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Mycoflora and chemotype characterization of *Fusarium graminearum* isolates obtained from corn and barley used as feedstuffs in the northeast of Iran

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Abstract: This study aimed to investigate the mycoflora and chemotyping of *Fusarium graminearum* isolates in corn and barley used in industrial dairy farms in Khorasan-Razavi Province, northeastern Iran. For this purpose, a total of 128 corn and barley samples were collected from 16 different large industrial dairy farms. Mean moisture of the samples collected in different seasons of the year was significantly different ($P < 0.05$). The most prevalent fungi isolated from corn samples were *Penicillium* spp. (90.62%) and *Aspergillus* spp. (82.81%), and that of barley samples was *Penicillium* spp. (89.06%). Frequency of *Fusarium* spp. in corn and barley samples was 85.93% and 26.56%, respectively. All isolates (100%) were confirmed as *Fusarium* using an ITS primer. Eight (11.11%) out of 72 *Fusarium* spp. isolates were confirmed as *F. graminearum* using species-specific primers. Five out of 8 *F. graminearum* isolates were positive in terms of the presence of the trichothecene-producing gene using a Tri5-specific primer. Three and 2 out of 5 toxin-producing *F. graminearum* isolates were positive using Tri13DON- and Tri13NIV-specific primers, respectively. In conclusion, the presence of toxigenic *Fusarium* in corn and barley samples used in feedstuffs in Iran is a potential risk to animal and human health.

Key words: *Fusarium graminearum*, deoxynivalenol, nivalenol, chemotype, polymerase chain reaction

1. Introduction

Food and feed commodities are usually contaminated by a range of different fungi during growth, harvesting, and storage stages. The most common fungi involved in the contamination and spoilage of feedstuffs belong to *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp. The local weather conditions as well as the environmental conditions in the storage facilities, especially temperature and relative moisture, are important for the fungal growth and consequently potential risk for mycotoxin production (1,2). Mycotoxins are secondary metabolites that have toxic effects on humans and animals. Approximately 20% to 45% of the world's grains are contaminated with mycotoxins produced by storage fungi (3). Transmission of the toxin to milk, meat, and eggs through farm animals feeding on contaminated feed poses an increasing health hazard.

Corn is a grain with a wide range of applications in food, pharmaceutical, and industrial fields. Barley seed has always played a special role in the nutrition of humans and farm animals. Previous studies have reported excessive amounts of toxic residues resulting from fungal growth in food products with grains including barley and corn, such as bread, pasta, baby formulas, fast foods, and similar products (3,4).

Fusarium species are commonly considered as field fungi invading more than 50% of corn grains before harvest (5). Several pathogenic species of *Fusarium* are found to be associated with maize, including *F. verticillioides*, *F. proliferatum*, and *F. graminearum* (6). *Fusarium* species are typically identified by morphological features such as growth form, nonsexual and sexual forms, and other features, which are often arbitrary and also time-consuming to identify and nonspecific (7). Studies based on mycotoxin chemotype production are very useful to characterize *Fusarium* isolates (8). Nevertheless, the methodology for carrying out the morphological and physiological characterization of toxigenic fungi is generally very time-consuming. Therefore, a rapid and reliable assay for the routine identification of toxigenic *Fusarium* spp. would benefit the food and feed industries. Polymerase chain reaction (PCR)-based methods are already having a significant impact on mycological research.

The important toxins produced by *F. graminearum* in small grains include 8-ketotrichothecens like deoxynivalenol (DON) and nivalenol (NIV) and their acetylation derivations such as 15-AcDON, 3-AcDON, and 4-AcNIV (9). DON causes reduced feed intake and weight

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gain when present in the feed at 1–3 ppm, especially in swine. Cattle and poultry can tolerate much higher levels of DON than pigs. The US Food and Drug Administration has recommended that total feed levels of DON not exceed 5 ppm for cattle and chicken and 1 ppm for swine. DON levels for human food should be less than 1 ppm (10).

Since there have been no studies regarding corn and barley contamination with *F. graminearum* in industrial dairy farms in Iran, and especially the northeast thus far, this research was conducted as the first study to investigate the moisture content and health of corn and barley grains used in animal feed in terms of contamination with seed-borne *Fusarium* spp. in different seasons and to detect DON and NIV toxin-producing species by the PCR method.

