Genetic structure in the perennial grasses
Lolium perenne and *Agrostis curtisii*

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Genetic variation was studied in unimproved grassland populations of two contrasting outbreeding perennial grass species. A total of 27 populations of *Lolium perenne* (perennial ryegrass) and 30 populations of *Agrostis curtisii* (bristle-leaved bent), sampled from seven and five regions spread across southern Britain, were assessed at three and four isozyme loci, respectively. The extent of genetic structure within and among populations was estimated using unbiased *F*-statistics. In *A. curtisii*, a nonagricultural species, populations from adjacent regions were found to be more genetically similar than those separated by greater distance. The reverse situation was observed within *L. perenne*, a species of major agricultural importance. It is suggested that the absence from *L. perenne* of the pattern of genetic variation found in *A. curtisii* is consistent with the occurrence of large-scale human-mediated gene flow via 'improved' ryegrass cultivars. If this is the case, then the disruption of natural patterns of genetic variation by the introduction of nonlocal genotypes may occur without apparent major ecological consequences.

Keywords: *Agrostis curtisii*, gene flow, genetic structure, isozymes, *Lolium perenne*.

Introduction

Genetic structure and morphological diversity within species can result from restricted gene flow (Wright, 1969; Slatkin, 1985, 1993). Direct methods of estimating gene flow are problematic, and can only give estimates for a relatively restricted area and over a short period of time. Over the past 30 years, isozyme markers have been used extensively in the study of genetic structure in plant populations. The forage grasses, which dominate many temperate agricultural and seminatural areas, have been no exception (Hayward *et al*., 1978; McNeilly & Roose, 1984; Ennos, 1985). Population genetic studies such as these have established indirect estimates of gene flow through the analysis of differences in the frequencies of alleles at various loci among two or more populations.

Although gene flow in flowering plants can occur via both pollen and seed, wind dispersal of pollen is considered to be more important in outbreeding grasses (Griffiths, 1950; Gleaves, 1973). However, this is likely to vary greatly between agriculturally important grasses, which experience considerable amounts of seed movement, and species less interfered with by man. The majority of studies of population-level genetic variation within grasses have looked at those species important to agriculture which are widely sown as forage, such as perennial ryegrass (*Lolium perenne*) (Hayward *et al*., 1978; McNeilly & Roose, 1984; Balfourier & Charmet, 1994). Knowledge of gene flow in grasses is dominated by studies of agricultural situations (Copeland & Hardin, 1970) or from experimental blocks (Griffiths, 1950; Gleaves, 1973; Giddings *et al*., 1997). Surprisingly little is known about gene flow and genetic structure in nonagricultural grass species. As a result of this lack of information, it has been difficult to infer how much of the genetic structure observed in agriculturally important species has resulted from human-mediated gene flow and what remains of natural patterns of variation. In this study we compare the genetic structure of two diploid species of outbreeding perennial grass, both sampled from apparently seminatural, agriculturally unimproved sites. The first of these, *L. perenne*, is probably the most important forage grass species of

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northern Europe and, as a consequence, it has experienced massive amounts of gene flow through the sowing of selected cultivars. In contrast, the second species, bristle-leaved bent (*Agrostis curtisii*) is a nonagricultural species, in the UK occurring in southern and south-western counties of England on well-drained acid soils of lowland heaths (Hubbard, 1984).

