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DEMOGRAPHIC *VERSUS* GENETIC (RAPD) VARIATION BETWEEN AND WITHIN TWO POPULATIONS OF THE CLONAL PLANT *PARIS QUADRIFOLIA* L. (LILIACEAE)

ABSTRACT: The two populations of *Paris quadrifolia* L. were studied in isolated habitats in a currently fragmented landscape. Both populations were located in deciduous forests, the first (A) – on fresh mineral and acidic soil, and the second (B) – on wet organogenic, less acidic and more fertile soil. We hypothesized that genetic variation should be higher within population of more fecund plants, and that genetic distance between the two populations that occupy different isolated habitats in a fragmented landscape should be high.

Demographic characteristics of populations were studied in the 2000–2005 period. In patches of both populations, 22 permanent plots measuring 1m^2 each were designated. For molecular testing 41 samples from both populations were selected.

The share of generative shoots was higher in the population A than population B (0.35 and 0.20, respectively). However, the fecundity of ramets expressed as the number of seeds in the ripe fruit was lower in A than in the B population (15 *versus* 21 seeds). The germination ability was significantly higher for the seeds from A than from the B population (79% *versus* 44%). The survival of the juveniles was high in both populations (54 and 76%). The Random Amplified Polymorphic DNA (RAPD) analysis with the application of five primers permitted identifying 91 loci. The estimation of genetic diversity was based on polymorphic loci, the share of which was average 44%. Nei's gene diversity (h) was higher in the A than B population (0.28 *versus* 0.22). The genetic diversity between the populations was not large (G_{ST} = 0.14). Clonal diversity was very high, G/N ratio = 1, and cluster analysis showed intermingling between samples from both the populations. There were quite a small genetic distance (D = 0.10), and a rather high level of gene flow (Nm = 1.51) between the populations from currently isolated habitats. The obtained results indicate that the genetic diversity was lower within population of more fecund plants from more productive habitat.

KEY WORDS: *Paris quadrifolia,* genetic diversity, generative reproduction, environmental conditions

1. INTRODUCTION

Genetic diversity in populations is often considered in an evolutionary context. Determination of the current population structure allows conclusions regarding the dynamics of demographic processes, primarily about fecundity and migration (Gabrielsen and Brochmann 1998, Auge *et al.* 2001, Wróblewska *et al.* 2003). Growth based on the production of genetically identical subsequent units (ramets), which are clones (Harper 1977), is a characteristic trait of clonal plants.

As a consequence of this type of growth, the genetic diversity in populations of clonal plants is expected to be low (McLellan *et al.* 1997). Populations of species whose reproduction is primarily vegetative with low levels of seedling recruitment are threatened by genetic erosion, which can diminish the ability of these organisms to adapt to changing environmental conditions and their resistance to pathogens (Tomimatsu and Ohara 2003). However, as early as in 1989 Eriksson called attention to the significance of the level of seedling recruitment in clonal plant populations and the likely large impact it has on the genetic diversity of these plants. Even a small, but repeated, inflow of individuals of generative origin can maintain this diversity at a substantial level (Watkinson and Powell 1993, Eriksson 1997). On the other hand, the established shoots of these plants, *i.e.* rhizomes, can prevent the loss of genetic diversity when environmental conditions are disturbed (Arnold and Emms 2000). Results of many studies indicate that the level of genetic diversity in populations of clonal plants is higher than that of annual plants (Nybom 2004).

Human activities lead to the fragmentation of the natural habitats of many species, and these results in the isolation and decreasing size of affected populations. The isolation and substantial diminishment of populations can, in effect, reduce their genetic diversity (Ellstrand and Elam 1993). Species with narrow ranges of distribution and narrow habitat requirements are especially vulnerable, as is the case in populations of *Saxifraga cernua* L. (Gabrielsen and Brochmann 1998), *Iris aphylla* L. (Wróblewska *et al.* 2003) and *Pedicularis palustris* L. (Schmidt and Jensen 2000). However, species that are currently common are also susceptible to fragmentation and isolation. In the near future, it may occur that species which are incapable of colonization will be especially threatened (Brunet and Oheimb 1998).

