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RESEARCH ARTICLE OPEN ACCESS

A PCR-RFLP Analysis for the Diagnosis of DAS and T-2 Genotypes in *Fusarium goolgardi*

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Abstract

Fusarium **pathogens and their mycotoxins are considered as the main threats to cereal production and food safety worldwide. However, due to the constant discovery of new** ***Fusarium*** **species especially along with mycotoxin production profile differentiation in certain species, efforts on their species composition, geographical distribution, and chemotype proportion are urgently required. In the** ***Fusarium goolgardi*** **species, two distinct trichothecene (TB) genotype populations have recently been identified. Previous studies have shown that the structural variance of TBs biosynthesized by the two genotypes is attributed to the** ***Tri1*** **gene. Polymorphisms of** ***Tri1*** **gene from type A TB-producers were investigated in different** ***Fusarium*** **species in the current study. According to these DNA sequence variations identified in** ***Tri1*** **gene sequences, a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) based diagnostic approach for the differentiation of T-2 and 4,15-diacetoxyscirpenol (DAS) genotypes in** ***F. goolgardi*** **was successfully developed. The PCR-RFLP assay will facilitate the studies on geographic distribution, frequency and other aspects of the two genotypes within** ***F. goolgardi*** **species.**

Keywords: Type A Trichothecenes, Genotype Identification, *Fusarium* mycotoxins, *Fusarium goolgardi*

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INTRODUCTION

Fusarium is one of the most economically destructive fungal genera with many species that cause variety of plant diseases. Members of *Fusarium* are the primary cause of *fusarium* head blight (FHB) or scab, a catastrophic disease that affects cereal production worldwide.[1-6] FHB epidemics in North America and China have resulted in huge economic losses.[1,4-9] As an example in the United States, FHB has caused more than 3 billion dollars losses in crop (include wheat and barley) since 1990s.[1,6] Likewise, frequent epidemics and huge yield losses induced by FHB were also reported in China, with the Yangtze River basin to be most severe.[4,5,7-10]

The fungi from *Fusarium* genera are also of concern because they can produce variety of mycotoxins such as TBs, a series of non-volatile sesquiterpenes have a common core skeleton. Currently, over two hundred TBs have been found, posing a serious risk to the safety of food and feed.[11] Among these *Fusarium* secondary metabolites, type A and type B TBs are the main contaminants of small grains and the two group compounds have aroused considerable public concern worldwide.[12]

Type A and type B TBs are mainly characterized by the presence or absence of a ketone functional group at carbon 8 (abbreviated as C-8). In Type A TBs, there is an ester functional group, or a hydroxyl, or no substituent at all at C-8 position of the core TB molecule, and T-2, neosolaniol (NEO), and 4,15-diacetoxyscirpenol (DAS), respectively, are representative of this group (Figure 1).[13,14] Whereas type B TBs are distinguished from type A by the appearance of a ketone functional group at C-8, and they are represented by deoxynivalenol (DON), nivalenol (NIV), as well as their acetylated derivatives (Figure 1).[14-16] *Fusarium* TBs of particular concern are type A groups, which are very poisonous.[17] It has been demonstrated that Type A TBs are strong inhibitors of DNA, RNA, and protein synthesis; they can cause immunosuppressive and pathological changes in liver cells.[18,19] Furthermore, these toxins can induce DNA fragmentation characteristic of apoptosis and even death.[20,21]

Traditionally, chemotyping of *Fusarium* strains have been carried out using gas chromatography/mass spectroscopy which are relatively time-consuming and expensive.[22] Nevertheless, PCR based molecular genetic assay approaches (genotype assays) facilitate the quick screening of *Fusarium* strain toxin potential. Different genotype analysis methods for the identification of type B TB producing *Fusarium* species have been developed during the last two decades.[23-28] All these methods are based on the polymorphisms of the specific *Tri* genes which are involved in TB biosynthesis. Genotyping is a useful tool for predicting TB production potential of *Fusarium* strains, and is commonly used for *Fusarium graminearum* species complex (FGSC) strain genotyping studies.[22,29,30] High-throughput diagnostic approaches for type A TB producers, on the other hand, have yet to be established.[31]

