



## Journal of Experimental Biology and Agricultural Sciences

<http://www.jebas.org>

ISSN No. 2320 – 8694

### MORPHOMETRIC AND MOLECULAR DIVERSITY AMONG THE ISOLATES OF COLLETOTRICHUM SPECIES CAUSING ANTHRACNOSE DISEASE OF CHILLI

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Received – October 31, 2017; Revision – December 15, 2017; Accepted – January 16, 2018

Available Online – February 20, 2018

DOI: [http://dx.doi.org/10.18006/2018.6\(1\).124.130](http://dx.doi.org/10.18006/2018.6(1).124.130)

#### KEYWORDS

Chilli

Capsicum

Anthracoese

*Colletotrichum*

Fruit rot

AFLP

#### ABSTRACT

A total of sixty *Colletotrichum* isolates were purified from anthracnose disease samples, collected from 15 chilli growing districts of Karnataka. The isolates were evaluated for their morphological and genetic characterization using AFLP marker assay. Based on the morphological characterization, 40 isolates were identified as *Colletotrichum capsici/truncatum* and 20 as *C. gloeosporioides*. Considerable morphological variability was observed in *C. gloeosporioides* isolates compared to *C. capsici* isolates. AFLP marker assay could clearly differentiate the *C. capsici* and *C. gloeosporioides* isolates at 43% genetic similarity, thus complementing species classification based on morphological characterization. However, morphological and AFLP grouping of isolates indicates no clear correlation between clustering in the dendrogram and morphological grouping of *C. capsici* and *C. gloeosporioides* isolates this suggested existence of wide variability in both the species.

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Peer review under responsibility of Journal of Experimental Biology and Agricultural Sciences.

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## 1 Introduction

Chilli (*Capsicum annuum* L.) is an important vegetable cum cash crop of Indian subcontinent. The crop is grown largely for its pungent fruits, which are used both green and ripe to impart pungency to the food. The sustainability in chilli production is threatened by many biotic stresses such as several insect pests and diseases (Isaac, 1992). Among these, anthracnose disease caused by *Colletotrichum* species is a major constraint to chilli production in major chilli-growing regions of the world and often results in high yield losses (Than et al., 2008). This disease caused by a complex of *Colletotrichum* species, results in both pre and post-harvest fruit decay (Liu et al., 2016). The species of *Colletotrichum* associated with anthracnose disease includes *Colletotrichum capsici*, *C. gleosporioides*, *C. truncatum*, *C. dematium*, *C. acutatum*, *C. siamense*, *C. fruticola* (Hong & Hwang, 1998; Gopinath et al., 2006; Sharma & Shenoy 2014), *C. coccodes* and *C. karstii* (Saini et al., 2016).

*Colletotrichum* infecting diverse hosts including cereals, legumes, vegetables, perennial crops and tree fruits have a high degree of pathogenic variability. Thus the accurate identification of the pathogen and species differentiation is imperative in development of appropriate management practices. Also studies on the variability of the pathogen populations are needed to direct breeding efforts towards long term resistance to anthracnose disease.

Classically, identification and characterization of *Colletotrichum* spp. was primarily relied on morphological characters such as colony color and growth rate, shape and size of conidia, optimal temperature for growth, presence or absence of setae and existence of the teleomorph (Adaskaveg & Hartin, 1997; Freeman et al., 1998). Although these criteria are valuable but alone are not always adequate for reliable differentiation among *Colletotrichum* spp. due to variation in morphology and phenotype among species under environmental influences (Cannon et al., 2000).

To overcome the inadequacies of these traditional schemes, molecular techniques combined with morphological studies have proven to be effective for characterization of *Colletotrichum* species (Sreenivasaprasad & Talhinhas, 2005; Van Hemelrijck et al., 2010). Therefore the present investigation was carried out to assess the variability in *Colletotrichum* spp. infecting chilli in Karnataka state of India using morphometric and molecular approaches

## 2 Materials and Methods

### 2.1 Collection and isolation of *Colletotrichum* spp.

Chilli fruits showing typical symptoms of anthracnose disease were collected from farmer's fields and chilli markets of 15 chilli growing districts of Karnataka, India (Table 1). The pathogen was isolated by following standard tissue isolation method (Karuna-