Paris quadrifolia L. is classified to the family *Liliaceae* (Tutin *et al.* 1980) or *Trilliaceae* (Takhtajan 1997). This species occurs in Europe, Asia Minor and Siberia (Meusel *et al.* 1965). This plant, which occurs in shady wet forests of central Europe, is a species characteristic of the order Fagetalia (Ellenberg *et al.* 1992). *P. quadrifolia* is a medicinal plant used in traditional and homeopathic medicine and contains many biologically active components (Harborne *et al.* 1999). *P. quadrifolia* is a herbaceous perennial with both underground and aboveground shoots. The apex of the underground rhizome sends up one shoot per year, with three to seven leaves and a single flower. Although the flowers are protogynous and cross pollinated, they are also capable of autogamy (Hegi 1909). Seed dispersal is endochorous (Eriksson and Ehrlen 1991), but this species has limited dispersal ability (Brunet and Oheimb 1998, Honnay *et al.* 1999). A small seed bank was confirmed in the soil, as was a low level of seedling recruitment in the populations of this species (Fröborg and Eriksson 1997, Panufnik-Mędrzycka and Kwiatkowska-Falińska 2001). However, the cause of weak recruitment of the juveniles of this plant was not determined (Baskin and Baskin 2001).

Several years of demographic studies of *Paris quadrifolia* indicated that there are significant differences in the traits characterizing the populations of this species. We hypothesized that genetic variation should be higher within population of more fecund plants, and that genetic distance between the two populations that occupy different isolated habitats in a fragmented landscape should be high. The aims of the studies were: (1) to estimate differences with regard to the size of the area occupied, abundance, and traits of generative reproduction between the populations; (2) to identify the genetic diversity within and between populations.

2. STUDY AREA

Two populations (A, B) of *Paris quadrifolia* in northern Poland were studied. They were located in the same region, six kilometers apart in different forest communities separated by agriculture land. The population A was located in a deciduous forest on fresh mineral soil that was fairly acidic but rich in nitrogen (Table 1). The trees present were *Alnus glutinosa* (L.) Gaertner, *Betula pendula* Roth, *Quercus petraea* (Mattuschka) Liebl. and *Carpinus betulus* L., and the forest floor cover was comprised of *Anemone*

Table 1. Location and soil properties in habitats of *Paris quadrifolia*.

Population:	A	B
Location	54°02´N 17°50´E	54°00´N 17°53´E
Organic matter (% d.w.)	30	88
pH in H_2O	3.9	5.9
N-NO_3 (mg dm^{-3})	35	7
N-NH_4 (mg dm^{-3})	2	27
P_2O_5 (mg 100 g^{-1} d.w.)	15	50
K_2O (mg 100 g^{-1} d.w.)	18	47
Mg (mg 100 g^{-1} d.w.)	19	119

nemorosa L., *Adoxa moschatellina* L., *Oxalis acetosella* L. and *Aegopodium podagraria* L.

The population B was located in a shady deciduous forest on organogenic, periodically flooded soil. The soil was less acidic, richer in nitrogen, phosphorus, potassium and magnesium than that of the population A (Table 1). The trees in this location were *Alnus glutinosa* and *Betula pubescens* Ehrh., and the forest floor cover was comprised of *Cardamine amara* L., *Cirsium palustre* (L.) Scop., *Caltha palustris* L., *Viola palustris* L., *Thelypteris palustris* Schott and *Solanum dulcamara* L. The nomenclature of species follows Tutin *et al.* (1964–1980). Soil samples were collected from 3 randomly chosen plots in both populations.

The soil was analyzed for pH and concentrations of nitrate, ammonium, phosphate, potassium and magnesium.

3. METHODS

3.1. Population studies

The spatial and demographic characteristics of the *P. quadrifolia* populations were obtained based on investigations in the 2000–2005 period. The population area was mapped to the nearest meter at the beginning of the study. In patches of both the populations, 22 permanent plots measuring 1 m^2 each were designated. For six years plots were visited in May when *P. quadrifolia* was in flower, and the generative and vegetative aboveground shoots as well as seedlings were counted. Every year in July ripe fruits were harvested, and after drying, the seeds were counted and weighed.