Fusarium goolgardi is a new species recently identified and the members of this species can produce type A TBs.[32,33] According to a recent investigation by Rocha LO *et al.*, *F. goolgardi* populations have at least two TB genotypes: DAS-NEO-T2 genotype (abbreviated as T-2 genotype) and DAS genotype.[33] Available data demonstrates that the enzyme encoded by *Tri1* is required to catalyze the hydroxylation reaction of type A TBs at C-8 position, and thus it is involved in the biosynthesis of T-2, DAS, and NEO.[17,33] Current study focused on: (1) the polymorphisms of *Tri1* gene from type A TB producers; (2) development of molecular genotyping methods for the diagnosis of T-2 and DAS genotypes in *F. goolgardi* based on their *Tri1* gene polymorphisms.

MATERIALS AND METHODS

Tri1 gene sequence analysis

The *Tri1* gene nucleotide sequences of different *Fusarium* species that produce type A TBs were obtained from GenBank database and evaluated for polymorphism. In total, eighteen *Tri1* DNA sequences from four *Fusarium* species, including *F. goolgardi* (8 strains: RBG5411, 6914, 6915, 5417, 5419, 5420, 5421, and 5422), *F. langsethiae* (3 strains: NRRL53410, 53417, and 53439), *F. sibiricum* (2 strains: NRRL53421 and 53427), and *F. sporotrichioides* (5 strains: NRRL3299, 53434, 26924, 29977, and 29978), were retrieved from the NCBI GenBank and submitted to multiple sequence alignment analysis with Clustal

W (version 2.0).[34] The species were chosen for their ability to synthesize type A TBs, and all of the strains included in the study had previously been determined by chemical analyses.[33,35,36] Table contains complete information on all representative sequences.

Fusarium strain culture and DNA extraction

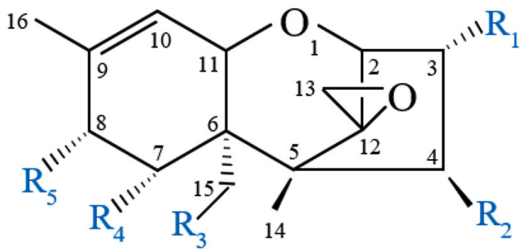
Mycelium of *Fusarium* strains grown on sterile glass-membrane paper overlaying potato dextrose agar, was scraped from the surface cultures and ground to a fine powder in the presence of liquid nitrogen. Homogenized mycelia were suspended in 650 µL pre-heated (65°C) CTAB buffer, mix well and then incubate in a water bath (65°C, 40 min). After incubation, total genomic DNA was extracted as previously reported.[28] Finally, DNA was diluted to ~50 ng/µL and kept frozen until required.

PCR amplification

Amplifications were performed in a final volume of 20 µL containing 1 × PCR buffer, 0.25 mmol/L of each dNTP, 0.2 mmol/L of primer A-Tri1F and A-Tri1R, and around 50 ng genomic DNA. The reaction was programmed as follows: 94°C 4 min; 30 cycles of 94°C 30 s, 60°C 20 s, 72°C 35 s; and 5 min final extension at 72°C. PCR products (2 µL) were separated through a 1.5% agarose gel electrophoresis and visualized under ultraviolet light after stained with nucleic acid fluorescent stain reagent (Transgen Biotech, Beijing, China). The PCR product sizes were evaluated according to known DNA standards.

Enzyme digestion

The *Tri1* amplicon was subsequently digested with *Bfa*I (NEB, Ipswich, MA, USA). The enzyme digestion was carried out in a 20 µL mixture containing 2 µL 10 × rCutSmart buffer, 3 µL *Tri1* amplicon, 1 µL *Bfa*I (10 U/µL), and 14 µL water. The digestion reaction was performed in a thermal cycler as previously described.[31] Immediately after digestion, the products were detected by electrophoresis on 1.5% agarose gel. The approach is illustrated in Figure 2.



