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Identification of reciprocal hybrids in citrus by the broadness of the leaf petiole wing

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ABSTRACT

Broadness of leaf petiole wing (WB) was investigated as a morphological marker for screening hybrids of the very narrow-winged species *Citrus limonia* and *C. sunki* with broad-winged species *C. aurantium* and *C. sinensis*. Controlled polinizations produced over 500 reciprocal hybrids with potential in the ongoing rootstock breeding program identified by the Pgi-1 and Prx_a-1 isozyme loci.

Measurement ratios WB/leaf length, WB/leaf broadness and WB/petiole length identified 86 to 91% of the reciprocal hybrids produced. However, visual classification of WB was an equally efficient but much easier and faster method. It can be very useful in breeding programs when large number of plants have to be screened or when isozyme, RFLP or RAPD laboratories are not available.

INTRODUCTION

Reproduction of most *Citrus* species is characterized by the occurrence of nucellar polyembryony in the seeds, a feature that leads to mixed offsprings containing nucellar clones (true-to-type maternal genotype) and zygotic (sexual) seedlings (Cameron and Frost, 1968). In breeding programs where hybridizations are involved, it is very desirable to identify the type of each seedling before the trials are set up in the field because in this way only the hybrids are evaluated. Early identification of hybrids from seed parents highly polyembryonic results in increased money and time savings because most of the plants produced are unwanted nucellar clones (Soost and Cameron, 1975).

Dependable methods to separate nucellar from hybrid seedlings in progenies rely on the evaluation of genetic markers. Certainly, in order to screen a large number of plants, the ideal markers are morphological traits, in view of the ease and quickness of the evaluations. For the identification of interspecific hybrids within the genus *Citrus*, these markers are unfortunately scarce. Apparently the only well-studied case that has a known genetic basis is the presence of anthocyanine pigment in some red colored *Citrus limon* cultivars (Toxopeus, 1962). Because this trait is dominant, it is useful only when *C. limon* is the male parent. Otherwise, as the seed genitor, both hybrid and nucellar plants have red shoots, being undistinguishable. This situation is similar to the intergeneric crosses involving the trifoliate leaf trait of *Poncirus trifoliata* (Toxopeus, 1962) and the red colored young shoots of *Severinia buxifolia* (Medina-Filho *et al.*, in press).

An attempt to use a morphological trait to identify hybrids was made by Teich and Spiegel-Roy (1972) who investigated the ratio of leaf length/width (L/W) in some *C. reticulata* cultivars and hybrids. Hybrids of cultivars having significantly different L/W ratio supposedly could be discriminated if the value was out of the range of the seed parent. On the other hand, the values in the mean range of the two parents were considered hybrids. However, the reliability of this method was not evaluated, due to the lack at that time of a *bona fide* marker to be used as a control.

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Although RFLP and more recently RAPD analysis are powerful techniques for hybrid identification (Roose *et al.*, 1992; Waugh and Powell, 1992), isozyme alleles are well known and still recognized as accurate markers in citrus. Since the study of Torres *et al.* (1978), they have been extensively used to distinguish nucellar from zygotic plants (Torres, 1983; Anderson *et al.*, 1991; Fatta Del Bosco *et al.*, 1994). The usefulness of isozymes has been demonstrated in comparison with other criteria, like trifoliate leaves (Manzocchi *et al.*, 1981), the polyphenol-oxidase browning and coagulation of young shoot homogenates (Geraci *et al.*, 1981), or visual observations of plant phenotype (Moore and Castle, 1988). A key attribute of the isozymes as markers is that they are coded by codominant genes which allow the unequivocal identification of nucellar and reciprocal hybrids, in most cases. A through discussion of the use of isozymes and their efficiency as markers in citrus is presented by Ballvé *et al.* (1991).

Toxopeus (1962) studied the broadness of leaf petioles as a marker. However, the understanding and usefulness of it was impaired by a complicated cross and hindered by very unexpected results. A cross of *C. maxima* with *C. hystrix*, two monofoliate broad-winged species, produced mostly plants with trifoliate leaves and, among the monofoliate ones, the great majority had narrow-winged petioles.