Materials and methods

Study populations

Twenty-seven populations of *L. perenne* were sampled from across Britain from old unimproved sites. The sampling occurred during the summer of 1976, when unimproved pastures were more common. The populations were chosen from within seven widely spaced regions whose locations can be seen in Fig. 1. Approximately 100 plants were collected from each population as isolated tillers spaced at least 1 m apart, taken from areas contain-
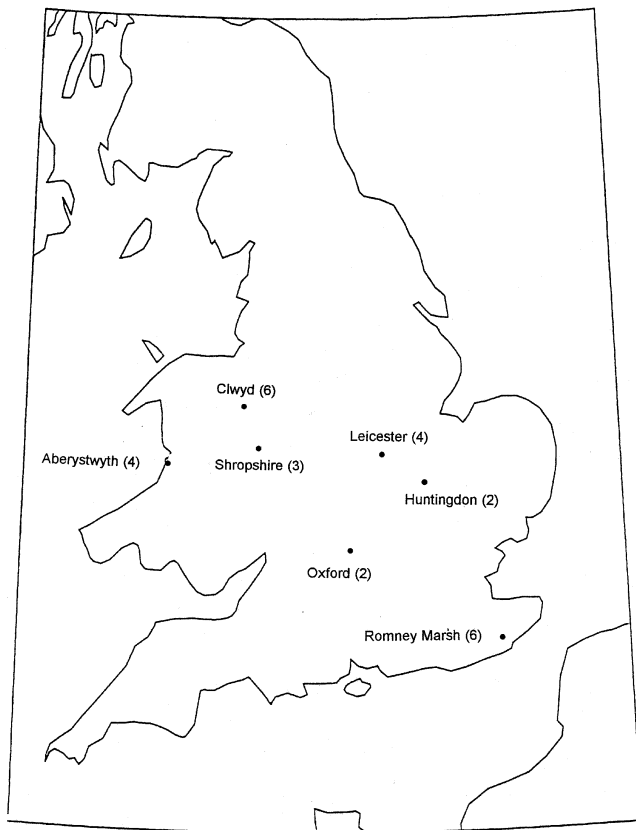
Fig. 1 A map of southern Britain showing the location of *Lolium perenne* sites. Isolated tillers were collected from 27 sites spread over seven regions. Values in parentheses are numbers of sites sampled per region.

ing no obvious ecological differences or gradients. The tillers were established in pots in the glasshouse prior to sampling for electrophoresis.

During 1979 seeds of *A. curtisii* were collected from five sites in each of six regions in southern Britain, whose locations are shown in Fig. 2. At each site, seed was collected from 100 randomly chosen individuals within an area of 100 m^2. Seeds from each plant were sown into lime-free potting compost, with one randomly selected seedling per parent being grown on in a 13 cm pot of the same compost until sampling for electrophoresis.

Isozyme electrophoresis

During 1977/78, leaf material was sampled from each *L. perenne* plant from all 27 populations. This material was crushed in 0.1 M Tris-HCL, pH 7.2 extraction buffer with 0.1% mercaptoethanol, and starch gel electrophoresis was performed following the method and staining procedures of Hayward & McAdam (1977). The gels were scored for the multi-allelic enzyme loci: phosphoglucoisomerase (*Pgi-2*, EC 5.3.1.9), acid phosphatase (*Acp-2*, EC 3.1.3.2) and glutamic-oxaloacetic transaminase (*Got-3*, EC 2.6.1.1). These loci show regular Mendelian inheritance and their enzyme products are dimeric.

During 1980 leaf samples of *A. curtisii* were taken from plants of a uniform age and homogenized in 0.1 M Tris-HCL, pH 7.0 with 0.1% mercaptoethanol. Electrophoresis, on 13% starch gels, used the buffer system and staining methods of Shaw & Prasad (1970). The following enzyme loci were scored: glutamic-oxaloacetic transaminase (*Got-3*) and phosphoglucoisomerase (*Pgi-2*), whose products are dimeric, and esterase (*Est-2*, EC 3.1.1.1) and peroxidase (*Per-1*, EC 1.11.1.7), which code for monomeric enzymes.

