The African Journal of Plant Science and Biotechnology ©2012 Global Science Books

Genetic Variation and Characterization of Swiss Chestnut Cultivars (*Castanea sativa* Mill.) Using RAPD and AFLP Markers

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ABSTRACT

In order to study the genetic variation and to provide specific markers for genetic characterisation and identification of Swiss chestnut cultivars, two sets of molecular markers were explored: Random Amplified Polymorphic DNAs (RAPDs) and Amplified Fragment Length Polymorphic DNAs (AFLPs). 98 RAPD and 222 AFLP polymorphic markers were amplified using 12 and 4 primer combinations, respectively. Clustering analysis performed with the two sets of markers to group cultivars according to their similarity coefficients separated the genotypes into clear groups with different grades of success. The genetic identification of cultivars was more correct by RAPD than by AFLP. AFLP generated the highest number of polymorphic bands and clustered fairly closely-related chestnut cultivars. These results allowed problems generated by synonyms and homonyms in different chestnut accessions to be resolved. Molecular marker results were comparable. The similarity matrices based on the two sets of data give a highly significant positive correlation between RAPD and AFLP (r = 0.78).

Keywords: AFLP, *Castanea*, fingerprinting, genetic relationship, RAPD

INTRODUCTION

European chestnut (*Castanea sativa* Mill.) is a species that, perhaps more than any other in Europe, has attracted a particular attention of man. During some historical periods, in various regions of Europe, chestnut cultivation became so dominant and indispensable for the survival of mountain populations, to the point that some authors do not hesitate to identify these cultures as "chestnut civilizations" (Lieutaghi 1969).

Southern Switzerland is a mountainous region (approximately 26,000 ha of chestnut forests) in which chestnut is wide spread (Conedera *et al.* 1997). Chestnut is one of the most common forest trees and has been cultivated in this region since nearly 2000 years. In Southern Alps, chestnut was considered as an important source of food for many human populations. Moreover, chestnut is widely known for its double usefulness. Its fruits satisfy the daily life need for nutrition (man and animal) and its wood is a means for providing either fire or stokes. For this purpose, chestnut has been subjected to a local diversification in the range of its products as well as in the range of its varieties; this is done by the majority of mountain populations. The choice of a large number of varieties was adopted by farmers to assure their personal needs. Some farmers possessed a typical combination of cultivars. Others, cultivated chestnut varieties for their quality and conserve of their fresh fruit as winter supply (Conedera *et al.* 1997). This resulted in a differentiation of the product and of the cultivated varieties.

Despite the great number of existing varieties, the need for a powerful determination method and the evaluation of genetic diversity and structure in cultivated varieties and in natural accessions is crucial for making conservation and management strategies and the utilization of the natural resources.

The genetic variability and the identification of European chestnut have been assessed both in natural populations and in cultivated varieties by the use of molecular markers.

DNA molecular markers techniques based on *in-vitro* enzymatic amplification of specific fragments of DNA via PCR (polymerase chain reaction) are of greater suitability in genetic diversity estimations and identification of genotypes (Thomas *et al.* 1993). They are used in several studies of genetic population such as RAPDs (Storchova *et al.* 2002; Suh *et al.* 2002), AFLPs (Waugh *et al.* 1997; Evans *et al.* 2001). Previous studies have assessed the genetic variability of chestnut using Random Amplified Polymorphic DNA (RAPDs) (Galderisi *et al.* 1998; Fineschi *et al.* 1993; Oraguzie *et al.* 1999), Amplified Fragment Length Polymorphism (AFLPs) (Yamamoto *et al.* 1998) and microsatellites (SSRs) (Buck *et al.* 2003; Martin *et al.* 2010; Nishio *et al.* 2011).

The aims of this study were (i) to study the genetic diversity within and among Swiss clones and cultivars of chestnut, (ii) to characterize genetic variation between cultivars and natural chestnut of coppice shoots so that we can manage the utilization of the natural resources in southern Alps and (iii) to study the usefulness of RAPD and AFLP for the identification of chestnut varieties and clones.

MATERIALS AND METHODS

Plant material

In the present study, plant material was collected from morphologically different varieties of different areas in southern Switzerland. The origin of studied accessions of Swiss varieties and coppice shoots are listed in **Table 1**.

