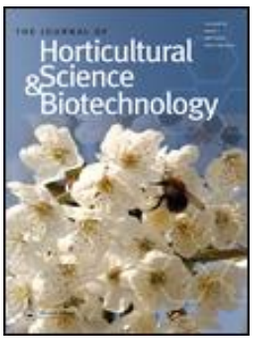

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Application of five DNA marker techniques to distinguish between five apple (*Malus* × *domestica* Borkh.) cultivars and their sports

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SUMMARY
Genetic variation between five apple cultivars ('Golden Delicious', 'Gala', 'Jonagold', 'Šampion', and 'Idared') and ten of their sports ('Golden Delicious Reinders', 'Goldrosio', 'Gala Must', 'Gala Schniga Schnitzer', 'Jonagored', 'Jonagold Excel', 'Szampion Arno', 'Szampion Reno Malinowy', 'Idaredest', and 'Red Idared') was investigated using five types of DNA markers: Inter-Simple Sequence Repeats (ISSR), Simple Sequence Repeats (SSR), Amplified Fragment Length Polymorphism (AFLP), Sequence-Specific Amplified Polymorphism (S-SAP), and Inter-Primer Binding Site (iPBS) amplification. In total, 941 polymorphic amplified fragments were obtained using 12 ISSR, 12 SSR, ten AFLP, 19 iPBS, and 15 S-SAP primers or primer pairs. Four of the above-described techniques (except for SSRs with the primer pairs used in this study) were able to distinguish between the sports and their parental cultivar. The most effective technique to distinguish between the genotypes analysed was S-SAP, which detects variations in DNA regions flanking retrotransposon insertion sites. The combined use of ISSR, AFLP, iPBS, and S-SAP markers identified and distinguished all of the sports tested.

Apple (*Malus* × *domestica* Borkh.) is a perennial woody plant belonging to the family Rosaceae and is one of the most widely-cultivated species in temperate regions, with an annual global production of 64 million metric tonnes of fruit (http://faostat.fao.org/). Most apple cultivars have been obtained through cross-breeding, although seedlings of unknown origin are occasionally used to expand the gene-pool (Janick *et al.*, 1996; Harris *et al.*, 2002). The large number of *M.* × *domestica* cultivars currently available to breeders and fruit producers also include so-called "sports" or "bud sports", which arise from spontaneous somatic mutations that occur in meristematic cells (buds) and develop into a sector (branch) of the original plant. By grafting, such mutants can easily be preserved, propagated, and cultivated as an improved, or even as a distinct cultivar. Some apple cultivars such as 'Delicious', 'Gala', 'Jonagold', 'Rome Beauty', or 'Idared' are especially prone to spontaneous or induced mutations (Janick *et al.*, 1996), which may affect a single gene or alter the ploidy of the plant. Changes in the sequences of specific genes may be expressed as variations in some traits of the tree or the fruit such as fruit pigmentation, growth habit, time of flowering, and/or the concentrations of some chemical compounds (Pratt, 1983; Campeanu *et al.*, 2009). Some of these traits (especially those controlled by a single gene) can be highly desired by breeders and growers.

Considering the role of sports in the apple market, their reproduction, distribution, and cultivation raises several legal issues, particularly with regard to disputes between the breeder/owner (or licence holder) of the progenitor cultivar, and the owner of the new sport. Thus, there is an urgent need for an efficient technique to distinguish between sports and the original cultivars from which they were derived. Traditional phenotypic evaluation of genotypes is limited by the long juvenile period of *M.* × *domestica* plants, the subjective nature of the analysis, and its dependence on environmental conditions. On the other hand, because sports differ from their "parental" plant in only one or a few mutations, the majority of molecular tools are not capable of distinguishing between them (Zhu *et al.*, 1997; Gianfranceschi *et al.*, 1998; Pancaldi *et al.*, 1999; Galli *et al.*, 2005). A retrotransposon-based system has been used to identify some, but not all, sports (Antonius-Klemola *et al.,* 2006).

