The Contribution of Pollen Germination Rates to Uneven Paternity Among Polycrosses of *Cryptomeria japonica*

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Abstract

Polycrossing is a very useful and cost-effective approach when designing mating trials. However, since the maternal general combining ability (GCA) in polycross designs is estimated on the assumption of equal contributions from paternal parents; any unequal paternal contribution leads to biased GCA estimates. Progeny from polycrossing *Cryptomeria japonica*, in which three mixtures from three paternal donors were crossed with each of three maternal parents, were analyzed using microsatellite markers to detect departures from equal paternal contribution. The deviations from equal paternal contribution were evaluated using a χ^2 goodness of fit test. In this test, we examined the null hypothesis of equal paternal contribution for each mother tree. Two different methods were used to calculate paternal contribution: first, simply the number of seeds produced by each male parent, and second, the number of seeds produced by each male parent weighted according to the proportion of full seeds in single cross. The results of the χ^2 test showed that the contribution to seed production by each paternal clone differed significantly in all polycrosses. The average pollen germination rate was significantly different between paternal clones. The contribution of each pollen donor in a polycross was related to their pollen germination rate.

Key words: Male reproductive success, Paternal success, GCA, Molecular marker, Polymixes.

Introduction

Polycrossing is one of the mating designs employed to create progeny populations for genetic testing (ZOBEL and TALBERT, 1984). In polycross designs, mixtures containing pollen from many male parents are used in the controlled cross. Therefore for a particular offspring, although the maternal parent is known, the paternal parent is unknown. The advantage of polycross designs is that they are inexpensive and require little labor. However, because the paternal parent is unknown, it is not possible to estimate the general combining ability (GCA) of the paternal parent. Estimates of the maternal GCA from polycross designs assume equal contribution by paternal parents. Any unequal paternal contribution in polycross designs leads to biased maternal GCA estimates. The majority of studies have shown that paternal contributions in controlled polycross trials are unequal; this has been demonstrated for *Populus* spp. (WHEELER et al., 2006), *Betula pendula* (PASONEN et al., 1999), *Pinus radiata* (MORAN and GRIFIN, 1985), *Picea mariana* (ROGERS and BOYLE, 1991), *Picea abies* (SCHOEN and CHELIAK, 1987; SKRØPPA and LINDGREN, 1994; ARONEN et al., 2002) and *Pseudotsuga menziesii* (APSIT et al., 1989; NAKAMURA and WHEELER, 1992). A few studies have demonstrated equal paternal contributions in species including *Pinus taeda* (WISELOGEL and VAN BUIJTENEN, 1988) and *Chamaecyparis obtusa* (SEIDO et al., 2000).

Sugi (*Cryptomeria japonica*) is an allogamous, wind-pollinated conifer species; it is an important commercial forest tree in Japan. Seeds of high genetic quality and diversity are produced in seed orchards. A previous gene flow study in a *C. japonica* seed orchard identified the relatively high paternal contribution of clone 53 to the pollination of clone 6 (accounting for about 64% of its total fertilization) (MORIGUCHI et al., 2007). However, it is not possible to conclude, from existing studies, whether differential paternal contribution occurs in *C. japonica*. Though there are two previous reports that investigated whether paternal contributions in polycrosses are equal using Wogon-Sugi, a chlorophyll-deficient mutant (OHBA et al., 1971) with incomplete paternal inheritance, the conclusions are different between them (OHBA, 1972; ITOYA, 1978).

Therefore, the aim of present study was to investigate whether paternal contributions in polycross offspring are unequal. We used microsatellite markers, a powerful molecular tool for determining parentage. We also studied whether the in vitro pollen germination rate corresponds to the proportion of seeds sired by each pollen donor.

Materials and Methods

Pollen collection and pollen germination tests

In February 2004, pollen from nine clones was collected from the *C. japonica* clone orchard archive located at the Niigata Prefectural Forest Research Institute. Pollen was stored in dry conditions in a refrigerator at 4°C for one month. Pollen collected from each of nine clones was germinated. Each germination test was replicated three times. Pollen grains were germinated on 1% agar containing 5% sucrose, in Petri dishes under standard conditions, i.e. 96 h at 25°C in the dark as

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described by SAITO and YAMAMOTO (1977). Germination was terminated by staining the pollen with a lactic phenol-cotton blue, after which the samples were transferred onto glass slides. The germination percentage was determined by counting more than 600 randomly selected pollen grains per sample. We defined the germination of pollen grains as having occurred when the length of the pollen tube was at least half of the diameter of the pollen grain.