In the autumn of 2001, the sowing experiment was conducted; the seeds (N = 230) were sown on sieved soil in frames protected by netting within the area of each population but at a distance about 5 meters from the patches of *P. quadrifolia* individuals. The seedlings were counted in the second year after sowing and juvenile plant stages were observed for subsequent three years, up to 2006. Survival of juveniles assessed as a number (percentage) of individuals that grew from seedlings.

The samples used for molecular testing were collected in April 2003 and they were the young aboveground shoots with leaves in bud. In order to estimate the possible maximum diversity within the populations, the samples were collected along a linear transect across the population area. The patches of *P. quadrifolia* in the population A occupied an area measuring from 1 to 140 m^2, and those in the population B from 1 to 20 m^2. Single samples were selected from the center each of small patches. From large patches (over 20 m^2) two samples were selected on the opposite edges of the patch. The minimum distance between the patches was 3 m. There were 23 samples selected from 15 small and 4 large patches (samples: 35 and 178; 188 and 219; 220 and 236; 238 and 248) in the A population, and 18 samples from 18 patches in the B population. Shoots were dried in bags with silica gel and then, within 48 hours, stored at a temperature of -20°C.

3.2. Genetic (RAPD) analysis

Total genomic DNA was isolated from the 41 selected samples of *P. quadrifolia* using the GENOMIC Mini AX Plant Kit (A&A Biotechnology, Poland). The quantity and purity of the isolated DNA was quantified with a spectrophotometer (UV/Vis, λ40 Perkin Elmer, USA), and then the DNA was diluted

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Table 2. Demographic characteristics of *Paris quadrifolia* from the two (A, B) populations in the years 2000–2005. Number of samples (n), arithmetic mean with standard deviation and the results of tests; significant differences among values are shown in bold.

Population Characteristic:	n	A	n	B	Test
Density of ramets per m^2	45	170.4 ± 154.9	50	86.4 ± 71.1	M-W; *P* **<0.01**
Generative shoots (%)	45	35	50	20	M-W; *P* **<0.001**
Number of seeds per ramet	418	15.0 ± 4.5	195	20.6 ± 6.8	t; *P* **<0.001**
Seed weight (mg)	2970	3.9 ± 0.8	3370	3.3 ± 0.7	t; *P* **<0.001**
Density of seedlings per m^2	25	12.9 ± 11.0	12	21.2 ± 21.2	M-W; *P*> 0.05
Germination ability (%)	8	78.6 ± 23.7	8	43.8 ± 17.5	M-W; *P* **<0.01**
Survival of juveniles (%)	8	75.6	8	54.1	M-W; *P*> 0.05

Table 3. Primers used for Random Amplified Polymorphic DNA analysis; number and share (%) of polymorphic bands detected.

	Population:	A		B	
Primer	Sequence	Number	%	Number	%
RB 12	CCTTGACGCA	11	50	12	52
RE 03	CCAGATGCAC	2	14	2	14
RH 08	GAAACACCCC	5	50	5	50
RB 01	GTTTCGCTCC	13	54	8	36
RAPD14	GGGTAACGCC	5	28	4	22
	Total	36	41	31	36

with TE buffer to 20 ng μL^{-1}. The samples thus prepared were used in the RAPD-PCR reaction.

Of the 20 primers tested (Proligo, USA), five generated band patterns. Five decamers were used in the RAPD-PCR reaction tests (Table 3).

RAPD-PCR reaction conditions were optimized with the Taguchi method (Cobb and Clarkson 1994, Caetano-Anolles 1998). The composition of the reaction mixture was as follows: deionized water; 1× PCR reaction buffer ($MgCl_2$); 3.0 mM $MgCl_2$; 0.2 mM of each dNTP; 1.2 µM primer; 1 U Taq polymerase (Fermentas, Lithuania). The tubes containing the reaction mix were placed in a thermocycler MJ Research PTC-200 (USA) at 4°C and 2 µL of DNA at a concentration of 20 ng μL^{-1} was added. The final volume of the mixture was 25 µL. Initial denaturation was done at a temperature of 95°C (2 min), denaturation at 94°C (1 min), primer annealing at 47°C (1 min), elongation at 72°C (1.5 min) and final elongation at 72°C (10 min) (stages 2–4 were repeated 46 times). To provide a test for contamination with foreign DNA, a negative control comprised of all the components of the reaction mixture, with the exception of the matrix DNA, was added to each reaction. All RAPD-PCR reactions were repeated three times for each primer and sample.