Table 1 *Colletotrichum* spp. infecting chilli samples collected from 15 districts of Karnataka

District	<i>Colletotrichum</i> spp. identified	Isolates
Gulbarga	<i>C. capsici</i> (Cc), <i>C. gleosporioides</i> (Cg)	Cc 1 Cg 1
Hassan	<i>C. capsici</i> , <i>C. gleosporioides</i>	Cc 2 Cg 18
Bellary	<i>C. capsici</i>	Cc 3, Cc 4, Cc 40
Hubli	<i>C. capsici</i>	Cc 5, Cc 6, Cc 7
Gadag	<i>C. capsici</i> , <i>C. gleosporioides</i>	Cc 8, Cc 9, Cc 10, Cc 11 Cg 2
Haveri	<i>C. capsici</i>	Cc 12, Cc 13, Cc 14, Cc 15, Cc 16, Cc 17, Cc 18, Cc 19
Dharwad	<i>C. capsici</i> , <i>C. gleosporioides</i>	Cc 20, Cc 21, Cc 22, Cc 23, Cc 24, Cc 30, Cc 31 Cg 3, Cg 4, Cg 5, Cg 6, Cg 7, Cg 8, Cg 9
Chitradurga	<i>C. capsici</i>	Cc 25, Cc 26, Cc 27, Cc 28
Tumkur	<i>C. capsici</i>	Cc 29
Raichur	<i>C. capsici</i> , <i>C. gleosporioides</i>	Cc 32, Cg 10
Bijapur	<i>C. capsici</i>	Cc 33, Cc 34
Davanagere	<i>C. capsici</i>	Cc 35, Cc 36
Chikkamagalur	<i>C. capsici</i> , <i>C. gleosporioides</i>	Cc 37 Cg 14, Cg 15, Cg 16
Bangalore	<i>C. capsici</i> , <i>C. gleosporioides</i>	Cc 38, Cc 39 Cg 17
Belgaum	<i>C. gleosporioides</i>	Cg 11, Cg 12, Cg 13
Kolar	<i>C. gleosporioides</i>	Cg 19, Cg 20

vishunavat & Kolte, 1998). The pathogen was purified using single spore isolation technique (Karuna-vishunavat & Kolte, 1998). The pathogenicity of monoconidial cultures of all the isolates was conducted by inoculating on hybrid NS1701 by following detached fruit method (AVRDC, 2003).

## 2.2 Morphological variability

Morphological variability viz., colony characteristics (colony colour, texture, margin and radial growth) and conidial morphology of 60 isolates of *Colletotrichum* spp. was assessed on Potato Dextrose Agar (PDA) medium. Petri dishes containing 15 ml of PDA medium was inoculated centrally with 5 mm diameter mycelial disc taken from the periphery of 5 days old culture. Three replications were maintained for each isolate and the plates were incubated at 25±1°C. Conidial size, shape and acervuli size of 60 isolates were recorded using Leica bright field microscope. The colony morphology was recorded by following mycological chart (Rayner, 1970).

## 2.3 Molecular variability

Amplified Fragment Length Polymorphism (AFLP) molecular marker assay was used to study the variability among sixty isolates of *Colletotrichum* spp. of Karnataka. Two isolates each of *C. capsici* and *C. gloeosporioides* sampled from Andhra Pradesh which were earlier characterized for their virulence pattern were also included in the study (Nanda, 2011).

## 2.4 Isolation of DNA and PCR amplification

The DNA was extracted from five days old mycelia grown on Potato dextrose broth by following procedure of Sharma et al. (2005) with minor modifications. AFLP protocol outlined by Vos et al. (1995) was followed with minor modifications in some steps. A total of nine, three nucleotide selective primer combinations were used for the study of genetic diversity in 60 isolates of *Colletotrichum* spp. The primers were first screened using DNA and the combinations that showed good amplification were selected for final amplifications.