Experimental material produced by pollination

Pollen mixtures were prepared by combining equal weights of desiccated pollen from three clones. The compositions of the three pollen mixtures are shown in *Table 1*. The polycross was conducted using a mixture of pollen from three clones; a single-cross for each clone was also made (*Table 1*). Each cross was repeated on three grafts from each maternal clone. Before the flowers reached maturity, we removed the male ones and

Table 1. – Summary of pollination results, including nine polycrosses and 27 single crosses.

Maternal parent	Paternal parents	Rate of Cone set (%)	Number of seeds per cone	Proportion of full seeds (%)
C5	Pollen mixture 1 (C29, C67, C75)	95.4 (± 4.0)	58.6 (± 4.7)	37.0 (± 7.4)
C5	C29	84.0 (± 14.5)	63.6 (± 2.6)	34.3 (± 10.9)
C5	C67	84.1 (± 15.7)	58.0 (± 8.9)	31.0 (± 3.1)
C5	C75	80.1 (± 15.5)	61.5 (± 1.6)	38.7 (± 5.2)
C38	Pollen mixture 1 (C29, C67, C75)	94.2 (± 6.6)	71.3 (± 14.3)	42.2 (± 9.3)
C38	C29	43.5 (± 8.2)	74.5 (± 7.8)	52.3 (± 4.3)
C38	C67	97.8 (± 3.8)	73.3 (± 7.8)	36.2 (± 10.6)
C38	C75	86.2 (± 17.6)	68.6 (± 11.3)	32.4 (± 4.0)
C65	Pollen mixture 1 (C29, C67, C75)	86.2 (± 17.4)	41.3 (± 19.0)	55.3 (± 7.1)
C65	C29	79.0 (± 25.2)	46.1 (± 24.4)	67.1 (± 9.5)
C65	C67	93.5 (± 8.6)	63.7 (± 8.3)	44.8 (± 11.6)
C65	C75	96.6 (± 4.0)	57.6 (± 18.2)	42.8 (± 13.6)
C6	Pollen mixture 2 (C5, C37, C53)	96.5 (± 4.1)	33.9 (± 6.5)	30.6 (± 7.1)
C6	C5	95.5 (± 7.9)	48.3 (± 2.9)	23.6 (± 6.2)
C6	C37	98.5 (± 3.4)	50.2 (± 8.5)	41.3 (± 8.0)
C6	C53	99.5 (± 0.9)	44.9 (± 1.9)	26.8 (± 1.3)
C44	Pollen mixture 2 (C5, C37, C53)	89.4 (± 12.9)	20.5 (± 3.4)	47.3 (± 5.1)
C44	C5	94.7 (± 6.2)	28.9 (± 4.5)	37.0 (± 10.4)
C44	C37	98.6 (± 2.5)	35.2 (± 3.7)	43.3 (± 4.6)
C44	C53	90.0 (± 8.7)	21.3 (± 4.6)	42.8 (± 4.4)
C60	Pollen mixture 2 (C5, C37, C53)	98.9 (± 2.0)	47.9 (± 5.2)	41.1 (± 11.2)
C60	C5	92.1 (± 10.4)	35.6 (± 12.1)	33.9 (± 4.4)
C60	C37	98.6 (± 1.2)	57.2 (± 12.1)	54.3 (± 5.5)
C60	C53	98.9 (± 1.9)	48.5 (± 21.7)	50.3 (± 2.4)
C52	Pollen mixture 3 (C9, C58, C63)	77.7 (± 24.3)	25.1 (± 9.0)	15.7 (± 15.0)
C52	C9	75.8 (± 30.6)	23.6 (± 2.5)	15.5 (± 6.0)
C52	C58	77.6 (± 14.2)	21.5 (± 3.7)	23.3 (± 4.9)
C52	C63	81.1 (± 5.4)	20.7 (± 4.1)	17.8 (± 5.1)
C27	Pollen mixture 3 (C9, C58, C63)	91.8 (± 7.4)	46.6 (± 15.8)	24.1 (± 4.6)
C27	C9	96.1 (± 2.3)	34.0 (± 7.6)	27.6 (± 8.8)
C27	C58	93.2 (± 9.6)	27.4 (± 2.5)	44.1 (± 3.5)
C27	C63	93.4 (± 9.6)	36.4 (± 5.5)	37.3 (± 2.8)
C54	Pollen mixture 3 (C9, C58, C63)	93.8 (± 5.5)	25.6 (± 5.8)	32.7 (± 6.9)
C54	C9	87.5 (± 13.0)	27.6 (± 5.1)	41.3 (± 5.5)
C54	C58	91.3 (± 11.3)	26.3 (± 5.0)	37.4 (± 6.3)
C54	C63	96.7 (± 1.1)	25.4 (± 5.4)	37.0 (± 1.0)

