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Original scientific article

INVESTIGATION ON GENETIC DIVERSITY OF PEPPER (*Capsicum* spp.) PARENTS AND INTERSPECIFIC HYBRIDS USING ISSR MARKERS

Todorka A. SREBCHEVA* and Milena L. KOSTOVA

Department of Plant physiology, Biochemistry and Genetics, Agricultural University - Plovdiv, Faculty of Agronomy, Plovdiv, Bulgaria

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Pepper (*Capsicum*) is one of the most important and widespread vegetable crops in the Balkans. Some old indigenous forms are not the focus of modern breeding but are preserved in some areas and represent valuable genetic resources. Three Bulgarian varieties (Plovdivska kapiya, Familiya and IZK Delicates) *C. annuum*, and two representatives of the chili pepper *C. chinense* and *C. frutescens*. Interspecific hybrids were made between sweet (*C. annum*) and chili pepper plants. The genetic relationships of the varieties of *Capsicum* species were assessed using ISSR primers. PCR amplification of isolated DNA from parental lines and interspecific hybrids revealed 65 distinct polymorphic bands. Cluster analysis clearly distinguished the parental forms and individuals from the F_1 and F_2 populations. The applied ISSR molecular technique can be successfully used to analyze genetic diversity in cultivars, early-stage seedlings, and interspecific hybrids, as well as to detect differences in individuals whose genomes are highly homogeneous, such as those of the genus *Capsicum*.

Keywords: Capsicum spp., cluster analysis, genetic diversity, interspecific hybrids

INTRODUCTION

Pepper belongs to the family *Solanaceae*, genus *Capsicum*. Five species are domesticated: *Capsicum annuum*, *C. frutescens*, *C. shinense*, *C. pubescens* and *C. baccatum* (LIPPERT, 1966; ESHBAUGHT, 1993; POZZOBON *et al*., 2005; DE TEODORO-PRADO *et al*., 2007). There are three genetic complexes based on the degree of genetic similarity and hybrid compatibility between the five domesticated species and closely related wild species. The complexes are *C. annuum* (*C. annuum*, *C. chinense* and *C. frutescens*), the *C. baccatum* complex (*C. baccatum*, *C. praetermissum* and *C. tovarii*) and the *C. pubescens* complex (*C. pubescens*, *C. cardenasii* and *C. eximium*). In addition, *C. chacoenseis* is sometimes assigned to *C. annuum* complex or *C. baccatum* complex (SHIRAGAKI, *et al*., 2020) depending on the method of phylogenetic analysis.

Corresponding author: Todorka A. Srebcheva, Department of Plant physiology, Biochemistry and Genetics, Agricultural University - Plovdiv, Faculty of Agronomy, 12, Mendeleev, Blvd., Plovdiv 4000, Bulgaria, Tel: +359 32 654 343, E-mail: teasrebcheva@abv.bg

In the different countries of the Balkan region, alongside the main production varieties, many indigenous pepper populations are cultivated with specific characteristics in terms of shape, color, taste, biological value, and use of the fruit. Biodiversity loss and ecosystem degradation have become major problems worldwide, making the process of conservation and protection of available genetic resources an important strategy for maintaining biodiversity. Conservation and characterization of conserved accessions are paramount to ensure that genetic diversity is useful for bioeconomy and genetic improvement.

With the increase in the number of registered varieties (cultivated and wild species) and increased breeding work, registration of new varieties will become difficult if not impossible (FOOLAD, 2007). This situation will have a negative impact on breeding teams due to reduced confidence in the system for protecting breeders' rights to potential new varieties, which may lead to disputes between breeders, traders, and farmers.

DNA marker technologies are proposed to overcome these problems. Molecular markers provide accurate genetic information on many biochemical, cytological, and morphological traits and help to better understand the genetic relationships between different plant species. Genetic connectivity and variation assessment are critical for the effective management and improvement of crop plants (NADEEM *et al*., 2018).

Using molecular markers, pungency genes can be detected at an early stage of seedling development (MAZOUREK *et al*., 2009). Pepper fruits are also rich in the carotenoid capsanthin, an antioxidant that gives them their red colour (MATEOS *et al*., 2003; MOHD et al. 2019). Since the establishment of the antioxidant properties of pigments and their importance for human health, the colour of fruits has been given even greater importance (FOOLAD, 2007).