After PCR reaction, 5 µL 6× concentrated loading buffer solution containing bromophenol blue was added to the test tubes with the PCR samples. The PCR products were separated in a 1.5% agarose gel (Prona Basica LE, EU) with a 0.5× TBE buffer solution at a pH of 8.0. After electrophoresis was complete, the gel was stained in water solution of ethidium bromide at a concentration of 0.5 µg mL^{-1}. Bands were identified using a transilluminator (Ultralum, USA) and documented using a video camera. Gel pictures were analysed with the software UVIMap.

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Each PCR product (band) were treated as an individual locus with two alleles (Nybom and Bartish 2000). Presence/absence of bands in electrophoregrams was coded as the binary matrix (0; 1). Statistical analyses were based on polymorphic bands with a frequency 5–95% calculated for each locus. The matrices were used for calculating the Euclidean square distance between the samples, which were analyzed with the average unweighted linkages of genetic distances and presented as UPGMA dendrogram. Genotype diversity was estimated as the number of multilocus genotype relative to the number of stems sampled (P = G/N). Calculation of Nei's (1973) gene diversity (h), Shannon's Information Index (I), genetic variability among the populations (G_{ST}) according to Nei (1987), genetic distance D according to Nei (1978), and gene flow Nm according to Wright (1951) were carried out with Popgene software (Yeh *et al.* 1999).

For traits whose distribution was similar to normal (seed number, and seed weight) differences between arithmetic means for populations was tested with the t-test. For other traits (shoot and seedling density, share of generative shoots, germination and survival) the differences were tested with the Mann-Whitney test at a significance level of $P < 0.05$ (Sokal and Rohlf 1995) using Statistica software (StatSoft Inc. 2005).

4. RESULTS

4.1. Population characteristics

The size of the area occupied by the populations differed; the area of the population A was nearly two times smaller than that of the population B: 2600 *versus* 4400 m^2. In contrast, the shoot (ramet) density of the A population was nearly two-fold higher than that of the B population (Table 2). Therefore, the abundance of the compared populations was similar. The share of generative shoots was higher in the population A than population B (0.35 and 0.20, respectively; $P < 0.001$). The fecundity of ramets expressed as the number of seeds in the ripe fruit was lower