DNA extraction

Total DNA was extracted from small leaves of > 80-year-old trees using hexadecyltrimethyl ammonium bromide (CTAB) according

Received: 27 December, 2011. Accepted: 26 January, 2012.

Original Research Paper

Table 1 Studied accessions of chestnut cultivars and their origin.

Accession order	Accession N°.	Accession name	Genotype	Origin
1	1	C. sativa	Verdanesa	Calonico 01.CH
2	2	C. sativa	Verdanesa	Calonico 04.CH
3	3	C. sativa	Verdanesa	Giornico 03.CH
4	4	C. sativa	Verdanesa	Giornico 06.CH
5	5	C. sativa	Verdanesa	Giornico 04.CH
6	6	C. sativa	Verdanesa	Chironico 02.CH
7	7	C. sativa	Verdanesa	Chironico 06.CH
8	8	C. sativa	Verdanesa	Chironico 10.CH
9	9	C. sativa	Verdanesa	Chironico 12.CH
10	10	C. sativa	Verdanesa	Chironico 13.CH
11	11	C. sativa	Verdanesa	Lodrino 04.CH
12	12	C. sativa	Verdanesa	Torricella 10.CH
13	13	C. sativa	Verdanesa	Torricella 13.CH
14	1	C. sativa	Lüina	Calonico 02.CH
15	2	C. sativa	Lüina	Calonico 07.CH
16	3	C. sativa	Lüina	Giornico 01.CH
17	4	C. sativa	Lüina	Giornico 02.CH
18	5	*C. sativa*	Lüina	Chironico 01.CH
19	6	*C. sativa*	Lüina	Chironico 05.CH
20	7	*C. sativa*	Lüina	Chironico 08.CH
21	8	*C. sativa*	Lüina	Chironico 14.CH
22	9	*C. sativa*	Lüina	Lodrino 03.CH
23	10	*C. sativa*	Lüina	Lodrino 05.CH
24	11	*C. sativa*	Lüina	Lodrino 14.CH
25	12	*C. sativa*	Lüina	Torricella 08.CH
26	13	*C. sativa*	Lüina	Torricella 09.CH
27	14	*C. sativa*	Lüina	Torricella 17.CH
28	1	*C. sativa*	Bonè negro	Calonico 03.CH
29	2	*C. sativa*	Bonè negro	Calonico 05.CH
30	3	*C. sativa*	Bonè negro	Calonico 06.CH
31	4	*C. sativa*	Bonè negro	Calonico 08.CH
32	5	*C. sativa*	Bonè negro	Chironico 03.CH
33	6	*C. sativa*	Bonè negro	Chironico 04.CH
34	7	*C. sativa*	Bonè negro	Lodrino 02.CH
35	8	*C. sativa*	Bonè negro	Lodrino 11.CH
36	9	*C. sativa*	Bonè negro	Lodrino 12.CH
37	10	*C. sativa*	Bonè negro	Lodrino 13.CH
38	1	*C. sativa*	Berögna	Lodrino 07.CH
39	2	*C. sativa*	Berögna	Lodrino 08.CH
40	3	*C. sativa*	Berögna	Prosita 07.CH
41	1	*C. sativa*	Pinca	Vezio 21.CH
42	2	*C. sativa*	Pinca	Vezio 22.CH
43	3	*C. sativa*	Pinca	Vezio 31.CH
44	1	*C. sativa*: Coppice		CH
45	2	*C. sativa*: Coppice		CH
46	3	*C. sativa*: Coppice		CH
47	4	*C. sativa*: Coppice		CH
48	5	*C. sativa*: Coppice		CH
49	6	*C. sativa*: Coppice		CH
50	7	*C. sativa*: Coppice		CH
51	8	*C. sativa*: Coppice		CH
52	9	*C. sativa*: Coppice		CH

to the method described by Porebski *et al*. (1997). High salt concentrations and polyvinylpolypyrrolidone (PVP-40) were added separately to remove polysaccharides and polyphenol compounds. Extraction was performed by an extended RNase treatment and a phenol-chloroform method. The purification of DNA was achieved by prep-A-gene matrix (Biorad) and quantified spectrophometrically. DNA was resuspended in TE (pH 8) solution and stored at –20°C.