Trichothecenes		R1	R2	R3	R4	R5
Type A	T-2	-OH	-OAc	-OAc	-H	-OIsoval
	HT-2	-OH	-OH	-OAc	-H	-OIsoval
	NEO	-OH	-OAc	-OAc	-H	-OH
	DAS	-OH	-OAc	-OAc	-H	-H
Type B	DON	-OH	-H	-OH	-OH	=O
	3ADON	-OAc	-H	-OH	-OH	=O
	15ADON	-OH	-H	-OAc	-OH	=O
	NIV	-OH	-OH	-OH	-OH	=O
	4ANIV	-OH	-OAc	-OH	-OH	=O

Figure 1. Structures of common Type A and B trichothecenes (NEO, neosolaniol; DAS, 4,15-diacetoxyscirpenol; DON, deoxynivalenol; 3ADON, 3-acetyl-deoxynivalenol; 15ADON, 15-acetyl-deoxynivalenol; NIV, nivalenol; 4ANIV, 4-acetyl-nivalenol; OAc, acetyl function; OIsoval, isovalerate)

RESULTS

DNA polymorphism of *Tri1* gene in Fusarium

In this study, we first compared the sequences of *Tri1* homologs from three *F. langsethiae* (NRRL53410, 53417, 53439), two *F. sibiricum* (NRRL53421, 53427), and five *F. sporotrichioides* (NRRL3299, 53434, 26924, 29977, and 29978). The full-length *Tri1* DNA sequences of these ten strains are varied from 1857 bp to 1860 bp with four introns, while the coding sequence is 1629 bp in all of them. Differences in DNA length of the intact *Tri1* genes are due primarily to one and two nucleotides insertion/deletion in the third and fourth introns, respectively.

The coding sequences of these ten *Tri1* DNA sequences were further subjected to alignment assays, and the results indicated that their identity ranged from 96.07% to 100%. The *Tri1* coding sequences of *F. sibiricum* strain

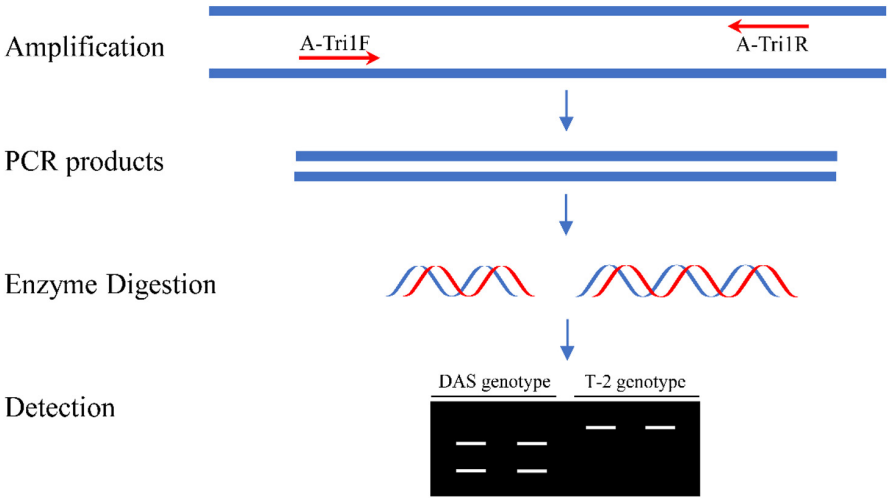


Figure 2. A schematic diagram of the PCR-RFLP analysis for the diagnosis of DAS and T-2 genotypes

Table. Species, strains, genotypes, and sequence accession numbers of *Fusarium Tri1* genes