Data analysis

The analysis of genetic structure was the same for both species. The extent of genetic structure within populations (F_{IS}) and among populations (F_{ST}) was estimated using the program FSTAT (Goudet, 1995), which calculates Weir & Cockerham's (1984) unbiased estimators of *F*-statistics. F_{ST} values were obtained between all pairs of populations and classified into those between populations in the same region (coded 1 in a variable termed 'region membership') and those between populations in different regions (coded 0) (Raybould *et al.*, 1996, 1997). The effects of distance and regional grouping were examined using partial regressions and partial
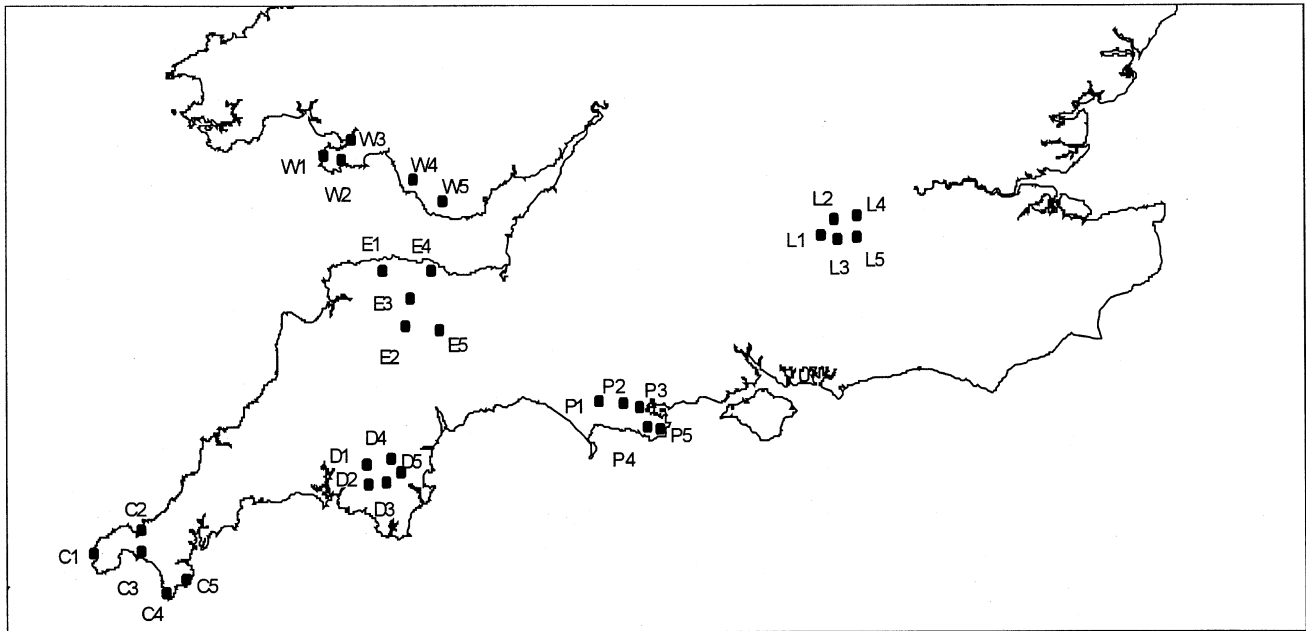
Fig. 2 A map of southern Britain showing the location of *Agrostis curtisii* sites. Seeds were collected from five sites in each of six regions: (C) west Cornwall, (D) Dartmoor, (E) Exmoor, (L) London, (P) Purbeck and (W) south Wales.

matrix correspondence tests (e.g. Manly, 1991; Thorpe & Baez, 1993) of pairwise genetic distances (F_{ST}) and geographical distance, region membership and the interaction between distance and region membership (distance multiplied by the region membership variable). The interaction tests for the uniformity of the effect of distance within and between regions (Raybould *et al.*, 1997).

Results

Isozymes detected significant nonrandom mating in several *Lolium* populations (Table 1), and in only one population (Romney Marshes 4) was there a slight, but nonsignificant, heterozygote excess. The over all populations mean F_{IS} was significantly greater than zero, both over all loci and at each locus individually. There was some variation among loci; heterozygote deficits occurred frequently at *Acp*, but rarely at *Got* and *Pgi*.

Compared with *Lolium*, a lower proportion of *Agrostis* populations showed significant heterozygote deficit, and a number showed significant heterozygote excess (Table 2). The mean over all populations F_{IS} over all loci was slightly negative. There was more variation among loci than for *Lolium*. Most populations had a significant excess of heterozygotes at *Pgi* and a significant deficit of heterozygotes at *Est*. At *Got* and *Per*, F_{IS} was not significantly different from zero in most populations.

In both species, F_{ST} was significant at all loci (Table 3). The over all loci estimate of F_{ST} was higher in *Lolium*, but this relates specifically to the high value for *Acp*. Estimates of F_{IT} were significantly greater than zero for all loci in *Lolium*, and for *Est*, *Got* and *Per* in *Agrostis*. There was a significant heterozygote excess in the whole sample at *Pgi* in *Agrostis*, which gave a much lower value of F_{IT} over all loci than for *Lolium*.