Genomic DNA was digested with 4 units of *MseI* and 10 units of *EcoRI* restriction endonucleases at 37° C for 3 hours in a PCR machine. The digested product was loaded on to 1.2 per cent agarose gel to confirm the complete digestion of the genomic DNA. The digested DNA was ligated with *MseI* and *EcoRI* adapters with T4 DNA ligase. Ligation reaction mix was incubated at 37°C for overnight. The digested / ligated product was diluted to 1:10 with T<sub>10</sub>E<sub>1</sub> (pH8) and stored at -20°C.

An initial round of PCR (Pre-amplification) was carried out to enrich a subset of the AFLP template; the primers used in pre-amplification have a single base selection. In the

Pre-amplification reaction, a 3.0 µl diluted ligation product, 2 µl each of *MseI* and *EcoRI*, 2 µl dNTPs, 1µl 10X PCR buffer were mixed in 1.0 unit of *Taq* polymerase and used in total volume of 10.2 µl. Pre-amplification was performed with an amplification profile of 94°C for 30 s, annealing at 56°C for 1 min, extension at 72°C for 1 min, repeated for 20 cycles and then at 10°C for 30 min. The pre-amplified product was loaded on to 1.2 % agarose gel to check the amplification. Depending on the amplification intensity, the amplified product was diluted to 1:3 in TE buffer and used as template for re-amplification using AFLP primers, each containing three-selective nucleotides. Re-amplification PCR was performed in 10.2 µl reactions containing of 3.0 µl template DNA, 2.0 µl each of *EcoRI* and *MseI* selective nucleotide primer, 2.0 µl dNTPs, 1µl 10X PCR buffer and 1.0 unit of *Taq* polymerase. The re-amplification reaction was carried out with cycling parameters of 94°C for 30s, 65°C for 30s reducing by 0.7°C / cycle to 56°C, 72°C for 1 min for 11 cycles, and 94°C for 30 s, 56°C for 30 s and 72°C for 1 min for 24 cycles followed by 10° C for 30 minutes.

PCR were carried out separately for each primer pair and the products were denatured immediately by adding eight microlitres of stop loading dye to each sample. Denaturation was carried out at 94°C for 5 min and then cooled to 10°C for 5 min. Electrophoresis of the samples was carried out on 6% polyacrylamide gels, by loading 3 µl of each final PCR product. Electrophoresis was carried out at 1,200 V for 3.0h until the dark blue dye ran off. The gels were then separated and developed silver staining technique.

## 2.5 Analysis of AFLP profiles

The amplicons generated in AFLP, behave as dominant markers. Therefore, the score '1' was assigned for the presence of band and '0' for absence of band at each loci. The variation in band intensity is not taken into consideration to avoid confusion in scoring. The binary data was used to estimate pair wise genetic distance based on Jaccard's coefficients using NTSYS-pc version 2.0 software. Dendrogram was constructed using Unweighted Paired Group Arithmetic Mean (UPGMA) algorithm based on distance matrix.

## 3 Results and Discussion

In total sixty isolates of *Colletotrichum* species were purified after isolation from infected chilli fruits collected from 15 chilli growing districts of Karnataka (Table1). Among the sixty collected isolates 40 fitted the description of *C. capsici/truncatum* and twenty fitted the description of *C. gloeosporioides* (Table 6). All the 60 isolates of *Colletotrichum* produced typical anthracnose symptoms such as sunken circular spots on fruit surface on 3<sup>rd</sup> day after inoculation.

Table 2 Colony growth rate of *Colletotrichum capsici* (Cc) isolates on Potato dextrose agar (PDA) and Chilli fruit extract agar (CFEA) media

Sl. No.	Isolate No.	Colony diameter (mm)	
		PDA	CFEA
1	Cc 1	67	75
2	Cc 2	70	80
3	Cc 3	70	74
4	Cc 4	68	72
5	Cc 5	71	75
6	Cc 6	70	83
7	Cc 7	71	87
8	Cc 8	70	79
9	Cc 9	72	80
10	Cc 10	69	75
11	Cc 11	70	76
12	Cc 12	68	90
13	Cc 13	67	86
14	Cc 14	64	80
15	Cc 15	69	82
16	Cc 16	70	80
17	Cc 17	73	78
18	Cc 18	72	88
19	Cc 19	62	81
20	Cc 20	65	78
21	Cc 21	70	89
22	Cc 22	62	84
23	Cc 23	61	83
24	Cc 24	68	82
25	Cc 25	69	80
26	Cc 26	65	80
27	Cc 27	62	74
28	Cc 28	64	83
29	Cc 29	68	84
30	Cc 30	65	80
31	Cc 31	69	81
32	Cc 32	64	73
33	Cc 33	65	80
34	Cc 34	64	84
35	Cc 35	62	83
36	Cc 36	60	82
37	Cc 37	65	74
38	Cc 38	64	75
39	Cc 39	68	72
40	Cc 40	70	74
CD @ 1%		2.76	