In the present study, the genetic relationship within and between *Capsicum* species was evaluated using ISSR markers to better understand their species affiliation and the characterization of interspecific hybrids.

MATERIALS AND METHODS

Plant material

Local populations and cultivars belonging to *Capsicum annuum*, *Capsicum frutescens*, and *Capsicum chinense* were used in this study. The description of parental forms is presented in Table 1.

Table 1. Pepper varieties used in this study

Pepper varieties	Species	Collection source	Fruit type	Pungency
Plovdivska kapiya	*C. annum*	Breeders variety	Blocky/bell	Non-pungent
Familiya	*C. annum*	Breeders variety	Italian/bell	Non-pungent
IZK Delikates	*C. annum*	Breeders variety	Long horned	Non-pungent
Specimen 4	*C. chinense*	Wild variety	Habanero	Highly pungent
Specimen 5	*C. frutiscens*	Wild variety	Conical	Highly pungent

In 2018-2020, F_1 parental forms were bred from six crosses between sweet and spicy parents and a selected F_2 population. Three sweet Bulgarian varieties (Plovdivska kapiya, Familiya and IZK Delicates) - *C. annuum*, were selected as non-pungent parental forms, and *C. chinense* and *C. frutescens* as pungency species. The parental forms were cross-pollinated to

obtain F_1 plants from 6 crosses (Table 2). For this study, 13 randomly selected samples (f) of the F_2 population from all 6 crosses were also analyzed.

Table 2. The analyzed crosses from the samples set in 2019 as follows

II	1x2	♀ Plovdivska kapiya x ♂ *C. chinense*
III	1x4	♀ Plovdivska kapiya x ♂ *C. frutiscens*
IIII	3x2	♀ Familiya x ♂ *C. chinense*
IIV	3x4	♀ Familiya x ♂ *C. frutiscens*
V V	5x4	♀ IZK Delikates x ♂ *C. chinense*
VVI	5x2	♀ IZK Delikates x ♂ *C. frutiscens*

Genomic DNA Extraction

DNA was isolated from the last young, fully developed leaf of plants selected and tagged for the study and grown outdoors. Three hundred milligrams of leaf material from each specimen were sheared after freezing with liquid nitrogen to produce a fine light green powder. For DNA extraction, the method using the innuPREP plant DNA extraction kit (Analiticjena) was applied following the manufacturer's instructions. The Epoch microplate spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) was used to examine the quality and quantity of DNA. Gels were stained with ROTI GelStine (Roth, Germany) and visualized under UV light.

ISSR analysis

The primers used to perform the ISSR assay (Table 3) were selected from a set of primers that have shown high levels of reproducibility and potential for polymorphism identification in previous studies. ISSR PCR reactions were performed in a 25 µl reaction volume using the following primers per reaction: 1.5 µl ISSR primer; 12.5 µl Master Mix My Taq Red Mix (Bio line), H2O -10 µl; 1 µl genomic DNA. ISSR PCR reactions were performed under the following amplification regime: denaturation at 94ºC for 3 min, 40 cycles at 94ºC - 1 min, primer melting temperature - 45 sec, extension 72ºC - 45 sec, followed by a final extension at 72ºC - 4 min, where the melting temperature of each primer was calculated according to KOCHIEVA *et al*. (2002a). The size of the ISSR allele is determined by the position of the bends relative to the ladder (100 bp Rainbow). The total number of alleles was reported for each ISSR marker in all genotypes tested. Amplified alleles were recorded as 1 (presence of band) and 0 (absence of band) in a binary matrix. After separation of the products, the gel was stained with ethidium bromide and visualized by illumination with UV light.

Table 3. Characteristics of primers used to perform ISSR analysis.

Primer	DNA sequence 5' – 3'	Lenght (bp)	Melting temperature (ºC)
ISSR P7	AC(8)YG	18	53.9
ISSR P14	AG(8)YT	18	51.4
ISSR P11	GA(8)YC	18	53.9
ISSR P8	AC(8)G	17	52
ISSR PE6	AC (8)CTG	19	55.4

Genetic diversity and cluster analysis

Overall polymorphism levels were estimated based on the resulting multilocus anonymous dominant markers. The amplification profile of ISSR genotypes was used to estimate genetic similarity based on the number of total bands amplified. The resulting molecular data were used to calculate relative genetic distances and to create hierarchical clusters with the statistical package "SPSS for Windows".