The proportion of variation in pairwise F_{ST} values that was explained by distance or region membership was low in both species ($r^2 < 0.07$, Table 4). However, significant effects and differences between the species were detected. In *Agrostis* there was a significant positive relationship between distance and F_{ST}, whereas in *Lolium* there was a significant negative relationship between distance and F_{ST} (Table 4). In *Lolium* there was no significant region membership effect, but region did have a negative effect in *Agrostis*.

In both species there was a significant distance effect when the region effect was removed from the analysis. When the over-regions negative distance effect was removed in *Lolium*, a significant effect of distance was detected within regions. Populations of *Lolium* within regions were more similar to each other than were populations in different regions, but apart from this effect, populations generally were more similar at greater distances apart. In *Agrostis*, the significant region effect was not detectable when

the effect of distance was removed. In other words, there was no greater similarity of populations within regions than would be expected given their geographical proximity. In neither species was there a significant interaction between distance and region membership (Table 4).

Discussion

The results obtained indicate a number of differences between the genetic structure of *L. perenne* and *A. curtisii* populations, some of which may result from the agricultural sowing of *Lolium* whereas other differences may relate to biological differences between the species. The significant positive F_{IS} over all populations of *Lolium* and significant negative F_{IS} over all *Agrostis* populations, may be indicative of a violation of the assumptions of Hardy–Weinberg in *Lolium*. Although the collecting of *Lolium* material occurred during the autumn following the drought

summer of 1976, if we assume the markers to be neutral, the fact that the positive F_{IS} was observed for all populations and all loci is difficult to reconcile with chance linkages to non-neutral loci. However, if the markers are not neutral, then the observed nonrandom mating in old pasture populations of *Lolium* may result from the nonrandom sampling of genotypes. McNeilly & Roose (1984), using isozyme markers, demonstrated that old unimproved ryegrass pastures can be dominated by very few genotypes, with some clones being much larger than others. If homozygotes were over-represented in the larger clones at the end of the summer of 1976, then spatially, randomly sampling such a turf would be likely to result in multiple samples being taken from homozygous individuals. The observed deficit of heterozygotes, nonrandom mating in all populations of *Lolium*, over all loci, could thus result from such a biased sampling strategy. In contrast, *A. curtisii* has a densely tufted growth form

Table 1 Estimates of F_{IS} in 27 populations of *Lolium perenne*. One-sided significance of estimates obtained by a randomization test with 10 000 permutations of alleles within samples (Goudet, 1995)

Population	N	F_{IS} all loci	F_{IS} *Acp*	F_{IS} *Got*	F_{IS} *Pgi*
Aberystwyth 1	106	0.172***	0.412***	0.142	−0.074
Aberystwyth 2	96	0.222***	0.517***	−0.002	0.035
Aberystwyth 3	93	0.061	0.196	−0.027	−0.010
Aberystwyth 4	93	0.149***	0.312***	−0.087	0.104
Clwyd 1	80	0.155**	0.268**	−0.018	0.162
Clwyd 2	103	0.195***	0.474***	−0.042	0.058
Clwyd 3	95	0.032	0.097	0.045	0.016
Clwyd 4	97	0.207***	0.500***	−0.142	0.012
Clwyd 5	97	0.061	0.041	0.332***	−0.021
Clwyd 6	97	0.062	0.017	0.343***	−0.053
Shropshire 1	97	0.117*	0.387***	−0.101	0.053
Shropshire 2	74	0.117*	0.306**	−0.085	0.012
Shropshire 3	89	0.196***	0.307**	0.139	0.101
Leicestershire 1	96	0.171**	0.428***	0.098	0.008
Leicestershire 2	72	0.052	0.389**	−0.045	−0.159
Leicestershire 3	82	0.083	0.279*	−0.125	0.006
Leicestershire 4	95	0.077	0.106	0.058	0.070
Huntingdonshire 1	76	0.171***	0.351**	−0.151	0.184*
Huntingdonshire 2	72	0.039	0.112	0.080	−0.052
Romney Marshes 1	104	0.104*	0.280**	−0.001	−0.027
Romney Marshes 2	93	0.115*	0.185	0.401***	−0.036
Romney Marshes 3	101	0.098*	0.213*	−0.054	0.030
Romney Marshes 4	92	−0.029	−0.038	0.058	−0.063
Romney Marshes 5	95	0.095*	0.042	0.137	0.138
Romney Marshes 6	103	0.246***	0.371***	−0.070	0.223*
Oxfordshire 1	94	0.092*	0.214	−0.038	0.047
Oxfordshire 2	97	0.182***	0.429***	0.037	0.031
Over all pops.	2489	0.121***	0.264***	0.035*	0.032*

$*P<0.05$, $**P<0.01$, $***P<0.001$.