### 3.1 Morphological variability

The colony growth rate of both *C. capsici* and *C. gloeosporioides* isolates varied and results of study revealed that isolates of *C. gloeosporioides* grew faster than *C. capsici* isolates (Table 2 & 3). All 40 *C. capsici* isolates were produced whitish grey colony with irregular margin and flat texture. However, twenty *C. gloeosporioides* isolates were categorized into three morphological groups (CgI, CgII, CgIII). CgI produced white colony with circular margin and fluffy textured while CgII and CgIII formed whitish grey colony with fluffy textured but differed in colony margin (Table 4 & 5).

Earlier researchers also reported such morphological variations among *Colletotrichum* spp. Sharma et al. (2005) categorized 37 isolates of *C. capsici* into five morphological groups based on variation in morphological traits. Than et al. (2008) differentiated isolates of chilli anthracnose from Thailand into three species, viz., *C. capsici*, *C. accutatum* and *C. gloeosporioides*. Similarly, Thind & Jhooty (1985) categorized 150 isolates of *C. capsici* and *C. gloeosporioides* causing anthracnose of chilli into eight groups on the basis of colony diameter and number of acervuli.

Table 3 Colony growth rate of *C. gloeosporioides* (Cg) isolates on Potato dextrose agar (PDA) and Chilli fruit extract agar (CFEA) media

Sl. No.	Isolate No.	Colony diameter (mm)	
		PDA	CFEA
1	Cg 1	78	90
2	Cg 2	76	78
3	Cg 3	80	90
4	Cg 4	89	89
5	Cg 5	90	90
6	Cg 6	90	90
7	Cg 7	89	90
8	Cg 8	90	88
9	Cg 9	90	89
10	Cg 10	85	90
11	Cg 11	90	90
12	Cg 12	88	90
13	Cg 13	82	90
14	Cg 14	83	90
15	Cg 15	85	90
16	Cg 16	90	90
17	Cg 17	78	90
18	Cg 18	88	90
19	Cg 19	80	74
20	Cg 20	79	85
CD @ 1%		3.03	

Table 4 Colony characters of *C. capsici* and *C. gloeosporioides* isolates on Potato dextrose agar (PDA) medium

Group	<i>C. capsici</i> (Cc) and <i>C. gloeosporioides</i> (Cg) isolates	Colony characters		
		Colour	Texture	Margin
CcI	Cc 1 to Cc 40	Whitish gray	Flat	Irregular
Cg I	Cg 1, Cg 2, Cg 3, Cg 9, Cg 13, Cg 14, Cg 17, Cg 19, Cg 20	White	Fluffy	Circular
Cg II	Cg 4, Cg 7, Cg 10, Cg 12, Cg 18	Whitish gray	Fluffy	Irregular
Cg III	Cg 5, Cg 6, Cg 8, Cg 11, Cg 15, Cg 16	Whitish gray	Fluffy	Circular

Table 5 Colony characters of *C. capsici* and *C. gloeosporioides* isolates on Chilli fruit extract agar (CFEA) medium

Group	<i>C. capsici</i> (Cc) and <i>C. gloeosporioides</i> (Cg) isolates	Colony characters		
		Colour	Texture	Margin
Cc I	Cc 1 to Cc 40	Grayish Black	Flat	Irregular
Cg I	Cg 6, Cg 7, Cg 8, Cg 9, Cg 10, Cg 11, Cg 12, Cg 13, Cg 14, Cg 15, Cg 16, Cg 17, Cg 18	Grayish olive green	Fluffy	Circular
Cg II	Cg 1, Cg 2, Cg 3, Cg 4, Cg 5, Cg 19	Whitish gray	Fluffy	Circular

