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V. L. Semerikov · A. Y. Belyaev · M. Lascoux

The origin of Russian cultivars of red clover (*Trifolium pratense* L.) and their genetic relationships to wild populations in the Urals

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Abstract Propagation and breeding of red clover (*Trifolium pratense* L.) in Russia was initiated about 200 years ago but the origin of present-day cultivars is disputed. Some authors argue that most Russian cultivars were derived from western European ones, whereas others support a Russian origin of the cultivars from local wild populations. In the present study we assessed the genetic variation at 17 allozyme loci in seven Russian cultivars, bearing the names of localities of the Urals, two American ones that have been used in Russia for scientific experiments and seven wild populations of the Urals and Western Siberia. Variation at the 17 protein loci supports the western European origin of the cultivars and also indicates that gene flow between cultivars and wild populations was limited or has not acted sufficiently long to affect the genetic composition of the red clover wild populations of the Urals.

Keywords *Trifolium pratense* · Allozyme polymorphism · Gene flow · Population structure · Introgression · Urals

Introduction

The planned release of genetically modified organisms (GMO) has led to a renewed interest in measuring the amount of gene flow occurring between domesticated plant taxa and their wild relatives, or simply between cultivated plants and their wild counterparts. In a recent literature review of the world's 13 most important food crops, Ellstrand et al. (1999) showed that 12 of these crops hybridize with their wild relatives in some part of their agricultural distribution. Hence, gene flow between cultivated plants and their wild relatives can take place, although its amount may vary greatly among species. Somewhat surprisingly, given its economic importance and wide use, the breeding history and population genetics of red clover (*Trifolium pratense*) is poorly documented. To the best of our knowledge only a few studies have assessed the genetic variation in red clover cultivars (Kongkiatngam et al. 1995, 1996) and in naturalized populations of the USA (Milligan 1991; Hagen and Hamrick 1998), but no studies were carried out on the natural range of red clover apart from the work of Semerikov and Belyaev (1995).

Red clover apparently originated in southeastern Europe and Asia Minor (Mukhina et al. 1993). In northern Europe it was first cultivated around 1650 and subsequently introduced to North America by European colonists (Merkenschlager 1934; Taylor 1985). Today its natural distribution ranges from the Atlantic coast eastward to southwest Siberia, and from Scandinavia southward to North Africa. *T. pratense* is a self-incompatible, insect-pollinated diploid (Mukhina et al. 1993) and its seeds are dispersed by horses, cattle, humans and birds.

In the present study we examine the genetic relationship between red clover cultivars used and/or originating in the Urals from which their names are derived, and local wild populations. In Russia the cultivation of red clover started in the central and western regions in the 18th century and by the end of 19 century it was already widely cultivated in the Urals (Khrebtov 1925; Khoroshailov 1964; Mukhina et al. 1993). The breeding history of some of the cultivars and landraces is documented but, generally, the early stages of the domestication process are very poorly understood (Mukhina et al. 1993). It is not known for instance which populations were used as a source of the material for the first cultivars, nor is it known where these cultivars were later

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V.L. Semerikov · A.Y. Belyaev
Institute of Plant & Animal Ecology,
620144 Ekaterinburg, Russia

V.L. Semerikov · M. Lascoux (✉)
Evolutionary Biology Centre,
Department of Conservation Biology and Genetics,
Uppsala University, Norbyvägen 18D,
75236 Uppsala, Sweden
e-mail: Martin.Lascoux@ebc.uu.se
Fax: (46)-18-471-64-24

used. According to some authors the first Russian cultivars were derived from unknown west European cultivars (e.g. Lisitsin 1947), whereas others believe that they were created by local farmers in Central Russia through mass selection of individuals collected in local wild populations (Khoroshailov 1964). Assuming that gene flow between the cultivars and the wild populations has not been too strong, or that the time since their introduction has been too short for gene flow to have a strong impact, we would expect under the first hypothesis that the Russian and foreign cultivars will be genetically similar and to form a clade separated from local wild populations. Alternatively, if the cultivars were indeed derived from local populations they should still be genetically close to the wild populations and they would not cluster separately, unless selection was strong enough to alter allele frequencies. This is unlikely since selection intensity has probably been low and of little effect on allele frequencies at either quantitative trait loci or independent loci (Latta 1998). Further, in a previous study Semerikov and Belyaev (1995) investigated the genetic relationship between three natural populations and two cultivars using 17 isozyme loci, and found that genetic differences among groups of cultivars and native populations was rather large and significantly exceeded the variation within groups. Hence, these preliminary results supported the first hypothesis rather than the second one. The purpose of the present study is to test this hypothesis using a larger number of populations.