Table 2 Estimates of F_{IS} in 30 populations of *Agrostis curtisii*. One-sided significance of estimates obtained by a randomization test with 10 000 permutations of alleles within samples (Goudet, 1995). See Fig. 2 for region locations and codes

Population	N	F_{IS} all loci	F_{IS} *Est*	F_{IS} *Got*	F_{IS} *Per*	F_{IS} *Pgi*
C1	93	−0.052	0.260	−0.044	0.047	−0.350***
C2	94	−0.029	0.408**	−0.087	0.410**	−0.552***
C3	85	0.074	0.489***	0.177	0.135	−0.393***
C4	97	−0.061	0.352**	−0.285**	−0.006	−0.192*
C5	97	−0.018	0.551***	−0.165	−0.112	−0.292***
D1	69	−0.147**	0.415**	−0.148	−0.225	−0.479***
D2	83	0.172***	0.505***	0.100	0.204	−0.040
D3	66	0.032	0.679***	−0.054	−0.047	−0.287
D4	58	−0.008	−0.021	0.146	0.282	−0.366**
D5	67	0.001	0.307*	0.142	−0.107	−0.323***
E1	65	−0.225***	0.045	−0.133	−0.280	−0.438***
E2	78	−0.079	0.181	−0.099	0.043	−0.309**
E3	85	−0.040	0.457***	0.025	−0.089	−0.411***
E4	91	−0.053	0.681***	−0.034	−0.233	−0.306***
E5	86	0.138**	0.288*	0.383***	0.082	−0.180
L1	64	−0.136*	0.475**	−0.037	0.307	−0.778***
L2	79	0.238***	0.703***	0.198	0.080	0.074
L3	71	0.087*	0.654***	0.234*	−0.082	−0.348***
L4	65	−0.126*	0.430**	−0.052	−0.256	−0.559***
L5	55	−0.071	−0.022	−0.189	0.300	−0.123
P1	45	0.090	0.522**	0.356**	−0.162	−0.292*
P2	83	−0.033	0.290	0.113	0.032	−0.322***
P3	94	−0.113**	0.371**	−0.090	−0.057	−0.430***
P4	99	0.146**	0.652***	0.289**	−0.122	−0.219*
P5	88	0.032	0.740***	0.175	0.041	−0.430***
W1	87	0.058	0.315*	0.202	−0.015	−0.220*
W2	75	−0.221***	0.051	−0.112	−0.313*	−0.446***
W3	91	−0.231***	0.124	−0.153	−0.298*	−0.473***
W4	86	0.060	0.400*	0.290*	0.094	−0.407***
W5	60	−0.105*	0.108	0.093	0.018	−0.486***
Over all pops.	2356	−0.022*	0.374***	0.023	−0.009	−0.339***

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 3 Estimates of F_{ST} and F_{IT} for *Lolium perenne* and *Agrostis curtisii* populations. One-sided significance of estimates obtained by a randomization test with 10 000 permutations of genotypes within the total sample (as F_{IS} is significant) (Goudet, 1995)

	All loci	*Acp*	*Got*	*Pgi*	*Est*	*Per*
L. perenne						
F_{ST}	0.068***	0.129***	0.014***	0.031***		
F_{IT}	0.181***	0.359***	0.049***	0.063***		
A. curtisii						
F_{ST}	0.036***		0.029***	0.032***	0.025***	0.066***
F_{IT}	0.015*		0.052***	−0.295***	0.390***	0.058***

*$P < 0.05$, ***$P < 0.001$.