2. Materials and methods

2.1. Sampling

In this study, 128 samples including 64 corn and 64 barley samples were collected from 16 industrial dairy farms in Khorasan-Razavi Province in the northeast of Iran during winter, spring, summer, and fall (16 samples in each season). Considering the capacity of the warehouse, sampling was randomly performed from the middle part of the barn and the samples did not have apparent signs of mold contamination. The collected samples were kept in sterile plastic bags and were immediately transferred to the laboratory at 4 °C.

2.2. Determination of moisture content of samples

To determine the moisture content, the samples were first fully ground, and then 5 ± 0.1 g of the sample was weighed in a dry glass plate and kept in an oven at 100 °C for 2 h to completely dry out (11). For further assurance, moisture of the samples was also simultaneously determined at 110 °C using a hygrometer (PCE Instruments, UK).

2.3. Determination of mycoflora and identification of *F. graminearum*

To determine the fungal contamination of samples, 10 g of each ground sample was completely mixed in 90 mL of diluent solution to prepare serial dilutions from 10^{-1} to 10^{-6}. Each of the obtained dilutions was then cultured in potato dextrose agar (PDA) medium and incubated at 25 °C for 5–7 days (12). Fungal flora of samples was characterized and identified based on morphologic and microscopic characteristics using the keys of Pitt and Hocking (13) and Raper and Fennell (14). Grains samples were surface-sterilized for 1 min with a 5% sodium hypochlorite solution, rinsed twice with sterile distilled water, and dried in a laminar flow cabinet. The grains were then incubated in PDA at 28 °C in the dark for 7 days. All *Fusarium* spp. isolates were subcultured on PDA and incubated at 25 °C for 2–4 weeks (7). Isolates were also grown in carnation leaf agar (CLA, Merck) according to Fisher et al. (15). The morphology of macroconidia and chlamydospores was assessed from cultures grown on CLA. *F. graminearum* was identified according to Leslie and Summerell (7).

2.4. Molecular identification of *F. graminearum* isolates and chemotype determination

F. graminearum isolates were also identified by PCR with species-specific primers. To prepare the mycelial mass, all the *F. graminearum* isolates were cultured in potato dextrose broth (Merck) medium and incubated at 25 °C for 7 days. Fungal mycelium was then isolated with Whatman filter paper and freeze-dried. Genomic DNA of isolates was extracted with a commercial kit (Bioneer, Korea). The primer pairs of ITS-F/ITS-R and Fg16N-F/R for identification of *Fusarium* spp. and *F. graminearum*, which amplified a fragment containing 431 and 280 bp, respectively, were used (5,16).

To identify chemotypes of *F. graminearum* isolates, Tri5-F/R-specific primers related to tracking the *Tri5* gene (17) and Tri13DONR/Tri13F and Tri13NIVF/Tri13R primers related to the *Tril3* gene (18) and responsible for controlling DON and NIV production, which produced fragments containing 380, 282, and 312 bp, respectively, were used (Table 1). The thermal schedule of the thermal cycler related to PCR reactions is presented in Table 2. PCR products were electrophoresed by agarose gel (1.2%) and were visualized under UV light of 260 nm in gel documentation (Vilber Lourmat).

2.5. Statistical analysis

Data were analyzed using descriptive statistics as mean ± SD. One-way analysis of variance (ANOVA) was used to compare the fungal contamination at different seasons. To evaluate differences between two seasons, we used the Duncan test in post hoc multiple comparisons. In order to compare contamination with different types of *Fusarium* in barley and corn samples, the chi-square test was used, which led to obtaining significant results. The significance level of the tests was 5%.

3. Results

3.1. Determination of moisture content and fungal contamination of samples

Mean moisture of the barley and corn samples in different seasons is shown in Table 3. Mean moisture of the barley samples in winter, spring, summer, and fall was 11.23 ± 1.21%, 9.45 ± 1.18%, 8.61 ± 1.17%, and 10.93 ± 1.51% and that of corn samples was 13.7 ± 1.46%, 10.22 ± 1.21%, 9.12 ± 0.84 %, and 13.6 ± 1.36 %, respectively (Table 3). The mean moisture of corn and barley samples in winter and fall were statistically higher than that of the samples collected in spring and summer ($P < 0.05$). There were no differences between the mean moisture content of winter and fall samples ($P < 0.05$).

