing of the β-tub (619 bp) was achieved in carbendazim-resistant *F. solani* (SF0301) strains isolated from Indian agricultural fields (Tarafder and Datta, 2022). Phylogenetic analysis based on β-tub sequences effectively categorized *Fusarium* spp. into nine complexes, effectively distinguishing *F. solani* and *F. fujikuroi* into three distinct clusters. Intriguingly, *F. solani*'s β-*tub* featured five exons and four introns, with conserved intron positions but shorter intron lengths compared to other *Fusarium* species (Tarafder and Datta, 2022). It's noteworthy that the comparison of amino acid sequences revealed no variations between resistant and susceptible isolates, suggesting that carbendazim resistance in *F. solani* SF0301 does not result from a point mutation in the β-*tub* (Tarafder and Datta, 2022).

Moreover, the PCR-RFLP assay demonstrated efficiency in the differentiation of *Fusarium* spp., encompassing *F. graminearum*, *F. culmorum*, *F. cerealis*, *F. oxysporum*, and *F. solani*. However, limitations surfaced in distinguishing *F. armeniacum*, *F. acuminatum*, and *F. sportrichioides* from *F. langsethiae*. To surmount these challenges, further exploration of additional genes is recommended to enhance species differentiation capabilities, as highlighted by Nosratabadi *et al.* 2018).

**Calmodulin *(CaM)*.** Calmodulin, an intracellular calcium-sensing protein, interacts with numerous proteins, thereby regulating their biological functions (Yap *et al.,* 2000). Calcium (Ca2+) functions as a secondary messenger and plays a crucial role in adapting to both abiotic and biotic stress, as well as in growth and development (Li *et al.,* 2022). In *Fusarium* spp., the complete primary structure of calmodulin was determined using Tricine-SDS-polyacrylamide gel electrophoresis, revealing the presence of two trimethyllysine residues at positions 76 and 115. It also exhibited a significant degree of homology to *CaM* in *Aspergillus nidulans* (Hoshino *et al.,* 1994).

*F. proliferatum* and *F. verticillioides* are primary contaminants of wheat grains with fumonisins. Evaluating the fumonisin production capacity of *Fusarium* species in two populations of Argentine wheat (harvested between 2008 and 2011) using calmodulin-based analysis identified various species, including *F. proliferatum* and *F. verticillioides*, among others species (Li *et al.,* 2022).

Phylogenetic analysis indicated greater nucleotide variability in *F. proliferatum* strains compared to *F. verticillioides*, irrespective of their origin, host, or harvest year (Palacios *et al.,* 2015).

Furthermore, DNA analysis of the nucleotide sequence of calmodulin facilitated the characterization of *Fusarium* spp. causing root rot in maize in Mexico (2007–2011), successfully identifying four species *F. verticillioides*, *F. nygamai*, *F. andiyazi*, and *F. thapsinum*. Importantly, distinct distributions of *Fusarium* species in various maize organs were observed. *F. verticillioides* was predominantly isolated from maize seeds, whereas *F. nygamai* prevailed in maize roots. Mixed infections involving *F. verticillioides/F. thapsinum* and *F. verticillioides/F. nygamai* were also detected in maize seeds and roots, respectively. Utilizing calmodulin as a marker not only aided in species identification but also demonstrated that different *Fusarium* spp. colonize different parts of the maize plant (Leyva-Madrigal *et al.,* 2015).

**Molecular tools for gene identification - biosynthetic gene cluster (BGC).** In the last few years, attention has turned towards markers directly associated with secondary metabolism as an effective approach for identifying species and predicting the mycotoxigenic chemotype within *Fusarium spp.* (Proctor *et al.,* 2009). Research into the genetic and biochemical pathways responsible for mycotoxin biosynthesis has revealed that genes involved in trichothecene, zearalenone, and fumonisin production are clustered together and co-expressed as biosynthetic gene clusters (BGCs) (Villafana *et al*., 2019). The development of molecular tools based on these BGC genes relies on a comprehensive understanding of their arrangement, diversity, and role in encoding enzymes for mycotoxin biosynthesis.