Table 2 Sequence of the random primers tested in RAPD analysis.

Primer	Sequence 5'-3'
OPA02	TGCCGAGCTG
OPA04	AATCGGGCTG
OPA10	GTGATCGCAG
OPB08	GTCCACACGG
OPD20	ACCCGGTCAC
OPE01	CCCAAGGTCC
OPE04	GTGACATGCC
OPE16	GGTGACTGTG
OPE19	ACGGCGTATG
OPX17	GACACGGACC
OPA07	GAAACGGGTG
OPA15	TTCCGAACCC

Table 3 Details on primers used in AFLP analysis.

Primer	Sequence 5′-3′
*Eco*RI adapter	CTCGTAGACTGCGTACC
	AATTGGTACGCAGTCTAC
*Eco*RI-+1 Primer	GACTGCGTACCAATTCA
*Eco*RI-+3 Primer	GACTGCGTACCAATTCAGT
	GACTGCGTACCAATTCAAC
	GACTGCGTACCAATTCAGG
*Mse*I Adapter	GACGATGAGTCCTGAG
	AATTGGTACGCAGTCTAC
*Mse*I-+1- Primer	GATGAGTCCTGAGTAACAA
	GATGAGTCCTGAGTAACAT
*Mse*I-+3- Primer	GATGAGTCCTGAGTAACAT
	GATGAGTCCTGAGTAACTT

Primers used in PCR

For RAPD, a total of 30 primers were screened from set OPA, OPB, OPD, OPE, OPM and OPX (Operon Technologies, Alameda, CA), 12 10-mer oligonucleotides were selected (**Table 2**) according to the number and consistency of amplified specific markers. For AFLP, 4 sets of selective primer combinations were used (E-AGG/M-CTT, E-AAC/M-CTT, E-AGT/M-CAT and E-AAC/M-CAT) (**Table 3**) from the GIBCO BRL AFLP™ Core Reagent Kit to generate AFLP fragments.

DNA amplification

1. RAPD

PCR were performed in a total volume of 25 µl. The amplification reaction contained 1X PCR buffer, 1.4 mM $MgCl_2$, 0.2 mM dNTP, 0.4 µM primer, 1 U/µl *Taq* polymerase (Eurobiotaq) and 20 ng/µl template DNA. PCR was performed in a Hybaid PCR express thermal cycler (HBP × 220) with following cycling profile: an initial denaturation at 94°C for 4 min, followed by 38 cycles of 1 min at 93°C, 1 min at 45°C and 1 min at 72°C with a final extension at 72°C for 5 min. PCR products were mixed with loading buffer and separated on 1.6% (w/v) agarose gel containing 0.4 µg/ml ethidium bromide, in 1X TBE at 100 V for 90 min. The DNA fragments were then visualized under UV light.

2. AFLP

The DNA concentration was adjusted to 100 ng/µl, and digested in 40 µl and incubated for 2 h at 37°C. Digestion was done with 5 U of *Eco*RI (Biolabs) and 5 U of *Mse*I (Biofinex) in T4-buffer. For the ligation reaction, the following mixture was added to the restriction-reaction mixture; *Eco*RI adapter (40 p mol/µl), *Mse*I adapter (40 pmol/µl), 1 U T4 DNA ligase, 1X T4 DNA ligase buffer and incubated for 3 h at 37°C.

Preamplification was carried out in volumes of 20 µl. Reactions contained 1X PCR buffer, 1.5 mM $MgCl_2$, 1 mM dNTP, 10 pmol/µl *Eco*RI-primer adapter, 1 U *Taq* polymerase (Qiagen AG, Basel) and 100 ng/µl template DNA. Preamplification with primers having a single selective nucleotide, were performed in a Biometra I thermocycler with the cycling following profile: 2 min DNA denaturation step at 94°C, followed by 28 cycles of 45 sec at

94°C, 45 sec at 56°C and 1 min at 72°C. A final elongation step was done at 72 °C for 10 min. The reaction mixtures were diluted 10-fold for selective PCR.