This paper reports investigations on the broadness of the leaf petiole wing (WB) as a marker for screening reciprocal hybrids of the very narrow-winged species *C. limonia* and *C. sunki* with broad-winged species *C. aurantium* and *C. sinensis*. These hybrids were produced aiming at combinations of several agronomic attributes present in different rootstocks (Pompeu Jr., 1990). Plants were classified to the broadness of leaf petiole using several methods, with their efficiency determined by isozymes.

MATERIAL AND METHODS

Seedlings three to twelve months old were obtained from crosses of *C. limonia* Osbeck 'Limeira' x *C. aurantium* L. 'São Paulo', *C. sunki* Hort. ex. Tanaka '200' x *C. aurantium*, their reciprocals, and *C. sunki* x *C. sinensis* Osbeck 'Natal', 'Valencia', 'Pera' and 'Hamlin'. Hand pollinations were performed according to the procedures of Bordignon *et al.* (1990). Three criteria were used to classify the plants of each progeny as nucellar or hybrid: visual observation of petioles, measurements of petiole wings, and isozyme analysis of the plants.

Visual observation

The predominant type of leaf petiole of each seedling at the nursery was determined by direct observation. Among offspring of *C. sunki* or *C. limonia* as the mother parent in crosses with sour or sweet orange as the pollen parent, narrow-winged seedlings, as the mother plants, were classified as nucellars and those which showed broad-winged petioles, in various degrees, were considered hybrids. Conversely, when sour orange was the female genitor, seedlings with similar broad wings were considered nucellars and those with narrow-wings in various degrees, hybrids.

Measurements

C. sunki, *C. limonia* and *C. aurantium* parental trees were characterized in a sample of 100 leaves. Measurements of the petiole wing broadness (WB), leaf broadness (LB), petiole length (PL), and leaf length (LL) were taken (Figure 1), and the ratios WB/PL, WB/LB, and WB/LL were calculated. For WB and the above ratios, the range of variation, mean, standard deviation and confidence limits were determined. The confidence limits at the 5% level were considered the typical values of parental clones. Statistical analyses were performed using the SANEST computer program.

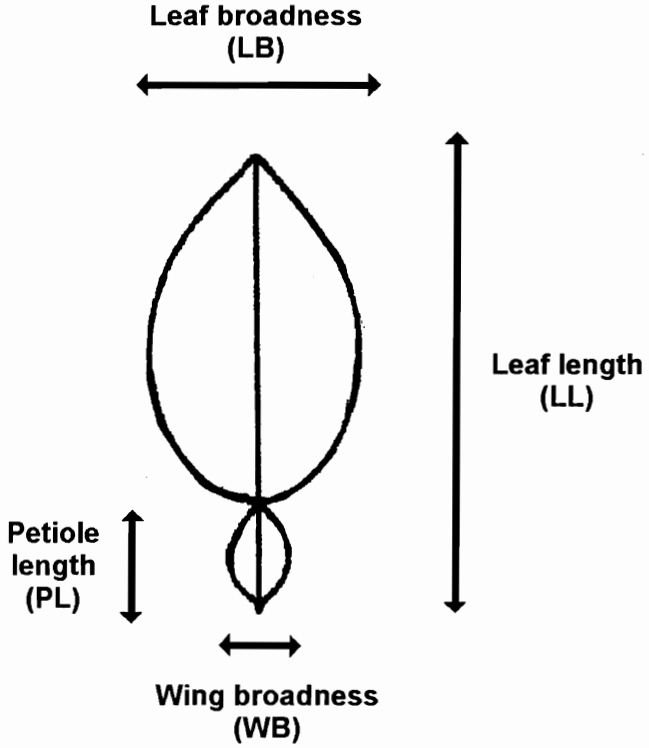

Figure 1 - Schematic representation of a citrus leaf showing the measures taken.

This same procedure was applied to 10 leaves of each seedling screened, and the confidence limit was calculated. When it fell out of the range of the seed parent, the seedling was classified as a hybrid. Otherwise, it was considered nucellar. Each measure was studied independently so that the same seedling could be indicated as nucellar by one measure but hybrid by another. Sweet orange was not included in this study, as explained later.