Species	Strains	Geno-types	Accession Numbers	References
F. goolgardi	RBG5411	T-2	KT597908	Rocha LO et al.
F. goolgardi	RBG5417	T-2	KT597907	Rocha LO et al.
F. goolgardi	RBG5419	T-2	KT597906	Rocha LO et al.
F. goolgardi	RBG5420	T-2	KT597905	Rocha LO et al.
F. goolgardi	RBG5421	DAS	KT597915	Rocha LO et al.
F. goolgardi	RBG5422	DAS	KT597916	Rocha LO et al.
F. goolgardi	RBG6914	DAS	KT597913	Rocha LO et al.
F. goolgardi	RBG6915	DAS	KT597914	Rocha LO et al.
F. langsethiae	NRRL53410	T-2	HQ594538	Yli-Mattila et al.
F. langsethiae	NRRL53417	T-2	HQ594539	Yli-Mattila et al.
F. langsethiae	NRRL53439	T-2	HQ594543	Yli-Mattila et al.
F. sibiricum	NRRL53421	T-2	HQ594540	Yli-Mattila et al.
F. sibiricum	NRRL53427	T-2	HQ594541	Yli-Mattila et al.
F. sporotrichioides	NRRL26924	T-2	HQ594535	Yli-Mattila et al.
F. sporotrichioides	NRRL29977	T-2	HQ594536	Yli-Mattila et al.
F. sporotrichioides	NRRL29978	T-2	HQ594537	Yli-Mattila et al.
F. sporotrichioides	NRRL53434	T-2	HQ594542	Yli-Mattila et al.
F. sporotrichioides	NRRL3299	T-2	AY040587	Meek et al.

NRRL53421 is identical to NRRL53427, and identical coding sequences were observed in the three *F. langsethiae* strains NRRL53410, 53417, and 53439. A total of 109 single-nucleotide polymorphisms were found in the coding sequences of the examined 10 *Tri1* sequences, while no other major differences were observed.

Portions of *Tri1* sequence from eight *F. goolgardi* strains with different genotypes were included for subsequent polymorphism analysis