Materials and methods

Material

Nine cultivars and seven wild populations of red clovers from the Urals were studied. Following Ellstrand et al. (1999) we have defined wild plants as those that grow and reproduce without being deliberately planted. Of course, that does not preclude the possibility that these wild populations could well be naturalized populations. The number of individuals per population, the putative geographic origin of the cultivars and the geographic location of the wild populations are given in Table 1 and Fig. 1. In both cases seeds obtained from a large number of parents were used. Wild populations were chosen to represent the main vegetation zones found in the Urals where red clover grows: dry steppes, forest steppe, southern and middle taiga (conifer forest), including plains and mountain valleys. In these areas it is possible to find habitats that have been only weakly affected by agricultural activity and *T. pratense* populations are more likely to be of native origin, especially since red clover cultivation was introduced rather late into the region. When collecting samples we tried to avoid as far as possible material that appeared to be of admixed origin: hence we avoided large fields and plants whose morphology was close from the cultivated types (for instance plants with a large number of short internodes), and sampled populations in areas where pasture cultivation was limited. Cultivar seeds were obtained from the collection of the All-Russian Institute of Plant Production and from the Urals Research Institute for Agriculture. Seeds were first germinated in Petri dishes and then transferred to field plots. When the plants were 3 to 4 weeks old, around 30 plants per population were randomly chosen for genetic analysis.

Fig. 1 Geographical locations of the investigated populations. *Squares* correspond to natural populations and *triangles* to sites that gave their names to some of the cultivars. The complete names of wild populations and cultivars are given in Table 1

Table 1 Cultivars and wild populations used in the present study. The location assigned to the cultivars is suggested by their names, but they were not harvested in these areas. The symbols used in Fig. 1 are given within parentheses

Item	Latitude	Longitude	N
Cultivars			
Permskii-c (*Pe*)	58°00′N	56°18′E	24
Krasnoufimsk-c (*Kr*)	56°38′N	58°01′E	30
Duvan-c (*Du*)	57°32′N	57°53′E	30
Kurgan-c (*Ku*)	56°10′N	65°10′E	28
Urdv-c (*Ud*)	56°51′N	60°36′E	33
Kyshtim-c (*Ky*)	55°41′N	60°40′E	30
Memmos-c	USA	–	30
Medium-c	USA	–	30
Serov-c (*Se*)	59°35′N	60°37′E	30
Natural populations			
Intzer-n (In)	54°12′N	57°36′E	32
Dzhabyk-Karagaiiskii-n (Dk)	54°07′N	59°34′E	30
Nizhnie Sergi-n (Ns)	56°37′N	59°13′E	30
Kytlim-n (Ky)	59°30′N	59°20′E	30
Tobolsk-n (To)	58°11′N	68°19′E	30
Bielsky/Talitsa-n (Bt)	57°01′N	63°41′E	33
Ivdel-n (Iv)	60°42′N	60°30′E	30

Isozymes

Ten enzyme systems representing 17 protein loci were examined: glutamate-oxaloacetate transaminase (GOT, EC 2.6.1.1: *Got-A, Got-B, Got-C*), leucine amino peptidase (LAP, EC 3.4.11.(13): *Lap*), shikimate dehydrogenase (SKDH, EC 1.1.1.25: *Skdh*), phosphogluconate dehydrogenase (PGD, EC 1.1.1.44: *Pgd-A, Pgd-B*), esterase (EST, EC 3.1.1.1: *f-Est, C-Est*), superoxide dismutase (SOD, EC 1.15.1.1: *Sod-A, Sod-B*), phosphoglucose isomerase (PGI, EC 5.3.1.9: *Pgi-A, Pgi-B*), diaphorase (DIA, EC 1.6.4.3: *Dia-A, Dia-B*), glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49: *G6pd*) and alcohol dehydrogenase (ADH, EC 1.1.1.1: *Adh*).