Isozyme analysis

Segregation and recombination of the alleles of phosphoglucoisomerase-1 (Pgi-1) and anodic peroxidase-1 (Prx_a-1) loci (Table I) allowed unequivocal identification of all nucellar and hybrid seedlings from crosses between *C. sunki* or *C. limonia* with *C. aurantium*. For the crosses of *C. sunki* with *C. sinensis*, only Prx_a-1 was useful since its genotype FF theoretically identifies 50% of the hybrids produced.

Table I - Genotypes of Pgi-1 and Prx_a-1 isozymic loci of *Citrus aurantium*, *C. limonia*, *C. sunki* and *C. sinensis*.

Species (cultivar)	Pgi-1	Prx_a-1
C. aurantium (São Paulo)	WS	FS
C. limonia (Limeira)	FS	MM
C. sunki (200)	FF	FM
C. sinensis (Natal, Valencia Pera, Hamlin)	FF	FF

Electrophoresis was accomplished according to Ballvé *et al.* (1995) and Tanksley (1979), with equipment for horizontal starch gels with the following specifications:

Samples: a leaf disk of 1 cm in diameter was crushed with five drops of distilled water;

Gel: hydrolized potato starch: 120 g/l;

Buffer: 25 ml/l Tris 0.73 M (0.018 M) and 25 ml/l citric acid 0.15 M (0.36 M), pH 8.2 adjusted with Tris (0.73 M);

Electrophoresis: electrodes buffer: 0.3 M boric acid, pH 8.3 adjusted with 4 N NaOH;

Electrical conditions: for inserting the extracts into the gel a current of 25 mA (not exceeding 150V) was applied for 25 min. After removal of wicks, the same electrical conditions were maintained for 50-60 min. Then, the amperage was adjusted to 30 mA, not exceeding 300 V, and gels ran for 30-40 min until they reached 300 V. After that, the gels were run for 2 h within the limits of 300 V and 30 mA.

RESULTS AND DISCUSSION

In *C. sunki* and *C. limonia* the petioles of all leaves are invariably very narrow-winged. Contrarily, *C. aurantium* and *C. sinensis* display leaves with broad wings (Figure 2). In these broad-winged clones, however, a few leaves with very reduced wings can be observed. This variation is represented in the measurements by the range of the histograms of distribution shown in Figure 3. *C. sunki* and *C. limonia*, in accordance with the visual observations, displayed very little variation. In *C. aurantium*, WB and its ratios showed wide variation. For all measurements, *C. aurantium* showed a certain proportion of leaves within the range of values of *C. sunki* and *C. limonia* (WB 9%; WB/PL 29%; WB/LB 34%; WB/LL 18%) indicating, in numbers, the aforementioned occurrence of narrow-winged leaves. However, as depicted by the modes, means, and confidence limits of the measurements and their ratios (Table II), the great majority of leaves are winged and appreciably different from *C. sunki* and *C. limonia*.

The isozyme analyses indicated (Table III) that from 1024 seedlings analyzed 561 hybrids were identifyed. As mentioned before, with exception of the *C. sunki* x *C. sinensis* cross, the seedlings classified as nucellar were, surely, of nucellar origin and, as expected, all of them showed exactly the same type of petiole of the female parental plant from which they ontogenetically originated.

The percentage of hybrids from the crosses shown in Table III were quite high: 29% for *C. limonia*, 22% for *C. aurantium* and 74% for *C. sunki*. This suggests a problem for the use of these species as rootstocks.

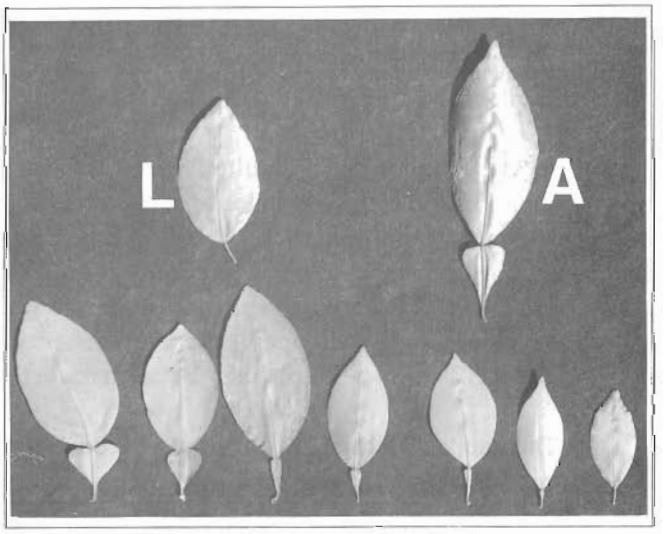

Figure 2 - Different typical leaf petioles of plants from a progeny of *Citrus limonia* (L) x *C. aurantium* (A) as male parent. Except for the rightmost leaf, which belongs to a nucellar plant, all of them are from hybrids identified by isozymes.