Table 1. Primers sequences used in this study.

Target species	Primer destination	Sequence (5'→3')	References	Size (bp)
Fusarium spp.	ITS-F ITS-R	AACTCCCAAACCCCTGTGAACATA TTTAACGGCGTGGCCGC	16	431
F. graminearum	Fg16N-F Fg16N-R	ACAGATGACAAGATTCAGGCACA TTCTTTGACATCTGTTCAACCCA	5	280
DON-producing *F. graminearum*	Tri13DONR Tri13F	GCTAGATCGATTGTTGCATTGAG CATCATGAGACTTGTKCRAGTTTGGG	18	282
NIV-producing *F. graminearum*	Tri13NIVF Tri13R	CCAAATCCGAAAACCGCAG TTGAAAGCTCCAATGTCGTG	18	312
Toxigenic *Fusarium* spp.	Tri5-F Tri5-R	GGGATGCTGGATTGAGCAG CATCACCTGAGGGTCCTTGT	17	380

Table 2. PCR conditions in this study.

Primer designation	Initial denaturation	Cycle	Denaturation	Annealing	Extension	Final extension
ITS F/R	94 °C, 5 min	35	94 °C, 60 s	58 °C, 60 s	72 °C, 60 s	72 °C, 10 min
Fg16N-F/R	94 °C, 5 min	35	94 °C, 60 s	60 °C, 30 s	72 °C, 60 s	72 °C, 5 min
Tri5-F/R	94 °C, 5 min	35	94 °C, 30s	60 °C, 45 s	72 °C, 30 s	72 °C, 7 min
Tri13DONR/Tri13F	94 °C, 5 min	35	94 °C, 30 s	58 °C, 45 s	60 °C, 30 s	72 °C, 7 min
Tri13NIVF/Tri13R	94 °C, 5 min	35	94 °C, 30 s	58 °C, 45 s	60 °C, 30 s	72 °C, 7 min

Table 3. Mean moisture content (%) of barley and corn samples.

Feedstuff	No.	Mean ± SD (%)			
		Winter (n = 16)	Spring (n = 16)	Summer (n = 16)	Fall (n = 16)
Barley	64	$11.23^{a,*} \pm 1.15$	$9.45^{b} \pm 1.12$	$8.61^{c} \pm 0.82$	$10.93^{a} \pm 1.07$
Corn	64	$13.71^{a} \pm 1.2$	$10.22^{b} \pm 1.19$	$9.12^{c} \pm 0.8$	$13.66^{a} \pm 1.15$

*Values with different letters are significantly different ($P < 0.05$).

Results obtained from culturing of the samples are presented in Table 4. The most common molds isolated from corn samples were *Penicillium* spp. (90.62%), *Fusarium* spp. (85.93%), *Aspergillus* spp. (82.81%), *Mucor* (75%), and *Rhizopus* (51.56%). Among the studied barley samples, the maximum level of contamination was related to *Penicillium* spp. (89.06%), *Aspergillus* spp. (73.43%), *Mucor* (68.75%), and *Rhizopus* (50%) (Table 4).

3.2. Determination of *Fusarium* spp. and *F. graminearum* isolates

Out of a total of 128 samples of corn and barley, *Fusarium* spp. were isolated from 55 (85.93%) and 17 (26.56%) samples, respectively. All of the isolates (100%) that were detected as *Fusarium* spp. using the culturing method produced a fragment containing 431 bp in PCR reaction with the ITS-F/R pair primer (Figure 1a). Of the 72 *Fusarium* isolates, 8 (11.11%) were identified as *F. graminearum* by Fg16N-F/R, which produced a fragment containing 280 bp (Figure 1b).

3.3. Chemotype determination of *F. graminearum* isolates

Chemotyping of *F. graminearum* isolates was done by PCR assay using a set of Tri5-F/R-specific primers for the presence of trichothecene-producing genes. The results

Table 4. Frequency of different genera of fungi present in corn and barley samples used in animal feed.