In the present study, we analysed the efficacy of five different PCR-based molecular marker systems including Simple Sequence Repeats (SSR), Inter-Simple Sequence Repeats (ISSR), Amplified Fragment Length Polymorphisms (AFLP), and DNA markers based on the sequences of retrotransposons [Sequence-Specific Amplified Polymorphism (S-SAP) and Inter-Primer Binding Site (iPBS) amplification to distinguishing between five apple cultivars ('Golden Delicious', 'Gala', 'Jonagold', 'Šampion', and 'Idared') and ten of their sports.

MATERIALS AND METHODS

Plant material

Five apple cultivars and two of each of their respective

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TABLE I
Numbers of PCR primers generating detectable DNA fragments and numbers of polymorphic PCR products generated for five apple cultivars and ten sports using the ISSR, SSR, iPBS, AFLP, and S-SAP techniques

Parameter	ISSR‡	SSR	iPBS	AFLP	S-SAP
No. of primer pairs or single primers used	12	12	19	10	15
No. of reproducibly detectable DNA fragments	95	47	441	191	557
No. (%) of amplicons distinguishing between cultivars	48 (51)¶	40 (85)	236 (54)	168 (88)	449 (81)
No. (%) of amplicons distinguishing between a cultivar and its sports	10 (11)¶	–	11 (3)	12 (6)	25 (5)

‡ISSR, Inter-Simple Sequence Repeat; SSR, Simple Sequence Repeat; AFLP, Amplified Fragment Length Polymorphism; S-SAP, Sequence-Specific Amplified Polymorphism; iPBS, Inter-Primer Binding Site amplification.
¶Values in parentheses are percentages of the total number of amplicons detected.

sports ('Golden Delicious' + 'Golden Delicious Reinders' and 'Goldrosio'; 'Gala' + 'Gala Must' and 'Gala Schniga Schnitzer'; 'Jonagold' + 'Jonagored' and 'Jonagold Excel'; 'Šampion' + 'Szampion Arno' and 'Szampion Reno Malinowy'; 'Idared' + 'Idaredest' and 'Red Idared') were obtained from the collection of the Research Institute of Horticulture, Skierniewice, Poland.

DNA extraction

DNA was extracted from fresh, young leaves according to Doyle and Doyle (1990). Three samples (2 g of leaf tissue per sample) were collected from each cultivar and each of its two sports.

DNA markers

Five DNA marker systems were applied according to previously described protocols; namely ISSR (Zietkiewicz *et al.*, 1994), SSR (Litt and Luty, 1989; Liebhard *et al.*, 2002), AFLP (Vos *et al.*, 1995), S-SAP (Waugh *et al.*, 1997; Yao *et al.*, 2001), and iPBS (Kalendar *et al.*, 2010).

Primers used for each type of marker

Twelve primers from UBC Kit No. 9 (810, 811, 818, 823, 836, 840, 841, 845, 846, 853, 864, and 890; University of British Columbia, Vancouver, Canada) were used for the ISSR markers. Twelve pairs of primers (CH01e01, CH05a04, CH02a08, CH03d08, CH04f06, CH02a03, CH01g05, CH03a04, CH02b12, CH03e03, CH02b12, and CH04e03; Liebhard *et al.*, 2002) were used for the SSR markers. Ten pairs of selective primers (E-CA/M-AG, E-CA/M-AT, E-ACA/M-CTG, E-ACA/M-CTC, E-ACT/M-CAG, E-CT/M-AT, E-ATG/M-CTC, E-ATG/M-CTG, E-ATG/M-CAG, and E-ATG/M-CAT; where E = *Eco* RI and M = *Mse* I) were used for the AFLP markers. Fifteen pairs of primers (LTR1/M-AG, LTR1/M-CA, LTR1/M-CC, LTR1/M-TC, LTR1/M-CT, LTR1/M-CAG, LTR1/M-CTC, LTR1/M-CTG, LTR2/M-CA, LTR2/M-CTG, LTR2/M-AG, LTR2/M-CCC, LTR2/M-CCT, LTR2/M-CTC, and LTR2/M-CAG; Yao *et al.*, 2001) were used for the selective S-SAP reactions. Nineteen primers (2374, 2378, 2076, 2081, 2075, 2077, 2083, 2085, 2087, 2095, 2074, 2270, 2072, 2226, 2241, 2255, 2278, 2277, and 2253; Kalendar *et al.*, 2010) were used for the iPBS markers.