The *TRI* gene cluster displays variations in terms of single nucleotide polymorphisms, the presence or absence of pseudogenes, and insertion/deletion events (Yörük, 2016). Among the most pathogenic trichothecene-producing species, *F. graminearum*, *F. culmorum*, and *F. cerealis*, each possesses a 12-gene core cluster (*TRI* cluster). The presence, absence, or functionality of specific *TRI* genes signifies their capacity to produce different trichothecenes, including nivalenol (NIV), deoxynivalenol (DON), or their acetylated forms (3- or 15-ADON) (Villafana *et al.,* 2019). Successful PCR methods have been developed for characterizing trichothecene production profiles of *Fusarium* strains using gene-specific primers targeting *TRI*12, *TRI7*, *TRI*13, and *TR*I3 genes based on nucleotide sequence variation (Chandler *et al*., 2003; Li *et al.,* 2005). Qualitative PCRs are used to demonstrate the capacity of isolates to produce type A and/or type B trichothecenes. Although many regions and genes in the core cluster can be used, Wei *et. al*. (2017) recommend protocols for *TRI3* and *TRI12* for gene assays. Chemotypic diversity among strains of *F. graminearum sensu stricto*, *F. asiaticum*, *F. meridionale,* and *F. cortaderiae*, isolated from wheat in Paraguay. The 15-ADON chemotype of *F. graminearum sensu stricto* was found to be the most prevalent among the isolates. The trichothecene genotype was identified using *TRI3* and *TRI12* and specially designed primers (Arrua Alvarenga *et al*., 2022).

The functions of the majority of genes within the fumonisin BGC and zearalenone BGC have been extensively studied in *Fusarium* spp. *F. verticillioides*, a well-known pathogenic species affecting maize and producing fumonisins, harbors a 16-gene *Fum* gene cluster, with key roles attributed to *FUM*1, *FUM*8, and *FUM*6 (Seo *et al.,* 2001). Similarly, for ZEN synthesis, four genes within this cluster have been identified as responsible, including *ZEB*1, *ZEB*2, *PKS*4, and *PKS*13. *ZEB*1 exhibits similarity to isoamyl alcohol oxidase, while *ZEB*2 acts as a regulatory protein gene (Nahle *et al.,* 2021).

El-Yazeed *et al.* (2011) successfully identified 21 *Fusarium* isolates, comprising 15 *F. verticillioides*, 3 *F. anthophilum*, and 3 *F. proliferatum* isolates, from animal feed sourced from Cairo farms. They employed specially designed primers targeting the ITS region to differentiate between fumonisin-producing and non-producing *Fusarium* species, with the *FUM1* gene serving as a pivotal target for fumonisin biosynthesis. Amplified fragments of 183 bp were detected in the isolates, whereas none were observed in the control. This study underscores the utility of PCR-based techniques for distinguishing *Fusarium* spp., identifying *F. verticillioides*, *F. proliferatum*, and *F. anthophilum*, and rapidly assessing fumonisin species.

By examining genes such as *TR*I5, *TR*I6, *FUM*6, *FUM*8, and the regulatory protein gene *ZEA*2, all involved in mycotoxin biosynthesis, the toxigenic potential for trichothecenes, fumonisins, and zearalenone within 96 previously identified *Fusarium* spp. isolates through molecular and morphological techniques was successfully determined. The presence and type of biosynthetic genes were further confirmed by direct sequencing of the amplification products (Dawidziuk *et al.,* 2014).