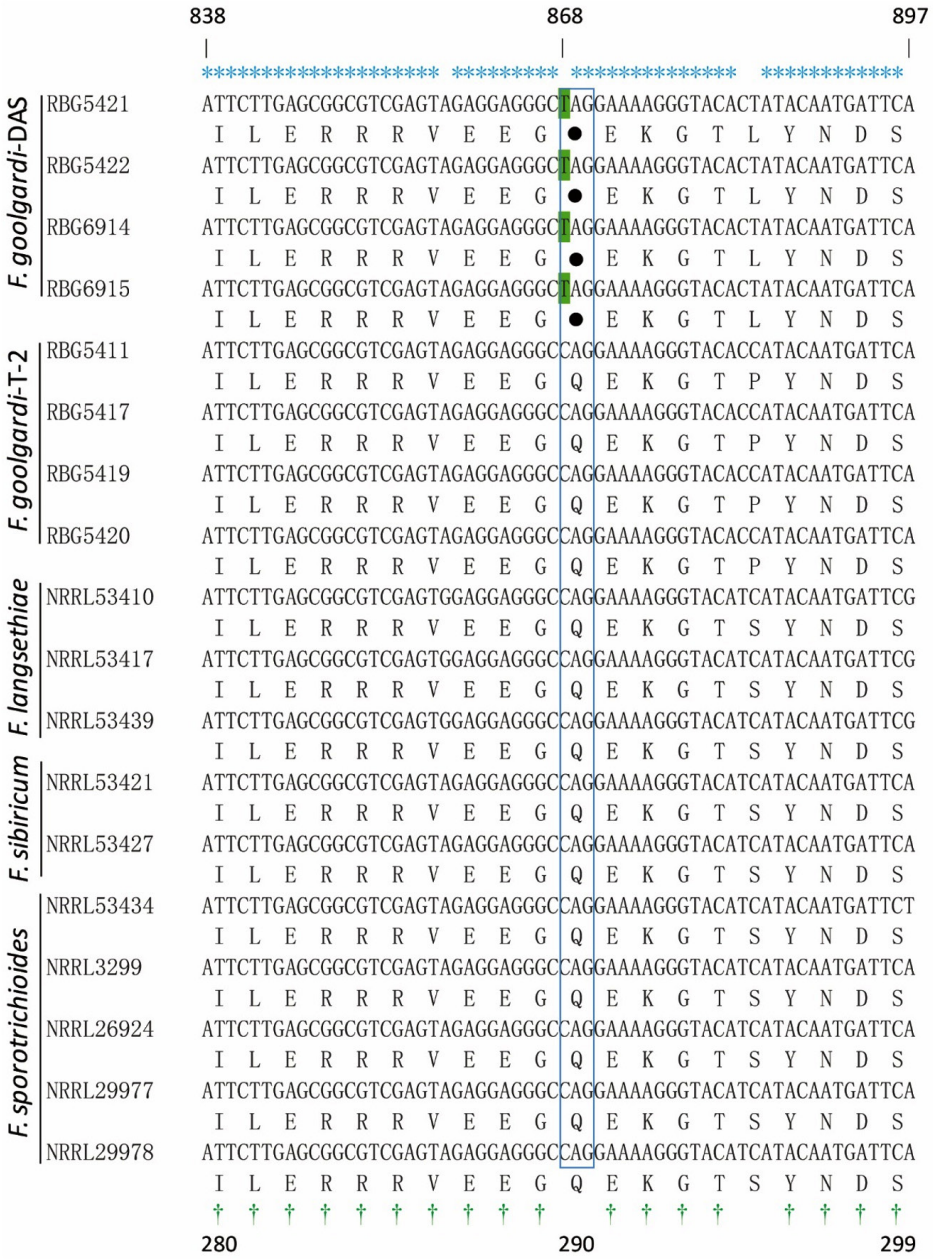


Figure 3. Alignment analysis of the nucleotides and corresponding protein sequences of partial *Tri1*. The conserved nucleotides and amino acids are indicated above and under the panel by * and †, respectively. The nonsense mutation sites in the CDS position 868 (C to T) of the *F. goolgardi* DAS strains indicated with green background, and the corresponding stop codon is represented by a solid circle (referred to strain NRRL29978)

and primer design. Nucleotide sequences of *Tri1* gene DNA sequences from four *F. goolgardi* strains (RBG5411, 5417, 5419, and 5420) with T-2 genotype and four *F. goolgardi* strains (RBG6914, 6915, 5421, 5422) with DAS genotype were aligned against reference sequences from the above-mentioned *F. langsethiae*, *F. sporotrichioides*, and *F. sibiricum* strains (Table). As shown in Figure 3, *Tri1* sequences in *F. goolgardi* strains with the DAS genotype differed significantly from those in T-2 producers. There is a nonsense mutation occurred at position 868 (C-to-T transition) in the middle of the *Tri1* coding region, resulting in a premature stop codon and the failure of normal transcription and translation of *Tri1* gene; therefore, it is considered as a pseudo-gene. While the *Tri1* sequences from T-2-producing *F. goolgardi* strains did not exhibit the nonsense mutations, which generally will expressed normally.

Primer design

The alignment allowed designing a pair of primers, A-Tri1F (5'-GTAGCAAATCACCTACGCAGAT-3') and A-Tri1R (5'-GAGGACGCATTCTCGTATATCT-3'). The expected amplicon size is 628 bp for all the type A TB-producing *Fusarium* strains, including *F. sporotrichioides*, *F. goolgardi*, *F. sibiricum*, and *F. langsethiae*.

PCR-RFLP approach for genotyping of *F. goolgardi*

A PCR-RFLP protocol targeting on the *Tri1* gene variations was designed to distinguish between DAS and T-2 genotypes (Figure 2). Due to a single-nucleotide mutation, the fragment amplified with A-Tri1F/R from the *F. goolgardi* DAS strains have been digested into two different fragments of varying sizes by *Bfa*I, *Mae*I, *Mth*ZI, and *Rma*I (the four restriction endonucleases recognize the same specific nucleotide sequence "CTAG"). However, the amplicons from all the other strains, including the *F. goolgardi* T-2 strains, can't be digested by any of these restriction endonucleases.