isolated the female ones using paper breeding bags. At the peak of receptivity, in March, we pollinated the appropriate clones. Each graft was pollinated twice (on two days). The breeding bags were removed in May, and the number of pollinated female flowers on each graft was counted. The mature cones were harvested in October, and the number from each graft was recorded. The rate of cone set for each graft was defined as the ratio of the number of mature cones in October to the number of pollinated female flowers in May. We extracted the seed from the cones, and counted the number produced, per cone, for each graft. Three hundred seeds were selected randomly from each graft and X-rayed (SOFTEX-EMB, Tokyo, Japan) to determine the proportion of full seeds.

DNA extraction and microsatellite genotyping

We collected needles from all clones that were used in the pollination trials. The total DNA was extracted from the needles using the modified CTAB method (TSUMURA et al., 1995). Seeds used for the X-ray analysis were sown on sterile paper on plastic plates, and DNA was extracted from germinated embryos using the CTAB method (MURRAY and THOMPSON, 1980). We used more than 90 seeds per polycross for DNA extraction, except for the two polycrosses, C52 x pollen mixture 3 and C54 x pollen mixture 3, which had produced few female flowers (*Table 2*). These two polycrosses were excluded from the subsequent analysis.

We used four microsatellite markers that exhibited high stability and polymorphism (*Table 3*; MORIGUCHI et al., 2003 and TANI et al., 2004) to determine the pollen donors and pollen contamination rates for seeds produced from the nine polycrosses. PCR amplifications were performed using Model 9600 and 9700 GeneAmp PCR Systems (Applied Biosystems). In each case a total volume of 10 µL was used, containing 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM of $MgCl_2$, 0.16 mM of each dNTP, 0.2 µM of each primer, 5 ng of template DNA, and 0.625 units of *Taq* polymerase (PROMEGA). The PCR program consisted of: 5 min denaturation at 94°C, followed by 30−35 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 60°C, and 30 sec extension at 72°C, with a final extension step of 72°C for 5 min. Genotypes of the paternal candidate *C. japonica* trees and the offspring from each polycross, based on the microsatellite markers, were determined using ABI prism 3100 genetic analyzers (Applied Biosystems).

Data analysis

From the genotype data for the paternal candidates, we calculated the paternity exclusion probability for each locus and the multi-paternity exclusion probability (WEIR, 1996) using the G-DIVERSE software developed by Hiroyoshi Iwata (NARC, Tsukuba, Japan). We determined the paternal parent of each of the germinated seeds after excluding pollen contamination and self-fer-

Table 2. – Number of offspring assigned to each paternal parent in the polycross and the pollen germination rate of each paternal parent. The two pollinations, C52 x pollen mixture3 and C54 x pollen mixture3, were excluded because the number of seeds produced was too low to analyze statistically. For each combinations, the relationship between the pollen germination rate and paternal contribution was examined by Spearman rank correlation test.