Detection

The ISSR and iPBS amplified fragments were visualised in 1.5% (w/v) and 2.0% (w/v) agarose gels, respectively, stained with 0.5 μg ml^{-1} ethidium bromide, and observed over UV light. The products of AFLP amplification were subjected to electrophoresis in denaturing 6% (w/v) polyacrylamide gels. The gels were analysed by silver staining (Chalhoub *et al.*, 1997). SSR fragments were separated in an Agilent 2100 Bioanalyzer (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) according to the manufacturer's protocol. The S-SAP products were detected by capillary electrophoresis using an ABI 3700 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

RESULTS

The five molecular marker techniques used made it possible to detect DNA polymorphisms within all 15 apple genotypes studied. Among the 1,331 PCR-amplified fragments obtained using 12 ISSR, 12 SSR, ten AFLP, 19 iPBS, and 15 S-SAP primers, 941 were polymorphic (Table I). The majority of these markers proved useful for cultivar identification. Forty-seven polymorphic SSR fragments (85% of the total number obtained) were useful for cultivar identification, but not for distinguishing between a cultivar and its sports. Forty-eight of the polymorphic ISSR fragments (51% of the total), 168 of the polymorphic AFLP bands (82% of the total), 236 of the polymorphic iPBS products (54% of the total), and 449 of the polymorphic S-SAP bands (81% of the total) permitted differentiation of all 15 cultivars tested.

These four types of DNA markers (not SSRs) varied in their effectiveness at distinguishing between the cultivars and their sports. For instance, the ISSR primers 823, 846, and 853 generated relatively high numbers of polymorphic products; however, they were only able to

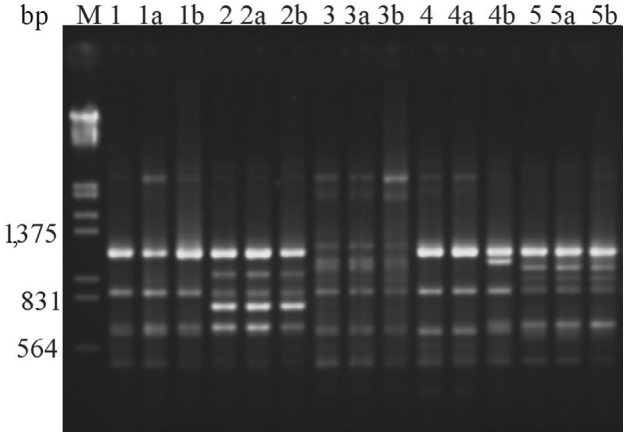

FIG. 1
Electropherogram of the ISSR products generated using the primer 823. Lanes represent: 1, 'Golden Delicious'; 1a, 'Golden Delicious Reinders'; 1b, 'Goldrosio'; 2, 'Gala'; 2a, 'Gala Must'; 2b, 'Gala Schniga Schnitzer'; 3, 'Jonagold'; 3a, 'Jonagored'; 3b, 'Jonagold Excel'; 4, 'Sampion'; 4a, 'Szampion Arno'; 4b, 'Szampion Reno Malinowy'; 5, 'Idared; 5a, 'Idaredest'; and 5b, 'Red Idared'. Lane M, DNA size markers (in bp).

TABLE II
Ability to distinguish between apple cultivars[‡] and sports using the AFLP, ISSR, iPBS, or S-SAP technique

Method (no. of primers)	Pairwise comparisons														
	1-1a	1-1b	1a-1b	2-2a	2-2b	2a-2b	3-3a	3-3b	3a-3b	4-4a	4-4b	4a-4b	5-5a	5-5b	5a-5b
AFLP (3)	+/-[†]	+/-	+/-	-/-	+/-	+/-	+/-	-/+	+/-	+/-	+/-	-/-	+/-	-/+	+/-
ISSR (3)	+/-	+/-	-/+	-/+	-/-	-/+	-/+	-/+	-/-	-/+	+/+	+/-	-/-	-/-	-/-
iPBS (4)	-/-	+/+	+/+	-/-	-/+	-/+	-/+	-/+	-/-	-/+	-/+	-/+	-/-	-/-	-/-
S-SAP (13)	-/+	-/-	-/+	-/+	-/-	-/+	+/-	+/-	+/-	+/-	+/-	+/-	+/+	+/+	-/-
	-/+	+/-	+/-	-/+	-/+	-/-	-/-	-/+	-/+	-/-	-/-	-/-	-/-	-/-	-/-
	-/+	-/-	-/+	-/+	-/+	-/+	-/-	-/-	-/-	-/+	+/+	-/+	-/-	-/-	-/-