To evaluate the reliability of the PCR-RFLP approach for identifying DAS and T-2 genotype strains, strains from *F. goolgardi*, *F. langsethiae*, *F. sibiricum*, and *F. sporotrichioides* were tested. TBs produced by these strains have been reported previously.[33,35,36] As shown in Figure 4, after digestion with *Bfa*I, the amplicons from *F. goolgardi* DAS strains were cut into two fragments with different sizes, 147 bp and 481 bp, respectively, in length. On the other hand, a single 628 bp band was observed from the amplicons of T-2 producers. The unique DNA profiles obtained from DAS and T-2 genotype strains generated by PCR-RFLP assays suggest that this method can be used for differentiation

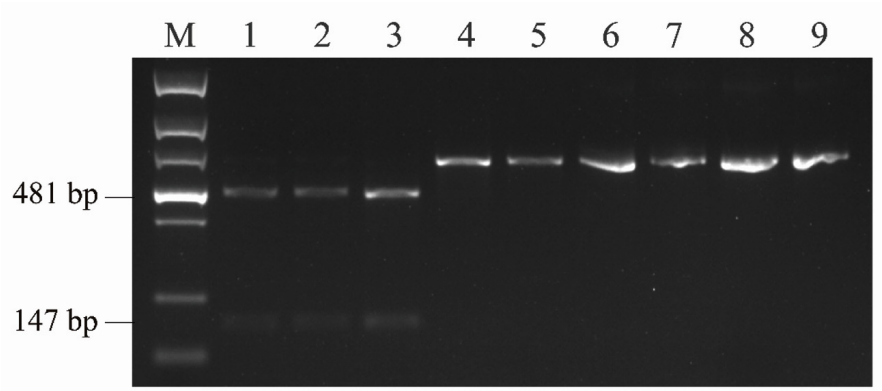


Figure 4. The restriction enzyme digestion products of *Fusarium* strains amplified by primer set A-Tri1F/R. M, DNA ladder marker; Lane 1 to lane 3, *F. goolgardi* strains with DAS genotype: RBG6914, 6915, and 5421; Lane 4 to lane 5, *F. goolgardi* strains with T-2 genotype: RBG5411 and 5419; Lane 6, *F. langsethiae* strain NRRL53410 (T-2 producer); Lane 7, *F. sibiricum* strain NRRL53421 (T-2 producer); Lanes 8 and 9, *F. sporotrichioides* strains NRRL29978 and NRRL3299 (T-2 producers), respectively

of these two type A TB producers in *F. goolgardi* populations. Furthermore, according to our analyses, the molecular genotyping detection method developed in the current study may also can be extended to identify DAS and T-2 genotypes in other species, such as *F. langsethiae*, *F. sibiricum*, and *F. sporotrichioides*.

DISCUSSION

TBs' structure diversity is caused by the variability of TB biosynthesis gene functions. As a result, *Tri* genes are often utilized as markers to assess the probable ability of *Fusarium* strains to produce TBs using PCR-based assays.[22-29] As we know, *Tri1* and *Tri16* genes are critical for the hydroxylation reaction and its immediate acylation of the TB skeleton structure at C-8 position in the biosynthetic pathway of T-2, respectively. However, at C-8 of the DAS, there are just two hydrogen atoms, and no further modification occurred. Thus, compared with T-2 producers, there is probably a nonfunctional *Tri1* gene or at least the gene can't be expressed normally in *Fusarium* strains which can only produce DAS toxin.

The occurrence of nonsense mutation in the coding region of *Tri1* gene from *F. goolgardi* strains with a DAS genotype renders the gene non-functional, thus leading to the production of TB homologues without C-8 modifications.[17,33] The findings also show that other polymorphisms may have occurred in the DAS lineage, possibly resulting in *Tri1* gene dysfunction. The precise scenario that resulted in these mutations in the *Tri1* gene is difficult to pinpoint; nonetheless, such changes can be exploited to distinguish between DAS and T-2 producers in the *Fusarium* genera.