Fungal genus	Corn samples (n = 64)				Barley samples (n = 64)			
	Winter	Spring	Summer	Fall	Winter	Spring	Summer	Fall
Aspergillus spp.	14	15	10	14	9	15	8	15
Fusarium spp.	16	13	13	13	2	3	4	8
Penicillium spp.	13	16	11	14	14	15	13	15
Cladosporium	1	8	2	1	5	7	6	10
Rhizopus	11	6	9	7	10	11	4	7
Mucor	13	9	12	14	9	8	13	14
Geotrichum	0	0	0	0	3	1	1	0
Alternaria	1	0	0	0	5	2	11	5
Monilinia	3	2	0	0	2	0	0	2
Syncephalastrum spp.	4	3	0	1	3	1	3	3
Nocardia	0	0	0	1	2	1	1	0
Yeast	6	5	10	10	9	12	6	5

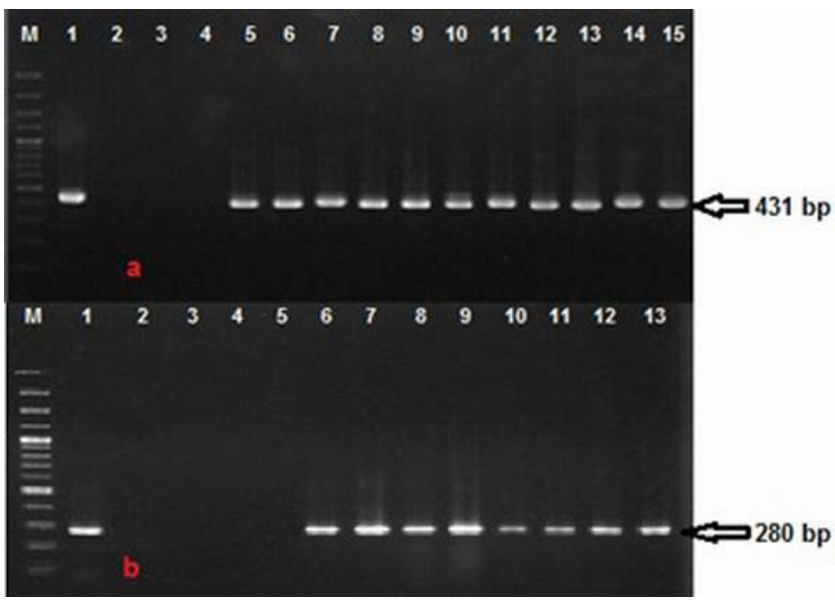

Figure 1. Agarose gel electrophoresis of species-specific PCR products. **(a)** M: 100-bp plus marker; Lane 1: Standard *F. graminearum* isolate (positive control); Lane 2: negative control (without genomic DNA); Lane 3: *Aspergillus flavus* with ITS primers; Lane 4: *A. niger* with ITS primers; Lanes 5–15: *Fusarium* spp. isolates. **(b)** M: 100-bp plus marker; Lane 1: standard *F. graminearum* isolate (positive control); Lane 2: negative control (without genomic DNA); Lane 3: *F. culmorum* with Fg16N primers; Lane 4: *F. oxysporum* Fg16N primers; Lane 5: *F. proliferatum* with Fg16N primers; Lanes 6–13: *F. graminearum* isolates.

showed that 5 out of 8 *F. graminearum* isolates were positive for trichothecene-producing genes (Figure 2a). Three and 2 out of 5 *F. graminearum* isolates were positive for DON- and NIV-producing genes, respectively, using Tri13DONR/Tri13F and Tri13NIVF/Tri13R primers (Figure 2b and 2c).

4. Discussion

Corn and barley are considered the major components of animal feed and might be infected with different types of fungi during the harvesting, collecting, and warehousing stages. Under optimal conditions, such as high temperature (25–35 °C), moisture content (13%–16%),