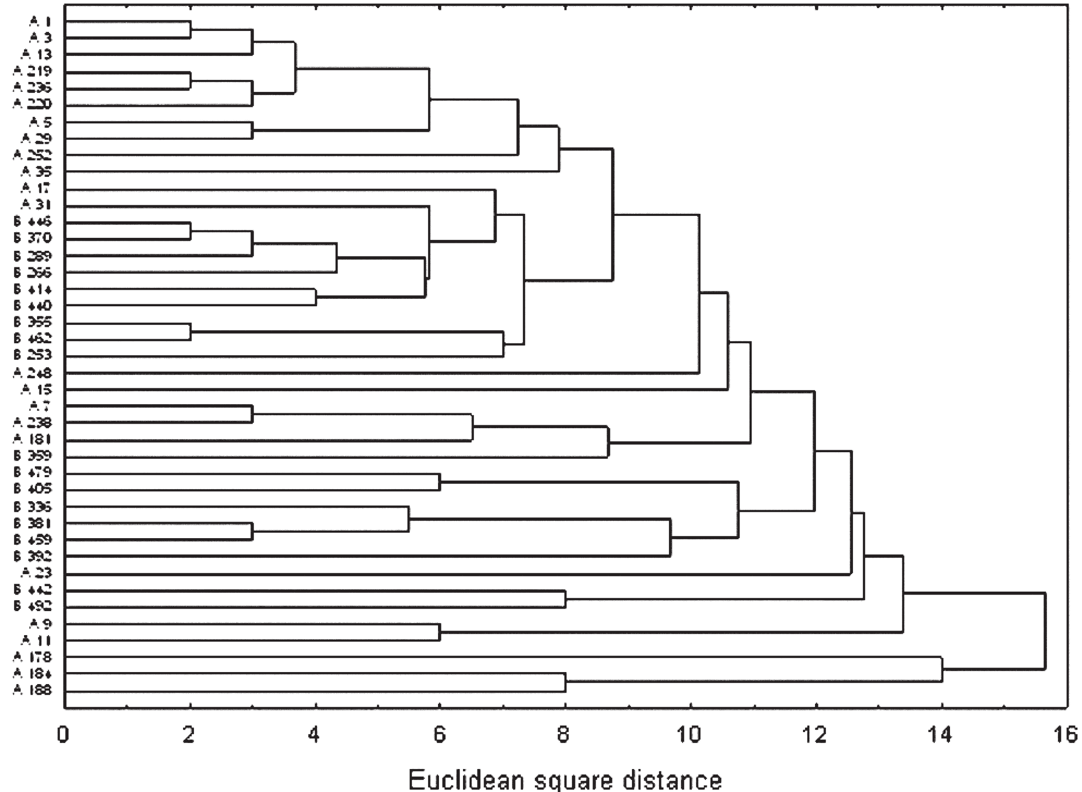

Fig. 1. Dendrogram derived with UPGMA based on the Euclidean distance between ramets from the A and B populations.

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in the population A than in the population B (15 *versus* 21 seeds; $P < 0.001$). The seeds from the population A were heavier than those from the population B (3.9 *versus* 3.3 mg; $P < 0.001$). Seedling density per 1 square meter of permanent plot in the areas of the populations was the most variable trait and differed between populations insignificantly (Table 2). Moreover, seedlings were present on plots in each year of the studies. In dryer and nutrient poor conditions plants from population A were more aggregated at higher density, and had fewer (but heavier) seeds per fruit (Table 1, 2). There was more reproductive shoots in those dryer conditions.

The germination ability of the seeds sown in the seed sowing experiment was significantly higher for the seeds from A than from B population (79% *versus* 44%; $P < 0.01$). In contrast the survival of the juveniles, up to four years following germination, insignificantly differed between the populations (54 and 76%).

4.2. Genetic diversity within and between the populations

As a result of the application of five selected RAPD primers, 91 bands (loci) were obtained from all studied samples of ramets from both the populations of *P. quadrifolia*. All of the primers applied yielded polymorphic bands, and their share across the populations was from 14% to 54% (Table 3). Genetic diversity within the population expressed as the share of polymorphic bands was 41% in the population A, and 36% in the B population. Genetic diversity estimated with the Shannon's Information Index was 0.40 within the A, and 0.34 within the B population. Moreover, Nei's gene diversity within the populations was 0.28 and 0.22 respectively.

Each sample (ramet) represented one unique RAPD phenotype, and the proportion of distinguishable genotypes was G/N = 1. The samples from the two populations could not be segregated based on UPGMA; this technique only permitted identifying several characteristic clusters in these two populations (Fig. 1). The fact that these clusters were mixed together indicates genetic relatedness between the two populations in question. Moreover, the pairs of samples 220–236 and 238–248 from smaller patches (25–45 m^2) were more similar than samples 35–178 and 188–219 from two large patches (84–136 m^2; Fig. 1). Genetic variation among the populations was $G_{ST} = 0.14$. The genetic distance between the populations was D = 0.10. The gene flow between these two populations was Nm = 1.51.

5. DISCUSSION

Repeated recruitment from seeds in natural stable populations of many clonal plants is low. However, the comparison of detailed long-term studies reviewed by Eriksson (1989) and Bierzychudek (1982) indicate that the level of seedling recruitment in many clonal plant populations is substantial. Our results indicate the presence of *Paris quadrifolia* seedlings in six consecutive years as well as a considerable survival rate to the juvenile stage in experimental conditions (Table 2). Germination ability is high (44–79%) in comparison with the results obtained by Jacquemyn *et al.* (2006) for seeds sown in a garden experiment (0–23%). Seedling density is lower in dryer and less productive environmental conditions similarly to Jacquemyn's *et al.* (2006) results from Belgium. Similarly to those results, also in dryer and less productive environmental conditions (population A) plants are more aggregated at higher density, and have fewer seeds per fruit. On the contrary to results from Belgium, there is more reproductive shoots, and seeds are heavier in dryer conditions.