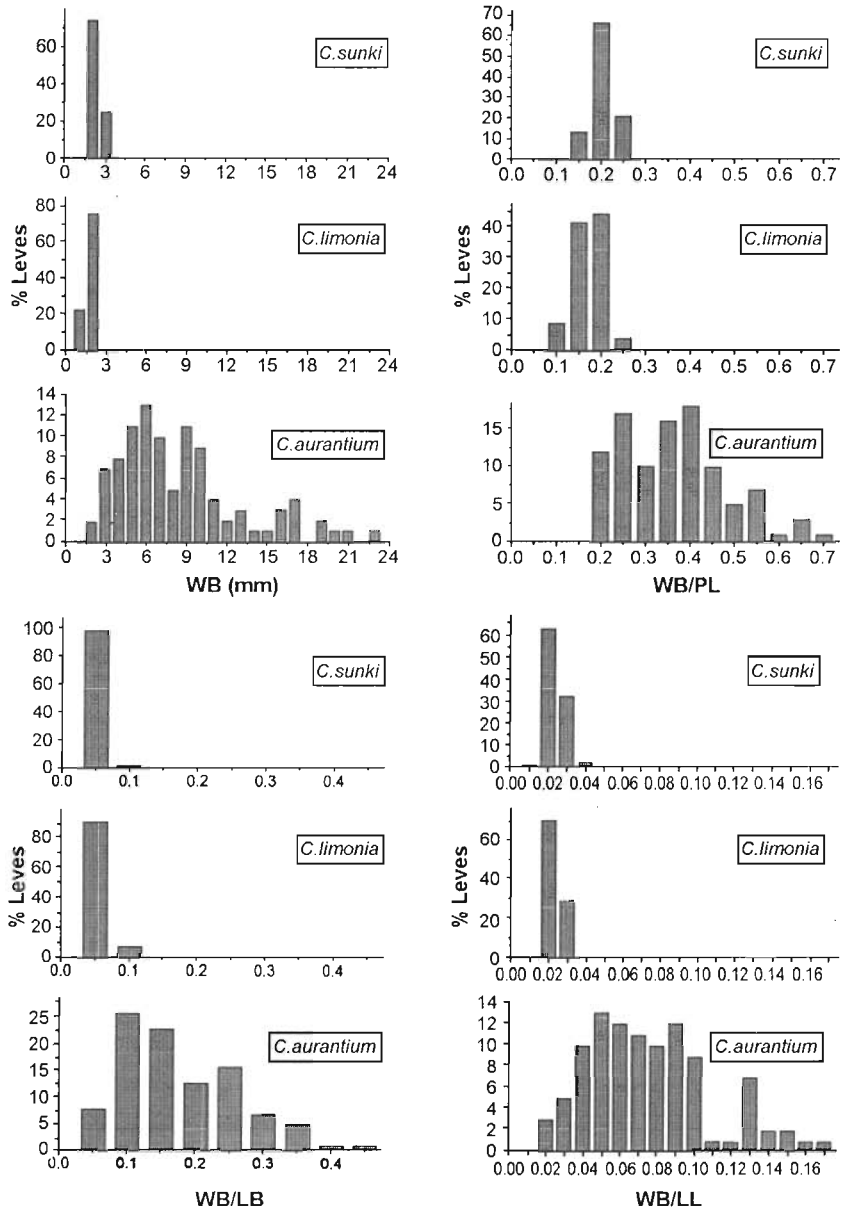

Figure 3 - Variation of broadness of petiole wing (WB) and its ratio with petiole length (PL), leaf broadness (LB) and leaf length (LL) in 100 leaves of citrus clones.