RESULTS AND DISCUSSION

Hybrid lines characterization

After conducting controlled self-pollination of selected plants from F_1 crosses (*C. annuum* - Familiya x *C. chinense* - Habanero and *C. annuum* - IZK Delicates x *C. frutescens*), seeds were collected and F_2 plants were grown in the following growing season. They showed a reduction in the expression of the fruit scaling trait. The data from the organoleptic analysis performed were used to compare the results obtained from the experiment and the predictions of the chosen hypothesis (one dominant *Pun1* gene controls fruit scaling (STEWART *et al*., 2007). In the cross, *C. annuum* - Familiya x *C. chinense* - Habanero, ¾ of the plants are expected to have spicy and ¼ sweet fruits. Organoleptic and laboratory analyses proved the hybrid nature of F_1 and found a 3:1 split of the pungent/non-pungent trait in the F_2 generation of the *C. annuum* - Familiya x *C. chinense* - Habanero and *C. annuum* - IZK Delicates x *C. frutescens*. The genetic analyses in our previous study proved that the hotness in the investigated Bulgarian pepper cultivars *Capsicum annuum* is due to the presence of the dominant *Pun1* allele and the absence of hotness is due to the homozygous state of the most frequent recessive allele *pun1-1* - (*pun1-1*/*pun1-1*) (data not shown). The *Pun1* gene has typical Mendelian inheritance (PICKERSGILL, 2007). The *pun1*-1/*pun1*-1 genotype phenotypically defines non-pungent peppers. *Pun1/Pun1* and *Pun1*/*pun1*-1 genotype phenotypically define pungent peppers SREBCHEVA and KOSTOVA (2022).

ISSR analysis

The importance of germplasm characterization is an important link between the conservation and utilization of plant genetic resources in various breeding programs (RANA *et al.*, 2014). ISSR molecular markers are now widely used for the taxonomic identification of closely related *Capsicum* species (OLATUNJI *et al*., 2019).

The genetic diversity of pepper in Bulgaria has not yet been analyzed in detail and this study provides valuable information to support vegetable breeding programs. It has been reported as a basis for accurate assessment of genetic distances among pepper genotypes from a large Bulgarian collection of local, indigenous, and modern cultivars using different types of DNA markers (TSONEV *et al*., 2017). BAHRAMI *et al.* (2009) in a study used RAPD markers to determine genetic diversity among 39 genotypes collected from different regions of Iran and other countries. One hundred random markers were initially tested on genomic DNA, which revealed a significant degree of polymorphism. Using RAPD and ISSR markers, Chen Xuejun et al. also investigated genetic diversity in pepper (XUEJUN *et al*., 2007). Their experience showed that the genetic differentiation rate (GDR) determined using ISSR markers was greater than that based on RAPD. All genotypes were clearly differentiated in the dendrograms prepared using the ISSR marker system of Greek landraces of pepper (TSABALLA *et al*., 2015). This means that

ISSR markers can better reveal the genetic diversity in pepper crosses and are more suitable for this type of evaluation (IBARRA-TORRES *et al*., 2015).

The rapid and accurate genetic purity test of F_1 hybrid plants from p. *Capsicum* is essential for seed production and in various breeding programs. DNA technology has great potential to improve hybrid purity assessment. MONGKOLPORN *et al*. (2004) determined the genetic purity of three F_1 hybrids of *Capsicum* using two marker system techniques, RAPD and ISSR (Inter-Simple Sequence Repeat). The choice of the optimum heat treatment temperature for the PCR reaction was determined based on the high purity of the bands obtained. Primers can produce amplified products at a specific hybridization temperature and can be used for the genotypic characterization of samples. To assess the heterogeneity present in each of the genotypes studied, a set of ISSR primers was used and all 24 samples were analyzed. Individual plants from each sample were examined to confirm the ability of the selected marker system to detect a sufficient number of polymorphisms. After initial screening of the primers, it was found that some of them did not result in amplified fragments. These results may be due to the absence or low number of the corresponding microsatellite sequence in the genome of the test samples. Running the reactions with the selected primers resulted in one or more polymorphic fragments for each specimen. For some samples, fewer numbers of polymorphic fragments were obtained. Obtaining such results is expected due to the low levels of genetic diversity in a self-pollinating plant such as pepper.