	C5 x PM1[a]	C38 x PM1[a]	C65 x PM1[a, ****]	C6 x PM2[b, ****]	C44 x PM2[b, ****]	C60 x PM2[b, ****]	C52 x PM3[c]	C27 x PM3[c, ****]	C54 x PM3[c]	Average pollen germination rate (%)
C29	120	127	63	-	-	-	-	-	-	73.5 (± 4.7)
C67	21	29	17	-	-	-	-	-	-	40.9 (± 4.9)
C75	42	35	10	-	-	-	-	-	-	38.1 (± 8.7)
C5	-	-	-	22	28	30	-	-	-	53.4 (± 10.0)
C37	-	-	-	87	71	90	-	-	-	68.7 (± 13.6)
C53	-	-	-	29	39	55	-	-	-	59.7 (± 11.8)
C9	-	-	-	-	-	-	3	23	4	52.0 (± 8.6)
C58	-	-	-	-	-	-	15	28	16	61.9 (± 13.6)
C63	-	-	-	-	-	-	11	50	4	68.5 (± 5.6)
Self-fertilization	8	3	0	0	3	0	0	0	0	-
Contamination	0	0	0	0	41	0	0	0	1	-
Total	191	194	90	138	182	175	29	101	25	-

[a] PM1 refers to pollen mixture 1 including the pollen from C29, C67 and C75.
[b] PM2 refers to pollen mixture 2 including the pollen from C5, C37 and C53.
[c] PM3 refers to pollen mixture 3 including the pollen from C9, C58 and C63.
****, P < 0.001.

Table 3. – Microsatellite markers used in this study.

primer	motif	Forward primer 5' to 3'	Reverse primer 5' to 3'	Q_i[a]
Cjgssr 78	$(GA)_{21}$	AAGAAGGCATCCAAGAGTGA	ACTGCCGATTAACTGATAGCTC	0,824
Cs 1525	$(CA)_{18}$	ATGAAGTGCCCTTGGTTTGT	ATCGCCTCCTCTTTTATCCT	0,843
Cjgssr 175	$(CT)_{16}$	ACCCTTTGAATTATTCCTTGAG	AGCGGAAAAAACTACATCAGA	0,688
Cs 1219	$(GT)_{10}$	AAGGTGTTGTTTTAAGGAGG	CAGCCATCTATTATTTGTGC	0,525

[a] Multi-paternity exclusion probability = 0.996.

tilization, following the simple exclusion method of MORIGUCHI et al. (2004). The deviations from equal paternal contribution were evaluated by a χ^2 goodness of fit test, as follows. First, we examined the null hypothesis of equal paternal contribution for each mother tree using the formula EPC1 (see below). Second, we examined the null hypothesis of equal paternal contribution for each mother tree, weighted according to the proportion of full seeds in a single cross, using EPC2.

EPC1 = (total number of seeds) / 3

EPC2 = (total number of seeds) x [(the proportion of full seeds of i-th paternal clone) / (sum of the proportion of full seeds in the pollen mixture)].

Differences in the proportion of full seeds and the average pollen germination rate among clones were examined by one-way ANOVA. The relationship between the pollen germination rate and paternal contribution was examined by Spearman rank correlation test. These analyses were done using JMP4 software ver. 11.5 (SAS Institute Inc., Cary, N.C.).

Results

The multi-paternity exclusion probability (WEIR, 1996), determined on the basis of the four microsatellite markers, was high (0.996). This indicated that the level of genetic polymorphism was sufficient to identify all pollen donors in this study unambiguously; this was because the number of parents was limited (only 17 clones). In fact, these four microsatellite markers unambiguously determined the pollen donors for each cross.

The average rates of cone set were generally high in all crosses, suggesting that pollination was generally successful in this study (*Table 1*). Although the C38 x C29 cross produced a large proportion of immature cones, we were able to use seeds from this cross for further analysis because of the sufficiently high average number of seeds per cone. For single crosses, there was no difference in the rate of full seed (examined using ANOVA) between the three paternal parents, with the exception of mother trees C38 ($p < 0.05$), C6 ($p < 0.01$) and C60 ($p < 0.005$). High contamination was detected in the C44 x pollen mixture no. 2 cross, and low rates of self-fertilization were detected in some crosses (*Table 2*).