[‡]Cultivars: 1, 'Golden Delicious'; 2, 'Gala'; 3, 'Jonagold'; 4, 'Šampion'; 5, 'Idared. Sports: 1a, 'Golden Delicious Reinders'; 1b, 'Goldrosio'; 2a, Gala Must'; 2b, 'Gala Schniga Schnitzer'; 3a, 'Jonagored'; 3b, 'Jonagold Excel'; 4a, 'Szampion Arno'; 4b, 'Szampion Reno Malinowy'; 5a, 'Idaredest'; 5b, 'Red Idared'.
[†]+/-, ability to distinguish using the following method-specific primers (respectively): AFLP (E-CA/M-AG, E-CA/M-AT, or E-CT/M-AT); ISSR (823, 845, or 853); iPBS (2072, 2087, 2226, or 2255); S-SAP (LTR1/M-AG, LTR1/M-CA, LTR1/M-CC, LTR1/M-TC, LTR1/M-CT, LTR1/M-CAG, LTR1/M-CTC, LTR1/M-CTG, LTR2/M-CCT, LTR2/M-CTG, LTR2/M-AG, LTR2/M-CA, LTR2/M-CTC.

distinguish 'Golden Delicious' from 'Golden Delicious Reinders' and 'Goldrosio', and 'Šampion' from 'Szampion Arno' and 'Szampion Reno Malinowy' (Table II; Figure 1). Several of the sports were also distinguished using AFLP markers. Each of the following pairs: 'Golden Delicious Reinders' and 'Goldrosio', 'Gala Must' and 'Gala Schniga Schnitzer', 'Idaredest' and 'Red Idared', and 'Jonagored' and 'Jonagold Excel', were distinguished from each other and from the parental cultivar using a combination of three primer pairs (E-CA/M-AG, E-CT/M-AT, and E-CA/M-AT). However, AFLP primers were unable to distinguish within the 'Šampion' group. Genetic discrimination between this cultivar and its two sports 'Szampion Arno' and 'Szampion Reno Malinowy', was only possible by iPBS using the primers 2087 and 2255. The use of iPBS primers 2226 and 2072 discriminated between the genotypes 'Golden Delicious' and 'Goldrosio' (Table II; Figure 2). 'Golden Delicious' was also distinguished from 'Golden Delicious Reinders' using the primers LTR1/M-CA, LTR1/M-CC, LTR1/M-CAG, and LTR2/M-CA, and from 'Goldrosio' using the primer LTR1/M-CT (Table II; Figure 3) and the S-SAP technique. 'Gala', and its sports 'Gala Must' and 'Gala Schniga Schnitzer', were distinguished using five sets of S-SAP primers: LTR1/M-CC, LTR1/M-CAG, LTR1/M-CTC, LTR2/M-AG, and LTR2/M-CTC. 'Jonagold' and 'Šampion' were distinguished from their respective sports, 'Jonagored' and 'Jonagold Excel', 'Szampion Arno' and 'Szampion Reno Malinowy', by S-SAP amplifications using the primers LTR1/M-AG, LTR1/M-TC, LTR1/M-CTG, LTR2/M-CCT, and LTR2/M-CA. None of the S-SAP primers were able to distinguish between the sports, 'Idaredest' and 'Red Idared'.

DISCUSSION

Breeders' Rights are based on the specific identification of the protected plant. In contrast to genetically diverse cultivars, which can easily be identified using molecular techniques, discrimination between sports and their progenitor cultivar is difficult. Isozyme electrophoresis (Weeden and Lamb, 1985), mini-satellites (Nybom, 1990; Watillon *et al.*, 1991) and Randomly-Amplified Polymorphic DNA (RAPD) markers (Harada *et al.*, 1993) could not distinguish between the sports of 'Golden Delicious', 'Gala', 'Idared', and other selected cultivars. Later, Galli *et al.* (2005) used 68 SSR markers to identify 70 apple accessions; however, they could not identify all the mutants (sports) tested.