Only one genotype has been documented for *F. langsethiae*, *F. sporotrichioides*, and *F. sibiricum*, and strains from the three species can produce T-2, as well as other type A TBs, including DAS and NEO.[18,20,33,35-37] However, the discovery of two different genotypes, DAS and T-2 types, within *F. goolgardi* strains has just been reported. The results from Rocha LO *et al.* demonstrated that some strains of *F. goolgardi* produced DAS and NEO in addition to T-2, while other strains produced only DAS.[33]

In the current study, a PCR-based approach was developed for the diagnosis of DAS and T-2 genotypes in *F. goolgardi*. In contrast to T-2 genotypes, a nonsense mutation occurred at position 868 (C-to-T transition) in the *Tri1* gene of the DAS genotype *F. goolgardi* strains. This mutation resulted in the emergence of a new enzyme cleavage site, C*TAG (* denotes the cleavage site), which can be recognized by restriction endonucleases *Bfa*I, *Mae*I, *Mth*ZI, and *Rma*I. Although a small number of *Fusarium* strains were evaluated with our PCR-RFLP method, findings reported in Rocha LO *et al.* provide significant support to our results.[33]

Different genotyping methods are extensively applied in species identification, molecular marker screening, and genetic diversity analysis. These fingerprinting techniques consider information distributed over the whole genome of an organism and generally permit discrimination of *Fusarium* strains with different phenotypes like different species or different TB profiles. Among these techniques, RFLP is famous for its robustness, reproducibility, and reliability.[38] Recently, four sets PCR-RFLP diagnostic methods for the differentiation of type B TB- and NX-producing FGSC were successfully developed by us relying on polymorphisms identified in *Tri1* sequences.[31] As far as we know, though, this is the first report on the genotyping analysis of T-2 and DAS genotypes in *F. goolgardi*. Further investigations are needed to determine the fungi's temporal and spatial distribution. The PCR-based RFLP assay developed in this work will make easier and faster the investigations on temporal and spatial distribution of DAS and T-2 populations.

Fusarium goolgardi is a recently identified *Fusarium* species.[32] Therefore, further research will be necessary to track and monitor the two genotypes' geographic distribution and spread as well as assess any potential variations in their competitive capacities, including pathogenicity and environmental adaptability. Moreover, the divergence of TB genotype within the *F. goolgardi* species also highlights the need to re-evaluate genotype and population diversity of the economically destructive fungal pathogens in *Fusarium* genera throughout different agricultural ecosystems worldwide.

On the other hand, subsequent identification of mutations (premature stop codons and frameshift) in *Tri16* coding region in

F. goolgardi strains carrying the DAS genotype give rise to new questions and perspectives that whether DAS genotype strains contain a functional *Tri16* gene exist or not in nature.[33] If so, what kind of TBs produced by such strains? It is still unclear whether strains that primarily produce NEO or coexistences of DAS and NEO, however, lack T-2 toxin exist or not.[17] Taking this into account, we recently conducted a comprehensive and systematic analysis of the advances on type A TB biosynthesis in *Fusarium* species, with respective to their biosynthetic pathway, gene evolution and other concepts, and the potential biosynthetic pathways for NEO and DAS toxins were proposed.[17] Based on the previous findings and current questions, further research is needed to discover the biochemical alterations or genetic basis changes in TB biosynthesis and related molecular regulation mechanisms. The settlement of these issues will undoubtedly give greater impetus to the understanding of evolution of secondary metabolites biosynthesis in *Fusarium* species, and provide new insights on the control and prevention of mycotoxins in food safety.

CONCLUSION

Identification of *Fusarium* strain toxin potential is essential with respect to food safety issues due to TBs' different toxicological effects. Previously, two distinct TB genotype groups, namely T-2 genotype and DAS genotype, were found in the species of *F. goolgardi*. In this study, the polymorphisms of *Tri1* gene from the two kinds of TB producers were investigated. A PCR-RFLP assay method was successfully developed to distinguish T-2 and DAS producers within *F. goolgardi*. This promising diagnostic method can be used for high-throughput genotype analysis of *F. goolgardi* strains as a step forward for plant disease management, TBs assessment and control in agriculture. Besides, this molecular diagnostic technology will facilitate the studies on its epidemiology, host range, and geographical distribution of the novel species.

ACKNOWLEDGMENTS

None.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This study was approved by the Shanghai Academy of Agricultural Sciences, Shanghai, China.

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