In this study, we corrected the expected equal paternal contributions on the basis of the proportion of full seeds in single cross (EPC$_2$). The χ^2 test for goodness-of-fit indicated that the null hypothesis, i.e. that every paternal clone contributed equally to seed production, should

be rejected for all polycrosses (*Table 4*). The average pollen germination rate was 57.4%, ranging from 38.1% to 73.5% for the different clones (*Table 2*), these differences were statistically significant (ANOVA, $p < 0.0001$). The pollen germination rates were significantly related to paternal contribution in all combination except for C5 x pollen mixture no. 1 cross and C38 x pollen mixture no. 1 cross (*Table 2*). Even the two combinations that have no statistical significance, the paternal contributions tended to be associated with the pollen germination rates (*Fig. 1*).

Discussion

The rate of cone set was generally high for the control crosses in this study (*Table 1*), suggesting that the timing of pollination, as well as the quantity of pollen supplied, was appropriate. The significant difference, for some mother trees, in the proportion of full seeds produced by each of the three paternal parents used in the pollen mixture seemed to be related to the pollen germination rates (*Table 1*, *Table 2*).

Paternal parentage was determined using four microsatellite loci. The high contamination detected in the C44 x pollen mixture no. 2 polycross could be the result of damage to breeding bags by snow, wind or birds (*Table 2*). Moreover, the low level of self-fertilization detected in some crosses might be explained by incomplete male flower removal before pollination. The significant deviation, in all polycrosses, from equal paternal contribution suggests that this is the general case in *C. japonica*. Similar results have been recorded previously in many forest tree species, as mentioned above.

The causes of unequal contribution have been studied in a large number of species. SNOW and SPIRA (1996), PASONEN et al. (1999) and ARONEN et al. (2002) reported that pollen-tube growth rate affects paternal contribution in *Hibiscus moscheutos*, *Betula pendula* and *Picea abies*, respectively. Moreover, SPIRA et al. (1996) reported that the timing of pollen arrival on the stigma affects paternal contribution in *Hibiscus moscheutos*. In addition, PARANTAINEN and PASONEN (2004) found pollen-pollen interactions in artificial crossing experiments using pollen mixtures in *Pinus sylvestris*. NAKAMURA and WHEELER (1992) reported that genetic incompatibility between male and female gametophytes affects paternal contributions in *Pseudotsuga menziesii*. In the current study, the average pollen germination rate was significantly different between clones and this difference seemed to influence the levels of the paternal contribu-

Table 4. – The chi-squared values of testing the null hypothesis of equal paternal contribution, based on seed number (EPC1) and seed number weighted on the basis of full seeds in single crosses (EPC2).

	C5 x PM1[a]	C38 x PM1[a]	C65 x PM1[a]	C6 x PM2[b]	C44 x PM2[b]	C60 x PM2[b]	C52 x PM3[c]	C27 x PM3[c]	C54 x PM3[c]
EPC$_1$(χ^2)	89.21[****]	94.79[****]	55.27[****]	55.35[****]	21.70[****]	31.14[****]	-	12.26[****]	-
EPC$_2$(χ^2)	89.58[****]	42.85[****]	42.81[****]	18.26[****]	16.45[****]	11.67[****]	-	14.15[****]	-

[a] PM1 refers to pollen mixture 1 including the pollen from C29, C67 and C75.
[b] PM2 refers to pollen mixture 2 including the pollen from C5, C37 and C53.
[c] PM3 refers to pollen mixture 3 including the pollen from C9, C58 and C63.
[*], P < 0.05; [**], P < 0.01; [***], P < 0.005; [****], P < 0.001.

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a) ☐ C29(73.5%) ☐ C67(40.9%) ■ C75(38.1%)

C65
C38
C5

b) ☐ C37(68.7%) ☐ C53(59.7%) ■ C5(53.4%)

C60
C44
C6

c) ☐ C29(73.5%) ☐ C67(40.9%) ■ C75(38.1%)