Table 6 Characters of Acervuli and conidia produced by *C. capsici* and *C. gloeosporioides* isolates on Potato dextrose agar (PDA) medium

<i>Colletotrichum</i> isolates	Conidia shape	Conidia size ( $\mu\text{m}$ )	Acervulli size ( $\mu\text{m}$ )
<i>C. capsici</i> (Cc 1 to Cc 40)	Falcate with acute apex	21.4- 25.0 x 3.8 -4.3	149.03 - 165.34
<i>C. gloeosporioides</i> (Cg 1 to Cg 20)	Cylindrical with obtuse end	10.8-12.1 x 3.1-3.9	135.20 -154.21

According to Jameel-Akhtar et al., (2008), reported that *C. capsici* produced falcate fusiform conidia with narrow truncated acute apices. They were normally one-celled, hyaline and uni-nucleate while, Prasanna Kumar (2001) reported that *C. gloeosporioides* produce hyaline, unicellular and either cylindrical with obtuse ends or elliptical conidia with a rounded apex or narrow truncate base conidia.

In the current study higher morphological variability was observed in *C. gloeosporioides* isolates on both tested media as compared to *C. capsici* isolates. These findings are similar to previous report, higher variability was observed within the *C. gloeosporioides* populations where the genetic heterogeneity may be explained by the presence of a perfect stage (Freeman et al.,1998).

### 3.2 Molecular variability

A total of 180 AFLP loci were sampled using nine primer combinations and all the primers were highly efficient in detecting polymorphism to an extent of 100%. Highest number of loci was sampled with the primer combination 5 (E+AAG, M+GCC). Clustering of *Colletotrichum* spp. isolates based on Jaccard's

dissimilarity co-efficient and UPGMA algorithm could clearly differentiate the two species of *Colletotrichum* at 43 per cent genetic similarity (Figure 1). In the cluster consisting of 40 Cc isolates, the isolates were sub grouped under 5 main clusters, 8 subclusters and 4 solitary clusters. Twenty Cg isolates were grouped under second subgroup with 3 main clusters, 5 subclusters and 2 solitary clusters.

Highly virulent *C. capsici* isolate, Cc 40 (sampled from Bellary district) formed a solitary cluster and highly virulent Cg isolate sampled from Dharwad district and Cg isolates sampled from Belgaum grouped into single cluster. Highly virulent *C. gloeosporioides* isolate of Andhra Pradesh formed a solitary cluster. The clustering in the dendrogram was not clearly correlated with geographical distribution and morphological characterization.

Considering that morphological character based assay alone is not always adequate for species identification due to overlap in morphological characters between species (as they evolve under different natural pressure) (Adaskaveg & Hartin, 1997). Application of both molecular diagnostic tools and morphological traits is an appropriate option for studying *Colletotrichum* species