Table 4 Regression and partial regression coefficients between a matrix of pairwise genetic distances (F_{ST}) and matrices of pairwise geographical distances, region membership and distance × region interaction. One-sided significance of regression coefficients was tested by a Mantel test with 10 000 randomizations (two-sided test for interaction term)

	Regressions		Partial regressions		
	Distance	Region	Distance	Region	Interaction
L. perenne	$b = -1.05 \times 10^{-4}$* $r^2 = 1.9\%$	$b = -1.71 \times 10^{-3}$ $r^2 = 0.0\%$	$b = -1.63 \times 10^{-4}$** $r^2 = 3.0\%$	$b = -5.07 \times 10^{-2}$* $r^2 = 1.2\%$	$b = 4.10 \times 10^{-3}$ $r^2 = 0.3\%$
A. curtisii	$b = 7.77 \times 10^{-5}$*** $r^2 = 7.0\%$	$b = -9.58 \times 10^{-3}$** $r^2 = 1.7\%$	$b = 8.38 \times 10^{-5}$*** $r^2 = 5.5\%$	$b = 1.60 \times 10^{-3}$ $r^2 = 0.0\%$	$b = 6.14 \times 10^{-3}$ $r^2 = 0.0\%$

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

(Hubbard, 1984), and is unlikely to form large spreading clones; it is thus more likely to have been sampled in an unbiased manner. The different sampling strategies used for the two species may also influence the apparent genetic structure of their populations, particularly so if the markers are not neutral and there is strong selection occurring during seedling establishment.

At the other end of the spatial scale, *L. perenne* and *A. curtisii* were seen to differ in their genetic structure as partitioned between regions. *Agrostis curtisii* was seen to exhibit classical isolation by distance (Wright, 1943, 1946; Slatkin, 1993) with a significant positive relationship between distance and F_{ST}; *A. curtisii* populations from adjacent regions were found to be genetically more similar than those separated by greater distance. In contrast, *L. perenne* showed the exact opposite relationship at the regional level, with a significant negative relationship between F_{ST} and distance. This relationship was observed over all loci. Although it may be possible to postulate a selective explanation for why populations of *L. perenne* separated by large distances are genetically similar (i.e. they are coastal populations), this is difficult to reconcile with the observation holding true over several neutral loci. A more plausible explanation is long-scale gene flow in *L. perenne* associated with agricultural usage. Much of the observed relationship can be accounted for by similarity between the *L. perenne* populations sampled around Aberystwyth and those collected from Romney Marsh, Kent. Indeed, if either of these regions is dropped from the analysis the relationship breaks down. There is some historical evidence (Beddows, 1953) that suggests that the fertile grasslands of Romney Marsh may have been sampled as a source of germplasm for early breeding work at the Welsh Plant Breeding Station, Aberystwyth. The resulting improved varieties of *L.*

perenne were widely planted around mid-Wales and may have been exchanging genes with local material for some time. This human-mediated long-distance gene flow may therefore be responsible for the observed genetic similarity between geographically separated populations. Similarly, Balfourier & Charmet (1994) suggested that the historical long-distance movement of *L. perenne* from Italy to Corsica, for agricultural use, was responsible for the current genetic similarities between these populations.

The observed difference between *L. perenne* and *A. curtisii* in genetic variation within regions may be a consequence of both natural and agriculture-related gene flow. The greater similarity of populations of *L. perenne* within regions than between regions may result from extensive gene flow between populations within regions, which is possible because the species is extremely abundant. This extent of gene flow may not be possible in the rarer *A. curtisii*. However, the same pattern of genetic similarity between populations of *L. perenne* within regions could also result from different regional preferences by farmers in selecting improved *L. perenne* varieties to sow.

Many of the patterns of genetic variation reported here within *L. perenne*, but not *A. curtisii*, are consistent with long-distance human-mediated gene flow associated with agricultural usage. This is perhaps surprising as this occurred in plants sampled from apparently old unimproved pastures and thus implies gene flow from neighbouring sown fields. This level of gene flow of nonlocal genes is relevant to current concerns regarding the release of genetically modified organisms and of the sowing of non-local provenance wild flower seeds. In the case of the very widespread and abundant *L. perenne* at least, considerable gene flow must have occurred, resulting from the agricultural sowing of nonlocal

types, apparently without disrupting the ecology of the seminatural plant communities in which it also is found. However, that is not to say that more subtle changes in the ecology of grasslands containing *L. perenne* have not occurred but are as yet unnoticed.

Acknowledgements

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