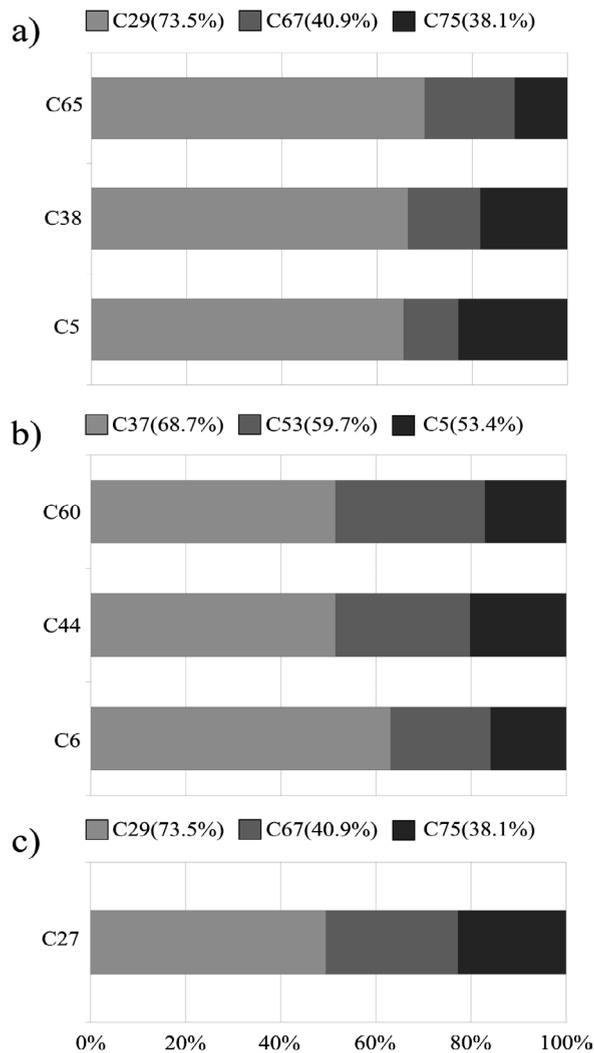C27

0% 20% 40% 60% 80% 100%

Figure 1. – The percentage of progeny assigned to each paternal parent in polycross-1 (a), polycross-2 (b) and polycross-3 (c). The values within parentheses are the pollen germination rate (%) of paternal parents. The two pollinations, C52 x pollen mixture3 and C54 x pollen mixture3, were excluded because the number of seeds produced was too low to analyze statistically.

paternal contributions only differed by a small amount. To obtain reliable GCA estimates from polycrosses in *C. japonica*, more equal paternal contribution is required. KUMAR et al. (2007) also suggested that polycrosses should make use of a large number of pollen donors in order to validate results, since large deviations from equal paternal contribution have been detected when using three or four pollen donors for *Pinus radiata* (MORAN and GRIFFIN, 1985) and *Picea abies* (SKRØPPA and LINDGREN, 1994). In contrast, small deviations from equal paternal contribution have been detected when using more than nine pollen donors for *Picea abies* (SCHOEN and CHELIAK, 1987) and *Pinus radiata* (KUMAR et al., 2007). Therefore, using a large number of pollen donors (KUMAR et al. (2007) recommended more than 15) in a polycross might decrease the variations in the level of paternal contribution. The use, in polycrosses, of pollen-donor clones with similar pollen germination rates is an alternative method, but this is technically difficult for artificial crosses.

Though clone no. 53 contributed disproportionately with clone no. 6 according to the pollen flow in a seed orchard (accounting for about 47% of the total fertilization) (MORIGUCHI et al., 2007), this was not demonstrated in the current work. NIKKANEN et al. (2000) reported a significant genotype-year interaction in pollen viability, which might account for our result. A specific investigation into the effect of pollen viability on paternal contributions in seed orchards is expected in the future study.

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tions (*Fig. 1*, *Table 1*). Therefore, the variation in pollen germination rate may affect paternal contribution in polycrosses of *C. japonica* (*Table 3*, *Fig. 1*); however, we did not observe the pollen-tube growth, nor any pollen-pollen interactions and selection in the postzygotic process. WHEELER et al. (2006) reported that pollen germination rate did not control paternal contribution in *Populus* spp.; however, they only recorded small differences in pollen germination rates between the pollen donors used in their polycrosses as a result of low pollen germination rates (< 25% in all paternal donors).

Generally, an unequal paternal contribution in polycross designs leads to biased maternal GCA estimates. However, KUMAR et al. (2007) reported that GCA estimates obtained from polycrosses were similar to those from female-tester mating designs. In their study, unequal paternal contribution was detected only in some families. Therefore, they suggested that precise GCA estimates could be obtained from polycrosses when

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