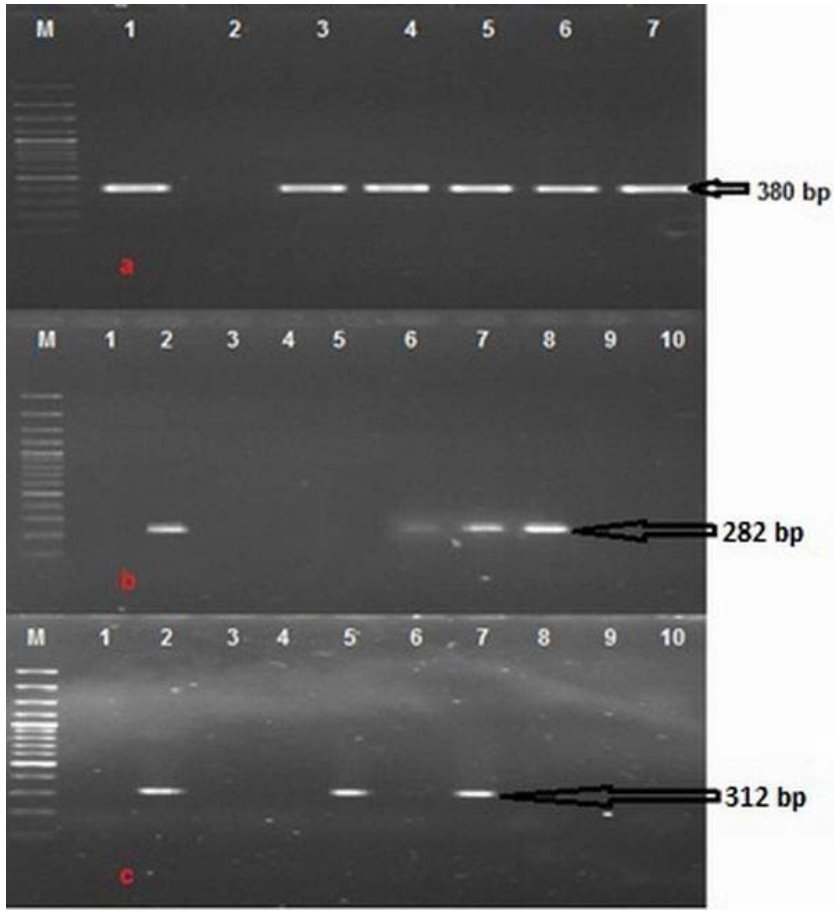

Figure 2: (a) PCR products of trichothecene-producing *F. graminearum*. M: 100-bp plus marker; Lane 1: positive control (trichothecene-producing *F. graminearum*); Lane 2: negative control (trichothecene-nonproducing *F. graminearum*); Lanes 3–7: trichothecene-producing *F. graminearum* isolates. **(b)** PCR products of DON-producing *F. graminearum*. M: 100-bp plus marker; Lane 1: negative control; Lane 2: positive control; Lanes 3–5: DON-nonproducing *F. graminearum* isolates; Lanes 6–8: DON-producing *F. graminearum* isolates. **(c)** M: 100-bp plus marker; Lane 1: negative control; Lane 2: positive control; Lanes 3, 4, 6, 8–10: NIV-nonproducing *F. graminearum*; Lanes 5, 7: NIV-producing *F. graminearum* isolates.

and water activity (0.70–0.90), toxigenic fungi can produce mycotoxins. Mycotoxins are fungal secondary metabolites produced by some fungi such as *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria*, which are detrimental to both humans and animals (19). Trichothecenes are compounds with a stable chemical structure and are considered one of the most important food contaminant mycotoxins in the world.

According to official Iranian standards, moisture content of corn and barley is below 14% and 12%, respectively (20,21). In the present study, the highest level of moisture in the corn and barley samples was found in winter and the lowest value was found in samples collected in summer, which was in agreement with the study by Jouany (22).

Mean total moisture content levels higher than 15% in cereals are usually required for the growth of fungi (22). In the present study there were a significant difference between corn and barley samples collected in winter, spring, and summer seasons separately ($P < 0.05$), but there were no differences between winter and fall seasons ($P < 0.05$). According to Birzele et al. (23), DON concentrations increase significantly in the presence of *Fusarium* spores if moisture level exceeds 17%–20% at 20 °C.