The results obtained here using SSRs, AFLP, and two retrotransposon-based marker systems (S-SAP and iPBS) were partially successful at discriminating ten sports from their parental cultivars: 'Golden Delicious', 'Gala', 'Jonagold', 'Šampion', and 'Idared'. Moreover, our results supported the conclusion that these ten new cultivars were indeed sports because all DNA markers were more efficient at distinguishing between cultivars than distinguishing each cultivar from its two sports. The successful application of these techniques to discriminate

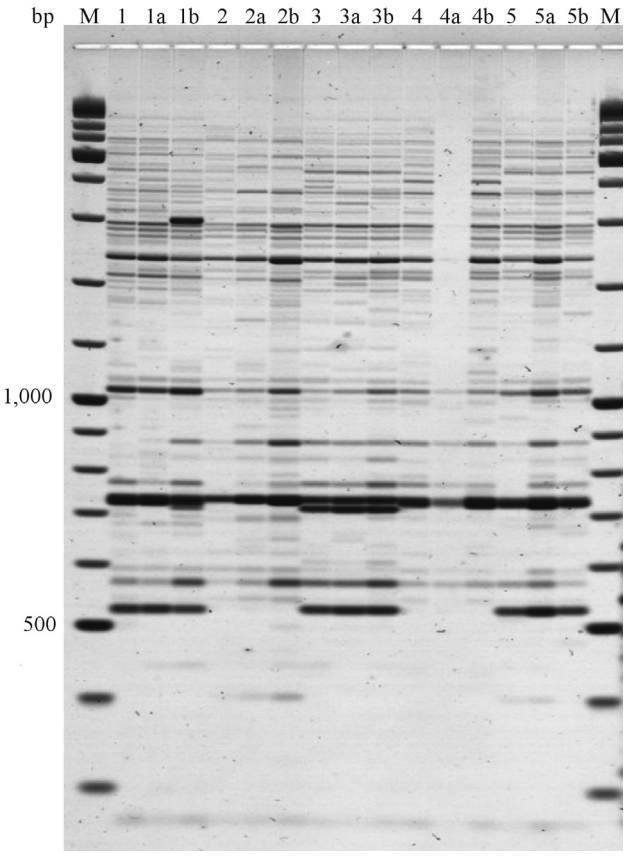

FIG. 2
Electropherogram of the iPBS products generated using the primer 2226. Lanes represent: 1, 'Golden Delicious'; 1a, 'Golden Delicious Reinders'; 1b, 'Goldrosio'; 2, 'Gala'; 2a, 'Gala Must'; 2b, 'Gala Schniga Schnitzer'; 3, 'Jonagold'; 3a, 'Jonagored'; 3b, 'Jonagold Excel'; 4, 'Sampion'; 4a, 'Szampion Arno'; 4b, 'Szampion Reno Malinowy'; 5, 'Idared; 5a, 'Idaredest'; and 5b, 'Red Idared'. Lane M, DNA size markers (in bp).