Table II - Values of modes, means, standard deviations (SD) and confidence limits (CL) of the wing broadness (WB) and its ratio to petiole length (WB/PL), leaf length (WB/LL), and leaf broadness (WB/LB) of 100 leaves of parental trees of *Citrus limonia*, *C. sunki* and *C. aurantium*.

	C. limonia	*C. sunki*	*C. aurantium*
WB (mm)			
Mode	2.0	1.7	6.0
Mean	2.2	1.6	7.5
SD	0.3	0.3	4.1
CL5% Min	2.1	1.5	8.3
Max	2.2	1.7	6.7
WB/PL			
Mode	0.20	0.17	0.40
Mean	0.20	0.17	0.34
SD	0.04	0.03	0.12
CL5% Min	0.19	0.17	0.32
Max	0.21	0.18	0.37
WB/LL			
Mode	0.020	0.020	0.050
Mean	0.023	0.023	0.070
SD	0.005	0.005	0.032
CL5% Min	0.022	0.022	0.064
Max	0.024	0.024	0.077
WB/LB			
Mode	0.050	0.050	0.100
Mean	0.053	0.054	0.161
SD	0.007	0.012	0.084
CL5% Min	0.051	0.052	0.145
Max	0.054	0.056	0.178

However, the present data resulted from artificial cross-pollinations with different clones, a situation not observed in commercial seed orchards. *C. sunki* bearing usually one to three embryos per seed produces 44-77% of hybrids when artificially cross-pollinated with compatible clones, but when open pollinated or selfed it yields over 98% of nucellar plants (Carvalho *et al.*, in press).

The data of Table III indicate that all criteria for evaluation of leaf petiole wing are quite satisfactory to identify hybrids, showing some variation when each cross is analyzed separately. Considering the crosses altogether, it is noteworthy that the visual observation criterion recognized 90% of hybrids. Only the WB measurement provided a better efficiency (91%). The 10% of hybrids not identified, thus confounded with nucellars, belong to a class of seedlings whose wing broadness overlap the limit range of the seed parents.

The frequency distribution observed in the F1 plants (Figure 4) suggests that petiole wing is controlled by polygenes and that parental clones must be heterozygous in some loci and/or some kind of epistasis is involved. Furthermore, broad-winged genotypes do not express the trait uniformly in all leaves of the plant. In fact, considerable variation was observed in different leaves of single plants as seen in the case of *C. aurantium* (Figure 3). The genetic basis of the trait must await a forthcoming study of specific F_2 progenies. This, however, does not preclude the herein suggested use of the broadness of petiole wings in the identification of hybrids.

Indeed, the efficiency of the visual evaluation was comparable to the laborious identification by measurements. Since it is the simplest of all methods, it should be preferred. In order to increase its efficiency, an alternative strategy would be to associate it with electrophoresis, checking by isozymes only those plants

Table III - Number of plants obtained in each cross, number of hybrids identified by isozymes, by visual scoring of broadness of leaf petiole wing, by actual measurements of it (WB) and by its ratios with petiole length (PL), leaf length (LL) and leaf broadness (LB). In parentheses, the percentage of plants relative to the total number identified by isozymes (100%).

	Number of plants	Number of hybrids identified by:					
		Isozymes	Visual	WB	WB/PL	WB/LL	WB/LB
C. limonia x *C. aurantium*	238	70	61 (87%)	65 (93%)	64 (91%)	67 (96%)	65 (93%)
C. sunki x *C. aurantium*	233	175	155 (89%)	159 (91%)	152 (87%)	158 (90%)	149 (85%)
C. aurantium x *C. limonia*	76	13	10 (77%)	10 (77%)	9 (69%)	8 (62%)	8 (62%)
C. aurantium x *C. sunki*	94	25	22 (88%)	23 (92%)	17 (68%)	20 (80%)	18 (72%)
C. sunki x *C. sinensis*	383	278	257 (92%)	—	—	—	—
Total	1024	561	504 (90%)	257 (91%)	242 (86%)	253 (89%)	240 (85%)