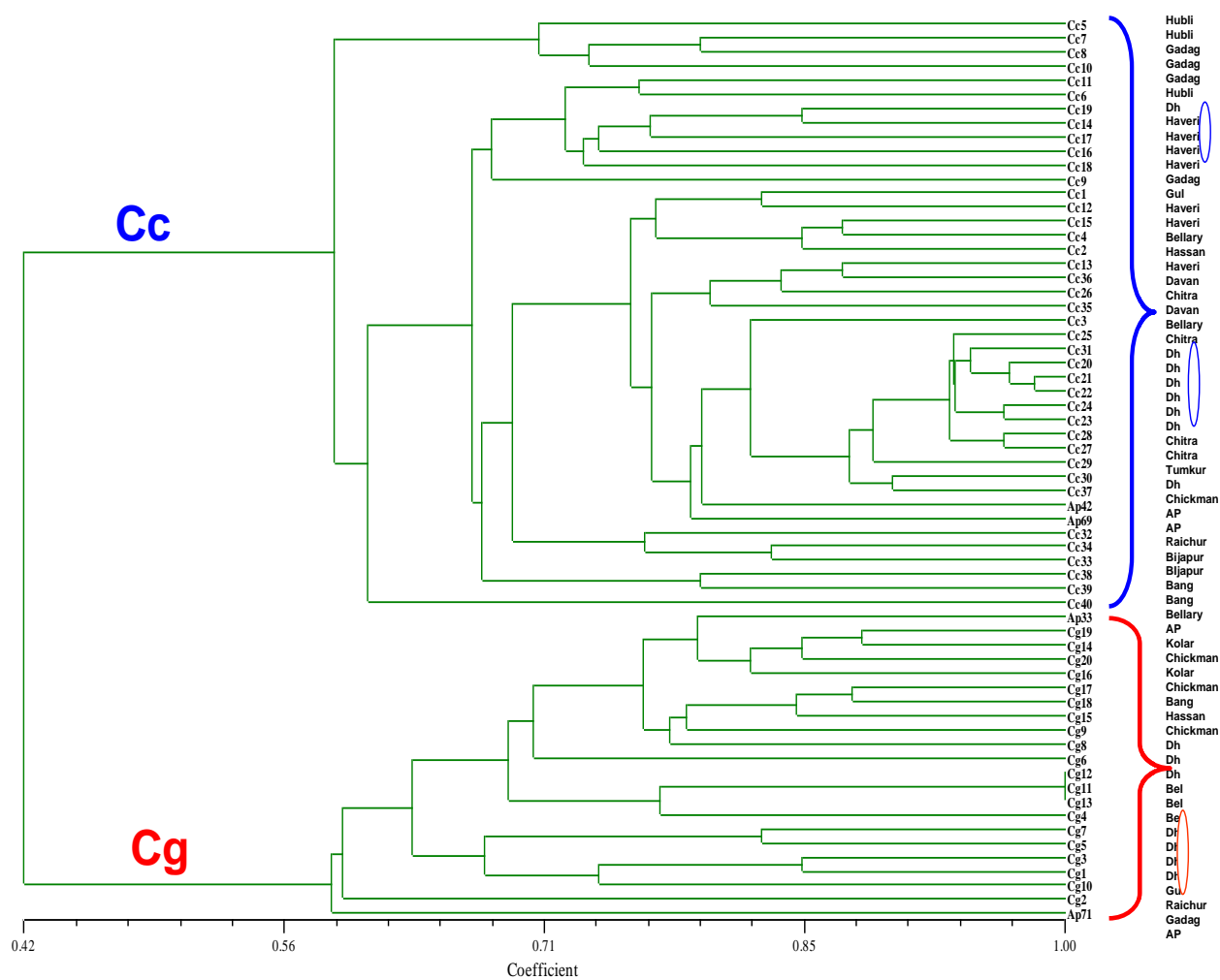


Figure 1 AFLP marker data-based dendrogram depicting grouping of *Colletotrichum* spp. isolates

complexes (Cannon et al., 2000). Thus, in the present study DNA-based assay using AFLP marker system was used along with morphological traits to confirm species identity of the *Colletotrichum* isolates.

Similarly Than et al. (2008) characterized three *Colletotrichum* spp viz., *C. acutatum*, *C. capsici* and *C. gloeosporioides* associated with anthracnose disease based on morphological characterization and by sequencing ITS region and beta tubulin gene and pathogenicity. Ratanacherdchai et al. (2010) analyzed the genetic diversity among isolates of *C. gloeosporioides* and *C. capsici* from Thailand by Inter simple sequence repeat (ISSR) analysis and reported that there were two distinct groups of *C. gloeosporioides* and *C. capsici*.

Morphological and AFLP based assays could clearly differentiate

the 60 isolates of *Colletotrichum* spp. into two species viz., *C. capsici* and *C. gloeosporioides*. Considerable inter and intra-specific variability was observed among the isolates of both *C. capsici* and *C. gloeosporioides*. The accurate taxonomic identification of the pathogen and their variability plays a major role in disease dynamics and consequently in the disease management strategies. Use of resistant cultivars is an economical and eco-friendly option for effective management of the disease. However, management of disease through breeding of pathogen-resistant cultivars has only had limited success due to frequent breakdown of resistance under field conditions because of higher variability, pathogen may adopt faster to fluctuating environment. Thus the variability among the isolates of *C. capsici* and *C. gloeosporioides* should be considered when these two species are used for screening of chilli genotypes for anthracnose resistance.

### Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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