Amplification with primers that have three selective nucleotides were conducted in 20 µl volume. Reactions contained 1X PCR buffer, 0.75 mM $MgCl_2$, 1 mM dNTP, 0.25 µM primer *Eco*RI, 0.30 µ*M Mse*I, 1 U *Taq* polymerase. PCR was performed for 36 cycles with the following cycle profile: a 30 sec DNA denaturation step at 94°C, a 30 sec annealing step and a 1 min extension step at 72°C. The annealing temperature in the first cycle was 65°C, and was subsequently reduced by 0.7°C each cycle for the next 13 cycles, and kept at 56°C for the remaining 23 cycles. 1 µl of PCR product was mixed with a 12 µl volume of deionised formamide and 0.5 µl Gene Scan 500 (ROX) size standard marker. The resulting mixture was heated for 2 min at 95°C and then quickly cooled on ice. Each sample was loaded and run on the ABI-310 automated DNA sequencer (capillary electrophoresis). GeneScan and Genotyper software (PE Applied Biosystems) were used to score the AFLP profiles.

Data scoring and analysis

Experiments were repeated in triplicate. For each individual and for each primer that yielded a clear pattern, polymorphic DNA fragments for the two types of markers were scored as present (1) or absent (0). For the two data sets, dendrograms were constructed by UPGMA (Unweighted Pair-Group Method with Arithmetic Averages) cluster analysis according to Jaccard's coefficient (Sneath and Sokal 1973) using cluster analysis software (http://www.biology.ualberta.ca/jbrzusto), and then visualized with the TREEVIEW program. The correlation between RAPD and AFLP techniques of genetic distance matrices was investigated by the Mantel test of matrix correspondence (Mantel 1967), based on Jaccard's similarity coefficient. Mantel's tests were performed with the R4 (Beta version) package (Casgrain P, Legendre P, Department of Biological Science, Université de Montréal) and statistical significance was determined by random permutation (999 permutations). The correlation between RAPD and AFLP methods was investigated by the Mantel test of matrix correspondence (Mantel 1967), based on Jaccard's similarity coefficient and was compared pairwise.

RESULTS

Molecular markers

All the molecular techniques used in this study were able to uniquely fingerprint each of the 52 cultivated chestnut accessions.

The 12 RAPD primers used on the whole set of plants produced a total of 98 polymorphic bands. For all the genotypes, the highest number of polymorphic and scorable bands was obtained by primer OPE-01 (10 fragments). An average of 8.0 bands per primer ranging from about 1000 to 5000 bp were produced.

Scoring of AFLP bands was difficult but polymorphism among cultivars was readily scored. The four primer combinations (E-AGG/M-CTT, E-AAC/M-CTT, E-AGT/M-CAT and E-AAC/M-CAT) yielded 222 polymorphic fragments with an average of 55 polymorphic bands per reaction, which ranged from 50 to 350 bp.

Intercultivar polymorphism

In the two dendrograms generated by the two methods, the species of *C. sativa* was clustered into two groups: the first cluster is composed of Swiss cultivars and the second is composed of coppice shoots (**Figs. 1, 2**). RAPDs ordered 'Pinca' and 'Berögna', as expected, according to the supposed affiliation to a variety. Discrepancies in forming clear groups within 'Lüina', 'Verdanesa' and 'Boné negro' varieties were observed in dendrograms produced by the two markers. Furthermore, RAPD and AFLP clustered clearly 'Lüina', 'Verdanesa' and 'Boné negro', though with exceptions, into separate groups. It seems interesting to mention