Determining whether individual fragments are specific to each genotype (sample) involves the use of pooled samples. On the other hand, the use of pooled samples would hide the level of heterogeneity of genotypes, which would interfere with the reproducibility of the results if there are heterogeneous individuals in the sample. Because the probability of such heterogeneity within each genotype is not negligible, as shown by the studies of COOKE *et al*. (2003) and BREDEMEIJER *et al*. (2002), and we had no prior information on the genotypes that were analyzed in this study, we resorted to comparing only individual plants from each sample. CHENG *et al*. (2016) described a large set of unique microsatellite primer pairs derived from in silico evaluation of the DNA sequences of six different pepper genomes (nuclei, mitochondria, chloroplasts) with a tested random set of 160 primer pairs, of which only 65 exhibited polymorphisms among the 21 pepper genotypes analyzed. It can be concluded that ISSR markers are an effective multilocus system for genetic diversity detection, genotyping (passporting), genome mapping, tracing evolutionary relationships, seed purity analysis, etc (LÓPEZ CASTILLA *et al*., 2019). Since they are PCR-based, a small amount of DNA is needed to perform this type of analysis. The lack of need for prior knowledge of genome sequences makes them easier to use than SSR markers while being more reliable and reproducible than RAPD (RAI *et al*., 2013). The ability to obtain many fragments with a single PCR reaction is also a plus, especially when working with sites that differ little from each other. Given the above facts and the positive results obtained in previous studies, the ISSR marker system was chosen to conduct the present study.

Phylogenetic analysis

The use of the ISSR primers resulted in the appearance of many polymorphic bands. Initial screening results demonstrated the potential of the chosen marker system to identify differences between individual samples as well. A greater number of polymorphic bands is a

desirable and necessary condition for more accurate detection of genetic diversity among samples. Further, it is suggested that molecular markers are valid tags for the assessment of genetic diversity in *Capsicum* spp. cultivars (ALAYACHEW *et al*., 2017).

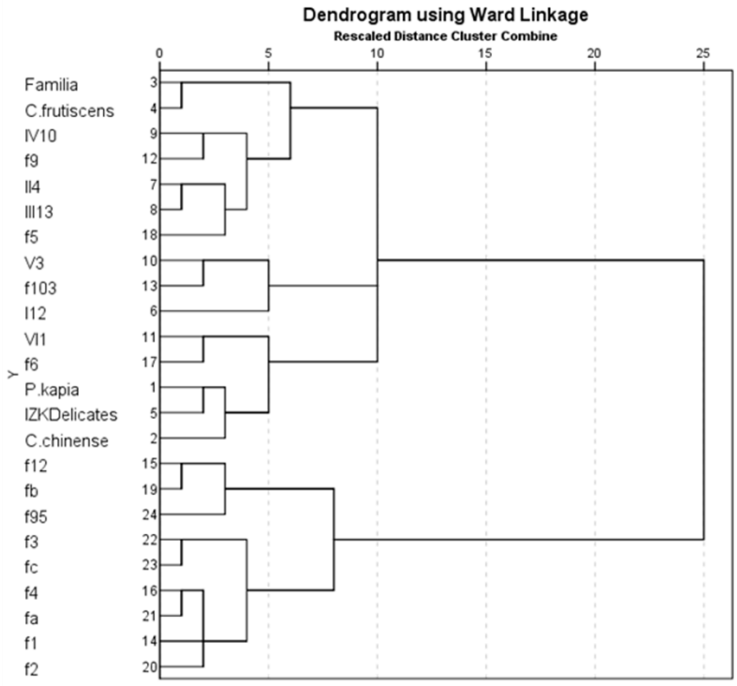

Figure 1. Clustering of samples based on identified ISSR polymorphisms of parental forms, F_1 and F_2 population

Based on the 5 polymorphic ISSR markers used, 65 polymorphic bands were obtained. We clearly distinguished fragments ranging in size from 350 to 2000 base pairs. The average number of bands generated with the polymorphic primer was 6, and the maximum was 9 for primer PE8. The average number of products for each primer was 8, and the reported polymorphism level was 4.4.