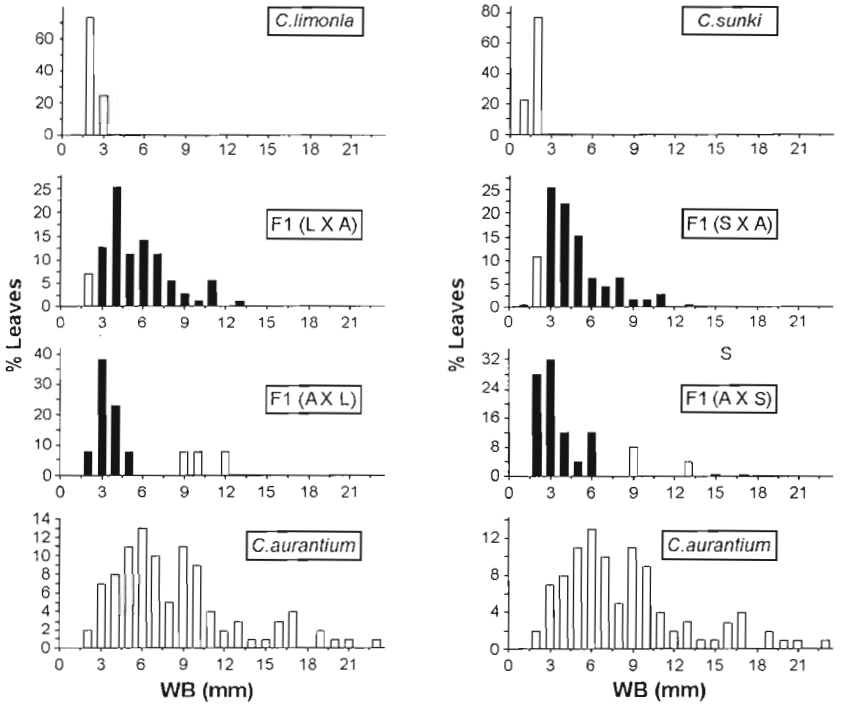

Figure 4 - Broadness of leaf petiole wing in *Citrus* species of Figure 3 and in 10 leaves of each reciprocal hybrid of Table III. L, *C. limonia*; A, *C. aurantium*; S, *C. sunki*.

that are visually classified as nucellars. Such a procedure would identify the remaining 10% of hybrids in the progenies, if necessary.

An important feature of the petiole wing as a marker is that it is useful to identify hybrids from both crossing directions with comparable efficiency, as observed in the reciprocal crosses between *C. sunki* and *C. aurantium*. A somewhat different proportion of hybrids were recognized in reciprocal crosses of *C. aurantium* x *C. limonia*. However, these results are not conclusive since a small number of seedlings were evaluated.

Concluding, the broadness of leaf petiole wing is a good marker for the identification of hybrids of sour (*C. aurantium*) and sweet orange (*C. sinensis*) in crosses with very narrow-winged species such as *C. sunki* and *C. limonia*. Although 10% of the hybrids cannot be identified on the basis of this easily screened trait, all seedlings visually classified as hybrids were in fact hybrids, as checked by isozyme analysis. This trait therefore can be very useful in extensive breeding programs if a large number of plants have to be screened, or when an isozyme, RFLP or RAPD laboratory is not available. The hybrids so produced and identified are being evaluated as to their potential as rootstocks for citrus in Brazil.

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RESUMO

Investigou-se a largura da asa do pecíolo da folha (WB) como um marcador morfológico para identificar híbridos recíprocos de *Citrus limonia* e *C. sunki*, que possuem a asa do pecíolo bastante reduzida, com *C. aurantium* e *C. sinensis*, cujas asas dos pecíolos são bem desenvolvidas. Polinizações controladas produziram mais de 500 híbridos identificados pelos loci isoenzímicos Pgi-1 e Prx_a-1. WB e a razão desta pelo

comprimento da folha, pela largura da folha e pelo comprimento do pecíolo identificaram, em média, 86 a 91% dos híbridos recíprocos produzidos. A simples observação subjetiva da WB é, entretanto, um processo igualmente eficiente porém muito mais fácil e rápido. Este poderá ser bastante útil e econômico em programas de melhoramento nos casos em que se necessite a seleção de grande número de plantas ou quando não se dispõe de laboratórios de eletroforese de isoenzimas RFLP ou RAPD. Os híbridos produzidos e identificados têm sido avaliados quanto ao seu potencial como porta-enxertos de citros no Brasil.

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