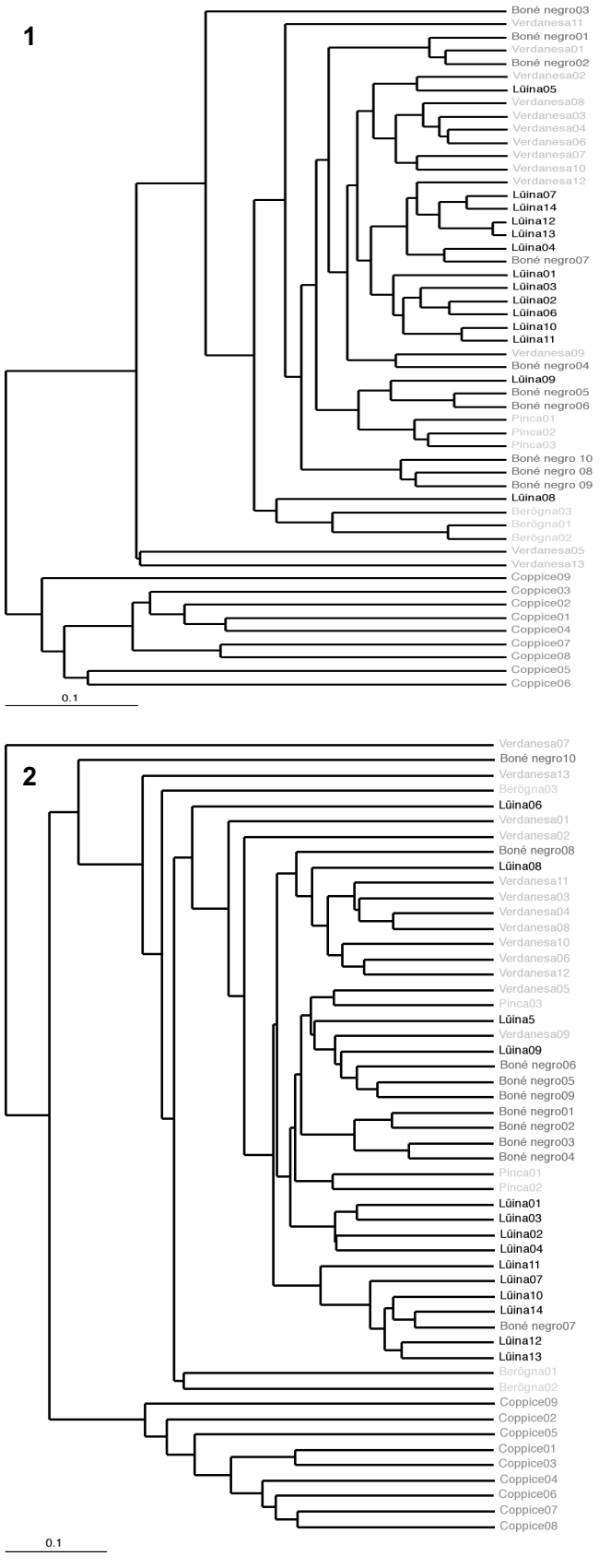

Fig 1 UPGMA dendrogram obtained using RAPD analysis (based on Jaccard's similarity coefficient). **Fig 2** UPGMA dendrogram obtained using AFLP analysis (based on Jaccard's similarity coefficient).

that several small groups of accessions have been formed that made identification very difficult. This was the case for three cultivars: 'Verdanesa', 'Lüina' and 'Boné negro'.

Intracultivar polymorphism

RAPDs and AFLPs data provided fairly a clear separation of cultivars with some extreme value of other cultivars, which were positioned within varieties. 'Boné negro 07' and 'Verdanesa 12' were closely related to 'Lüina', 'Lüina 05' was closely related to 'Verdanesa' and 'Lüina 09' was related to 'Boné negro' as shown by RAPD. For AFLP, 'Lüina 08', 'Boné negro 08' were clustered with 'Verdanesa' and 'Boné negro 07' was related to 'Lüina' cultivars. RAPD method could separate two cultivars ('Pinca' and 'Berögna') in a clear distinct group showing some similarity in clustering cultivars. Considering the 'Verdanesa' related accessions, the topology of each tree revealed with RAPD and AFLP sets is unique with some similarity.

Comparison between marker systems

Mantel test indicated a high correlation between matrices based on AFLPs and RAPDs ($r = 0.78$, $P < 0.01$) confirmed the similarity of clustering cultivars by these types of markers.

DISCUSSION

The two methods used in this experiment identified genetic variation in Swiss *C. sativa* cultivars with different grades of success. For the two methods used in this study, 98 and 222 polymorphic bands for (RAPD and AFLP respectively), being efficient in the characterization of chestnut cultivars. Our results divide all genotypes into two different groups: Swiss cultivars and coppice shoots. Swiss cultivars are used for nut production, whereas coppice shoots are basically used for wood production. The different uses for special ends relating to such group explain in a way the division formerly showed by dendrograms. The grouping of Swiss cultivars and coppice shoots into two different clusters by the two dendrograms may suggest that grafting methods, generally practised by farmers, for chestnut propagation, could have transformed natural forests into clonal or polyclonal fruit orchard (Fineschi 1988).