Cluster analysis was performed based on genotype profiling of all samples using the five ISSR primers. The 1/0 system generated dendrograms, with one representing polymorphic bands and zero representing monomorphic and missing bands in the corresponding genotype. The results of this analysis show a distribution in two main clusters. The cluster including the original parental forms and F_1 crosses is clearly distinguished.

The subclusters also show the relative genetic distances between individual samples (Fig. 2). The expected grouping of parental lines in the subclusters is also observed, with genetic proximity shown by the cultivars 'Familiya' and *C. frutiscens* respectively. and the cultivars

'Plovdivska kapiya', 'IZK Delicates', and *C. chinense*. The results of the phylogenetic analysis of *Capsicum* species from West Africa obtained from UPMGA reinforce the thesis that the four *C. annuum* and *C. frutescens* are varieties of a single species (OLATUNJI *et al.*, 2019). Figure 2 shows a dendrogram with the analyzed distribution of parental genotypes and F_1 generation crosses. The genetic relatedness among accessions determines the expected grouping into subclusters. Figure 3 shows a dendrogram of the analyzed crosses compared to the F_2 accessions. Two main clusters are distinguished, indicating the genetic distance between the accessions.

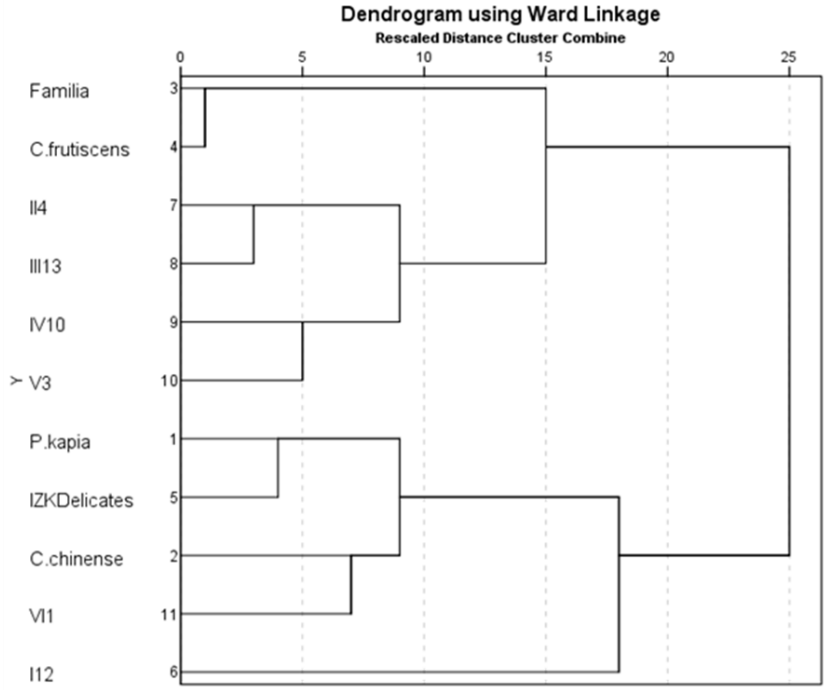

Figure 2. Clustering of selected parental forms and F_1 crosses based on ISSR mole

With the increasing number of registered varieties (of cultivated peppers and wild species) and increased breeding work, registration of new varieties will become difficult, if not impossible (FOOLAD, 2007). This would negatively impact breeding teams by reducing confidence in the system to protect breeders' rights on possible new varieties, possibly leading to disputes between breeders, traders, and farmers. DNA-based marker technologies have been proposed to overcome these problems.