**Collaborative methods.** In recent years, researchers have increasingly relied on a combination

of multiple genetic loci in conjunction with initial morphological identification. While the internal transcribed spacer region, previously the primary marker, effectively distinguished and assigned *Fusarium* species to a species complex, it had limitations in recognizing closely related species (Balajee *et al*., 2009; O'Donnell *et al.,* 2015). Consequently, alternative markers like Translation elongation factor 1-α *(TEF1-α)* and others have been recommended for precise species identification. According to O'Donnell *et al.* (2022), to achieve accurate species identification, phylogenetic analysis and sequencing of additional loci are necessary. They propose using a combination of three marker loci: *TEF1-α*, DNA-directed RNA polymerase II largest *(RPB1)*, and/or second largest subunit *(RPB2)* sequences for phylogenetic species recognition, rather than relying solely on ITS or LSU rDNA sequences.

In a study by Laurence *et al*. (2014), *Fusarium* species boundaries within the *Fusarium oxysporum* species complex (FOSC) were defined using portions of loci such as *TEF-1α*, mtSSU, *RPB1*, *RPB2*, the genes encoding Nitrate reductase *(NIR)*, phosphate permease *(PHO)*, *CAL*, and the *larger* subunit of ATP citrate lyase *(acl1)*. The Genealogical Concordance Phylogenetic Species Recognition (GCPSR) tool was employed for this purpose.

Wang *et al*. (2022) utilized a combination of morphological and phylogenetic methods to identify various *Fusarium* spp., employing molecular markers including the 5.8S nuclear ribosomal RNA gene with both ITS regions, the intergenic rDNA spacer region (IGS), partial calmodulin *(CaM)*, and partial *TEF1-α*.

In another study, Palacios *et al.* (2015) conducted a phylogenetic analysis based on the *CaM* and *TEF-1α* genes to identify and characterize 130 *Fusarium* strains obtained from durum wheat samples in Argentina. Their analysis revealed that 87 of the strains were identified as *F. proliferatum*, while 28 strains were identified as *F. verticillioides* through BLAST analysis on the *Fusarium*-ID database.

## Conclusion

The *Fusarium* genus, comprising approximately 200 species, has been a focal point for the research community for over two centuries due to its pathogenic properties and production of various secondary metabolites. This genus holds significant importance for ecosystems, agriculture, and public health. To mitigate the entry of mycotoxins into the food chain, it is imperative to develop timely and accurate methods for identifying pathogenic fungi. However, precise identification remains a formidable challenge, as traditional morphological methods are slow, subjective, and prone to errors due to overlapping characteristics among species.

Recent advances have introduced genomic regions within the *Fusarium* genus, such as IGS, *CaM*, *TEF1-α*, β-tub, and gene sequences, as effective markers for early diagnosis and reliable identification of *Fusarium* spp. isolated from economically important crops. The IGS region, known for its substantial sequence variation, is particularly valuable in phylogenetic analysis, providing enhanced resolution. *TEF1-α*, encoding a crucial component of protein translation, has emerged as the most informative gene for species identification. In fungal phylogenetic analysis, mitochondrial rRNA genes are favored over nuclear rRNA genes due to their higher rate of mitochondrial base substitution.

Recent studies have embraced the use of multiple molecular markers to enhance the reliability of species identification. Moreover, genes involved in the synthesis of mycotoxins, like trichothecenes, fumonisins, and zearalenone, particularly BGC genes, are becoming essential targets for establishing the chemotype of aggressive *Fusarium* strains.

To address the need for a comprehensive sequence-based database, *Fusarium*-ID was developed, facilitating the reliable identification of numerous *Fusarium* species and related genera. This regularly updated platform collates verified identification data from curated nuclear ribosomal DNA (rDNA) sequences. *Fusarium*-ID offers species descriptions, identification methodologies, and protocols, along with locus selection recommendations.

In conclusion, for robust *Fusarium* species identification and control strategies, the use of ITS, *TEF1-α*, and *RPB2* as primary markers, supplemented by *TUB2*, *CaM*, and *RPB1* as phylogenetic markers, is recommended. Furthermore, an increased focus on sequencing genomes will contribute to understanding phylogenetic relationships among closely related mycotoxigenic species and their interactions with host plants, ultimately aiding in the prevention of mycotoxin contamination in agricultural produce.

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