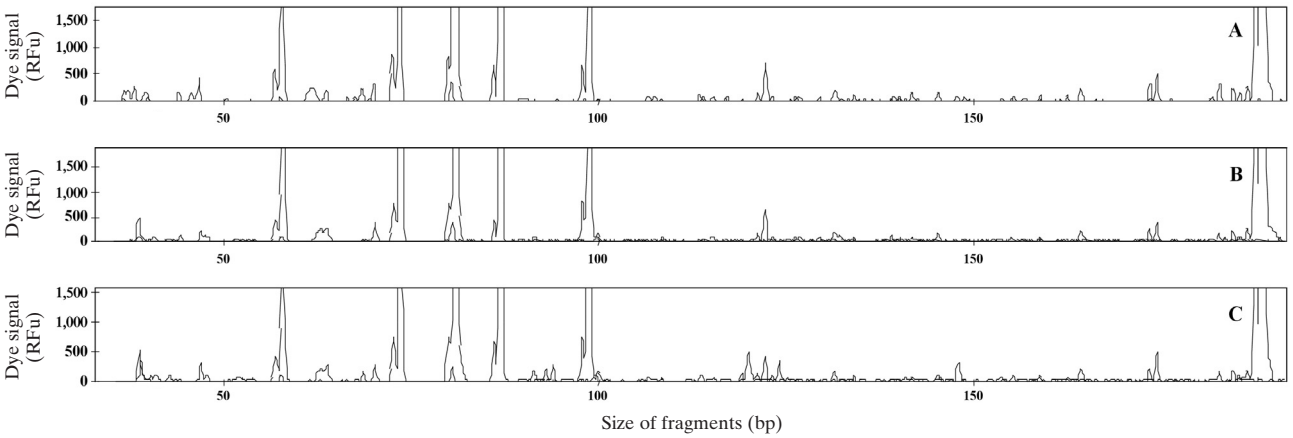

FIG. 3
Polymorphism of PCR-amplified fragments generated by the S-SAP primer pair, LTR1/M-CT from 'Golden Delicious' apple (Panel A) and its two sports 'Golden Delicious Reinders' (Panel B) and 'Goldrosio' (Panel C).

between sports requires identification of a single point mutation (one or a few nucleotides) in the genome of plant. Thus, only 6% of the polymorphic AFLP fragments, 11% of the ISSR fragments, and none of the SSR amplicons were found to be sport-specific. The percentages of these sport-specific bands ranged from 51 – 88%, depending on the marker system. ISSR markers only discriminated sports belonging to 'Golden Delicious' and 'Šampion'. AFLP markers distinguished between 'Golden Delicious' and its sports ('Golden Delicious Reinders' and 'Goldrosio'), between 'Gala' and its sports ('Gala Must' and 'Gala Schniga Schnitzer'), between 'Jonagold' and its sports ('Jonagored' and 'Jonagold Excel'), and between 'Idared' and its sports ('Idaredest' and 'Red Idared'). However, AFLP markers could not discriminate between the two sports and their original cultivar. The two sports of 'Šampion' were completely indistinguishable using AFLP markers. Our results suggested that AFLP primers having one or two selective nucleotides were more efficient at identifying sports than primers having three selective nucleotides, which were applied without success to a study on 'Gala' and 'Braeburn' sports (Venturi *et al.*, 2006).

The use of transposons (McClintock, 1950) to identify cultivars (Kumar and Bennetzen, 1999) resulted in the generation of DNA fragments from the multiple sites of retrotransposon insertion in the mutant (sport) genome (Schulman *et al.*, 2004). Among the numerous long terminal repeat (LTR) retrotransposons that have been isolated from apple, the majority were incomplete (Tignon *et al.*, 2001; Sun *et al.*, 2008), and only five full sequences have been submitted to GenBank (Yao *et al.*, 2001; Antonius-Klemola *et al.*, 2006; Zhao *et al.*, 2007; 2010). In spite of these limitations, many authors have described transposon-based systems as being useful for the identification of various genotypes of a single species (Breto *et al.*, 2001).

In our experiments, the iPBS and S-SAP primers generated many polymorphic DNA products which could identify the five progenitor cultivars (54 – 81% of amplicons depending on the technique), but only a few polymorphic bands were sport-specific (3 – 5%). Using the iPBS technique, only two sports could be distinguished from their original cultivar. Others have reported the unsuccessful use of iPBS markers to characterse mutants (Antonius-Klemola *et al.*, 2006; Kalendar *et al.,* 2010).

Using 13 combinations of S-SAP primers, we detected genetic differences between eight out of the ten sports tested (i.e., 'Golden Delicious Reinders', 'Goldrosio', 'Gala Must', 'Gala Schniga Schnitzer', 'Jonagored', 'Jonagold Excel', 'Szampion Arno', and 'Szampion Reno Malinowy'). The S-SAP technique has also been used to identify somaclones (Waugh *et al.*, 1997; Kumar and Bennetzen, 1999; Breto *et al.*, 2001; Tahara *et al.*, 2004; Venturi *et al.*, 2006; 2009). Nevertheless, the identification of some mutants remained impossible because single nucleotide polymorphisms, or small insertions/deletions, are difficult to detect even using the S-SAP technique (Galli *et al.*, 2008). Identification of such sports will probably be based on specific single nucleotide polymorphisms (SNPs) or specific genome sequence information.

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