The two methods mentioned above show that Swiss cultivars are genetically closely related, our results agreed with the results based on morphological traits (Conedera *et al.* 1993), this may be explained by the fact that southern Switzerland is a rich assortment of polyclonal varieties, which are the results of a clonal selection process, generally done by farmers in terms of their needs or in terms of their aspiration to superior quality of chestnut fruit (Conedera 1994). The agro-ecological adaptation area of cultivars (not wide geographical distribution), a possible exchange of plant material and seed among the different regions of southern Switzerland and the old cultural traditions may explain the homogeneity among cultivars. Nishio *et al*. (2011) showed in Japanese chestnuts some identical genotypes originated from different area and found some similar morphological traits among cultivars such as the similar shape of their nuts, similar harvesting dates and similar characteristics in tree vigour. The same authors showed that these cultivars might be probably carried among locations by human activities.

The results obtained with the two methods indicate that 'Verdanesa', 'Lüina', 'Pinca', 'Berögna' and 'Verdanesa' are genetically related. This result could be explained by the common origin of European chestnut trees. As reported by some previous studies (Fineshi *et al.* 2000), shown that European chestnuts were coming from the East and were widely diffused in Europe by human activities. Several hypotheses about the origin of European chestnut have been established in many studies and Fernández-López and Monteagudo (2010) by the use of set of isozyme loci demonstrate the possibility that Iberian populations gene pools have one unique ancestral population.

High genetic similarities between and within Swiss cultivars were shown by these markers analysis. Similar results are found by Müller-Starck *et al*. (1993) and show that Swiss varieties appear morphologically more uniform. The same authors confirm this genetic uniformity among cultivars by isozyme traits. The same findings were confirmed by Conedera *et al*. (1993), who showed that those varieties are morphologically homogenous and have a similar architecture of the crown. Also, the asexual propagation through grafting and crossing between trees affects the closeness of the genetic relationships among cultivars.

The 12 primers of RAPD and the 4 primer combinations of AFLP employed allowed the distinction of several accessions as cultivars for all the studied varieties. RAPD and AFLP sufficiently characterized the genetic diversity among chestnut accessions, although, some differences were observed between dendrograms generated from each data set.

The complementarity's between the two methods in the identification of cultivars was confirmed by a high correlation between the two types of markers ($r = 0.78$). RAPD and AFLP markers also allowed us to solve problems generated by synonyms and homonyms in different accessions. These methods allowed us to prove that some accessions with different names, which could have been considered as different cultivars, were actually closely related, showing a very similar or identical genotype, such as for example, in the case of 'Boné négro 07' that was suspected to be similar to 'Lüina'. Moreover, the use of additional primers of those two kinds of markers may contribute to better distinction among accessions (Cervera *et al.* 1998).

Our results suggest that RAPDs are more informative and more reliable than AFLP markers in clustering accessions belonging to the same cultivars. Also, RAPD technique has some advantages compared with the other techniques used, such as their ease of use and their cost in time and money. It is interesting to note that errors can occur while scoring AFLP bands because of the high number of bands in a single line, and the large number of fragments differing only by 1 bp make this method less reproducible (Sudupak *et al.* 2002).

The high correlation between RAPD and AFLP data has already been reported in several genetic diversity studies (Powell *et al.* 1996; Renganayaki *et al.* 2001). Both are dominant markers but AFLP can generate a larger number of polymorphic bands per primer combination (222 vs 98) in our study.

CONCLUSION

It may be concluded from this study that RAPD and AFLP markers provide consistent information for cultivars identification and are valuable tools for studying the genetic relationships between Swiss chestnut cultivars. However, for better discrimination among the studied cultivars of chestnut, it is required to test much more primers for the two methods and to attempt other methods of molecular analysis to detect polymorphism; such as the use of chloroplast DNA or sequencing methods.

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