Results obtained from culturing barley samples in PDA medium demonstrated that the highest level of contamination was related to *Penicillium* (89.06%) and then *Aspergillus* (73.43%), both of which are capable of producing toxins. Among the studied corn samples, the highest level of contamination was related to *Penicillium* (90.62%), *Fusarium* (85.93%), and *Aspergillus* (82.81%), respectively, which can produce mycotoxins (Table 4). Results of investigating fungal flora in corn and barley samples in this study were in agreement with those of

Roige et al. (24), Gerbaldo et al. (25), and Ghiasian and Maghsood (26). Roige et al. (24) concluded that the highest numbers of fungi infecting corn seed used in animal feed in Argentina were *Penicillium* (70%), *Fusarium* (47%), and *Aspergillus* (34%).

The study conducted by Ghiasian and Maghsood (26) on samples of wheat, alfalfa, and barley used as animal feed for 93 dairy farms in Hamadan Province in different seasons demonstrated that the most widespread isolated fungi were *Aspergillus* (37.4%), *Penicillium* (23.7%), *Fusarium* (17.5%), *Cladosporium* (9.1%), *Alternaria* (4.3%), *Rhizopus* (3.9%), and *Mucor* (3.4%), respectively.

According to the results obtained in this study, *Fusarium* spp. was isolated in 55 (85.93%) of 64 corn samples and 17 (26.56%) of 64 barley samples.

Fusarium contamination in grains can be produced by different factors including physicochemical properties of seeds, sensitivity to fungus, plant variety, and moisture content of seeds in different phases of harvesting, collecting, and warehousing. Corn is more sensitive to various environmental damaging factors than barley, due to being less thick; thus, it is more prone to fungal damage. The results of this study also showed that corn samples contained a higher level of moisture than barley samples, which can be the cause of higher contamination rates with seed-borne fungi such as *Fusarium*.

All isolates (100%) that were determined as *Fusarium* spp. by culturing method were confirmed by PCR method (Figure 1a). In order to determine the species of *F. graminearum*, Fg16N-F/R-specific primers were applied (27,28). Eight (11.11%) of 72 detected *Fusarium* isolates were confirmed as *F. graminearum* (Figure 1b). Yoruk and Albayrak (28) investigated 33 *F. graminearum* strains isolated from Turkish wheat and barley farms with Fg16N-F/R-specific primers. Twelve of 33 studied isolates were confirmed as *F. graminearum*.

In this study, 5 of 8 *F. graminearum* isolates amplified a 380-bp fragment using Tri5-F/R-specific primers and were positive for trichothecene-producing genes (Figure 2a). In order to quickly detect *Fusarium* spp. with the purpose of preventing the entrance of mycotoxins into the food chain via agricultural products such as wheat, Amar et al. (17) used a Tri5-specific primer, which targets the route of trichothecene biosynthesis, to directly identify toxin-producing *F. culmorum*. In the PCR assay, 3 of 5 trichothecene-producing *F. graminearum* isolates were positive as DON producers (Figure 2b). Two out of 5 toxin-producing *F. graminearum* isolates were also positive as NIV producers, one of which was isolated from barley and the other from the corn samples (Figure 2c). Qu et al. (29) investigated the detection of DON- and NIV-producing *F. graminearum* isolates in grain samples collected from China, Nepal, the United States, and Europe based on the PCR method. A Tri13 primer was used to amplify a fragment with 583 bp, which indicated the presence of a DON-producing gene, and a fragment with 644 bp, which indicated the presence of a NIV-producing gene. Results demonstrated that this method was faster and more reliable than other detection methods in detecting mycotoxins in *Fusarium* species and could control the safety of food products. Using the PCR method, Astolfi et al. (30) conducted a study on barley seeds collected from some Brazilian farms to evaluate their capability in producing 13-AcDON, 15-AcDON, and NIV mycotoxins by *F. graminearum*. Results showed that 66%, 29.3%, and 4.4% of *F. graminearum* isolates were capable of producing 15-AcDON, NIV, and 13-AcDON, respectively.

In conclusion, this study showed a high level contamination of corn and barley samples with toxigenic fungi. Although the presence of toxigenic fungi in feedstuffs does not certainly indicate that mycotoxins are naturally occurring in the feed, it alerts us to the potential risk of contamination. The presence of trichothecene-producing strains of *Fusarium* in Iranian grain samples indicates the possibility of large-scale contamination of the grains with these toxins and thus the need for proper screening of food and feed commodities for the detection of these toxins or toxigenic fungi.

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