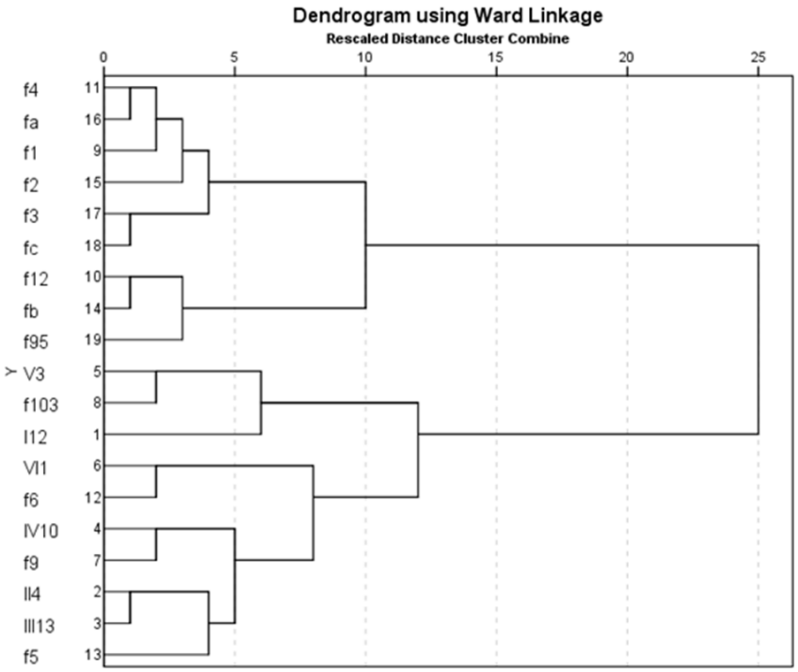

Figure 3. Clustering of F_1 cross and F_2 generation samples based on ISSR molecular markers.

The genome sequence of pepper, together with those of potato and tomato, elucidates the evolution, diversification, and adaptation of more than 3000 species of the Solanaceae family (TANKSLEY *et al*., 1988). Research on the pepper genome enables the development of new breeding technologies to select for horticulturally important traits such as fruit size, yield, pungency, abiotic stress tolerance, nutritional content, and disease and pest resistance.

CONCLUSION

The molecular analysis demonstrates the applicability of the ISSR marker system used in the identification of the different specimens and is suitable for studying genetic diversity. The applied ISSR molecular technique can be used successfully to analyze genetic diversity in cultivars, seedlings in the early stages of development, and interspecific hybrids. This marker system demonstrates the potential of this technology to detect differences even in specimens whose genomes are highly homogeneous, such as those in the genus *Capsicum*. The pungency in the studied Bulgarian pepper cultivars *Capsicum annuum* is due to the presence of the dominant *Pun*1 allele, and the lack of pungency - to the homozygous state of the most common recessive allele pun1-1 - (*pun1-1*/*pun1-1*). Through genetic analysis, it was proved that the crosses were carried out successfully and the hybrid plants were heterozygous with *Pun1*/*pun1-1* genotype. The results of the present study will be used as a basis for future research related to genetic diversity among *Capsicum* species.

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ISTRAŽIVANJE GENETIČKE RAZNOLIKOSTI RODITELJA PAPRIKE (*Capsicum* spp.) I INTERSPECIFIČNIH HIBRIDA KORIŠĆENJEM ISSR MARKERA

Todorka A. SREBCHEVA* i Milena L. KOSTOVA

Departman za biljnu fiziologiju, biohemiju i genetiku,
Poljoprivredni univerzitet - Plovdiv, Bugarska

Izvod

Molekularna analiza pokazuje primenljivost ISSR sistema markera koji se koristi u identifikaciji različitih uzoraka i pogodan je za proučavanje genetske raznovrsnosti. Primenjena ISSR molekularna tehnika može se uspešno koristiti za analizu genetičke raznolikosti sorti, sadnica u ranim fazama razvoja i interspecifičnih hibrida. Ovaj sistem markera pokazuje potencijal ove tehnologije da otkrije razlike čak i u uzorcima čiji su genomi vrlo homogeni, kao što su oni iz roda *Capsicum*. Oporost kod proučavanih sorti bugarske paprike *Capsicum annuum* je zbog prisustva dominantnog alela *Pun1*, a nedostatak ljutine - zbog homozigotnog stanja najčešćeg recesivnog alela *pun1-1* - (*pun1-1*/*pun1-1*). Genetskom analizom dokazano je da je ukrštanje uspešno obavljeno i da su hibridne biljke heterozigotne sa *Pun1/pun1-1* genotipom. Rezultati ove studije će se koristiti kao osnova za buduća istraživanja vezana za genetičku raznolikost među vrstama *Capsicum*.

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