The markers obtained permitted identifying 41 RAPD phenotypes, which indicates that the genotype diversity in the *P. quadrifolia* population is higher than the value reported from Belgium – G/N = 0.43 (Jacquemyn *et al.* 2006), and Lithuania from one fragmented population at botanical garden (0.68; Jogaite *et al.* 2005) as well as from other 13 populations (0.52; Jogaite *et al.* 2006). However, each sample of clonal *Iris aphylla* from 3 populations also represented a distinct RAPD phenotype (Wróblewska *et al.* 2003). The same RAPD genotype diversity of studied *P. quadrifolia* and *I. aphylla* (G/N = 1) may have been the result of similar sampling procedure which avoids sampling the same genotype. Moreover, such high geno-

type diversity may have resulted in part from somatic mutation (Fernando and Cass 1996). It is probably more likely that the high diversity results from both high germination ability and survival of the seedlings.

The genetic diversity of the studied populations of *P. quadrifolia* is substantial. Nei's gene diversity is higher than in the *P. quadrifolia* populations from Belgium (0.09; Jacquemyn *et al.* 2006), Lithuania (0.19), and Norway (0.097; Jogaite *et al.* 2006). The genetic diversity of the studied populations expressed by the Shannon's Index is higher than in other clonal plant populations such as *Iris aphylla* (Wróblewska *et al.* 2003) or *Lilium martagon* L. (Persson *et al.* 1998), the values of which have already been described as high. Substantially higher Shannon Index values were obtained from clonal populations of *Maianthemum bifolium* (L.) F.W. Schmidt (Arens *et al.* 2005), and *Cerastium fischerianum* Ohwi (Maki and Horie 1999).

Demographic simulations indicate that even low repeated recruitment of seedlings ensures that the genetic diversity of the population is not decreased (Watkinson and Powell 1993). In clonal species such as *Viola riviniana* Rchb. (Auge *et al.* 2001) and *Anemone nemorosa* (Stehlik and Holderegger 2000) the high recruitment of seedlings equalizes diversity among old and young populations. Investigated *P. quadrifolia* population with numerous generative shoots, and heavier seeds has the higher gene diversity similarly to results from Belgium's populations (Jacquemyn *et al.* 2006). On the contrary to those results, there is lower seedling density, and plants produce fewer seeds per fruit (*e.g.* less fecund plants) in population which has higher gene diversity. Moreover, higher gene diversity occurred in less productive environmental conditions.

Genetic variation between the *P. quadrifolia* populations is lower than among populations of this species from Belgium (Jacquemyn *et al.* 2006). The amount of genetic drift between these populations is higher in comparison to levels recognized as low estimates for highly isolated populations of *Iris aphylla* (Wróblewska *et al.* 2003) and *Ranunculus reptans* L. (Fisher *et al.* 2000). The samples from studied populations of *P. quadrifolia* could not be segregated based on cluster analysis, and the UPGMA dendrogram showed intermingling between samples from the populations. This result is similar to those results obtained by Jogaite *et al.* (2005) for 5 groups of one fragmented population of this species, as well as for 3 populations of *Iris aphylla* isolated by hundreds of kilometers were intermingled on the dendrogram (Wróblewska *et al.* 2003).

Paris quadrifolia is a plant that is both allogamous and autogamous (mixed species), but wind-carried pollen flow as well as seed dispersal by small rodents between these two populations are unlikely. Additionally, seedlings occurred only in the vicinity of fruiting ramets, which suggests that seed dispersion is limited. The estimated variation between the *P. quadrifolia* populations is lower in comparison with the value for mixed species of G_{ST} = 0.28 (Nybom 2004). This may suggest the prevalence of allogamy in the flowers of this plant, since there is a significant correlation between the values of G_{ST} and the breeding system: the highest variation is among populations of autogamous species (Nybom 2004). The application of codominant markers could verify this assumption.

In comparison with populations of species that propagate vegetatively and generatively, with close genetic distances and relatively high gene flow, the genetic variation between the two *Paris quadrifolia* populations is low. This may stem from the age of these populations. The area they currently inhabit is probably the remainder of a greater area of undeveloped landscape in the study region they occupied previously. This may suggest they are refuge populations. On the other hand, our results of polymorphism study are obtained from only two populations. Our results indicate the need for more detailed investigations of the spatial structure of genotypes within populations.

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