The green parts of each plant were ground in 200 µl of an extraction buffer composed of 69 mM Tris–HCl, 2.1 mM EDTA, 97 mM boric acid, 13 mM mercaptoethanol, 0.1 mM NADP and 470 mM sucrose. The supernatant was separated by centrifugation and frozen. Electrophoresis in 6.4% polyacrilyamide gel in tris-

borate-EDTA system was conducted. The electrode buffer (pH 8.0) was: 116 mM Tris–HCl, 3.5 mM EDTA, 161 mM boric acid. Gel buffer (pH 8.6) was the following: tris 118 mM, EDTA 3.5 mM, boric acid 118 mM. Histochemical staining was carried out by standard methods (Harris and Hopkinson 1976).

Data analysis

Data were analyzed with the programs Genepop (version 3.1.d) (Raymond and Rousset 1995), Fstat (version 1.2) (Goudet 1995), AutocorrG (Hardy 2000) and NTSYS-pc (Rohlf 1988).

Hardy-Weinberg expectations

The fit of genotypic distributions to Hardy-Weinberg expectations was tested by the exact test proposed by Haldane (1954). The overall significance for each locus was estimated by Fisher's combined probability test (Fisher 1954). According to this test, if *P*-values are obtained for each locus separately under the null hypothesis, then $-2\sum_{i=1}^{n}\log(P_i)$ is distributed according to a χ^2 distribution with $2n$ degree of freedom where n is the number of loci (Sokal and Rohlf 1994). F_{IS} values, where F_{IS} is the correlation between two uniting gametes within a subpopulation, were estimated according to Weir and Cockerham (1984). Heterozygote deficits or excesses were tested using an exact test (Rousset and Raymond 1995).

Linkage disequilibrium

For each population, the non-random association between pairs of loci, or linkage disequilibrium, was tested using Fisher's exact test on contingency tables. Contingency tables were created for all pairs of loci in each population and an unbiased estimate of the exact probability was obtained by using a Markov chain Monte Carlo method (Raymond and Rousset 1995). Each test is not affected by a potential departure from Hardy-Weinberg, because each contingency table considers the genotypic composition, not the allelic composition. For each pair of loci, a global measure was obtained by averaging across populations, and a global test was obtained through Fisher's combined test.

Population differentiation

Genetic differentiation between populations or groups of populations was tested for each locus separately using Fisher's exact test on contingency tables. As for linkage disequilibrium, the Markov chain Monte Carlo method permits the attainment of an unbiased estimate of the exact probability (Raymond and Rousset 1995). Wright's F statistics, F_{IS}, F_{IT} and F_{ST}, were estimated according to Weir and Cockerham (1984) and a 95% confidence interval was estimated by bootstrapping over loci. F_{IS} and F_{IT} are the correlations between two uniting gametes relative to the subpopulation and relative to the total population, respectively, and F_{ST} is the correlation between two gametes drawn at random from each subpopulation and measures the degree of genetic differentiation of subpopulations (Nei 1987). Only statistically independent loci that did not depart from Hardy-Weinberg proportions were retained.

Ordination of populations was performed by principal component analysis on the matrix of allele frequencies using NTSYS-pc (Rohlf 1988). Finally, genetic distances between populations were estimated with Nei's genetic distance (Nei 1987) and a UPGMA dendrogram was drawn using procedures from NTSYS-pc.

Isolation by distance

Isolation by distance among natural populations was analyzed according to Rousset (1997) and Hardy (2000). The distance between populations was estimated using simple trigonometric formulas under the assumption that the Earth is a sphere with a radius of 6,360 kilometers.

Results

Genetic diversity and Hardy-Weinberg equilibrium

Although differences were not significant, the genetic diversity measured by expected heterozygosity values, polymorphism and the average number of alleles was lower in wild populations than in cultivars (Table 2). Loci *Pgi-A, Dia-A, Dia-B* and *Sod-B* were monomorphic in all populations, and cultivars, and the locus *Adh* was only polymorphic in the American cultivar Medium. Exact tests detected a significant heterozygote deficit in the wild populations Kytlim and Tobolsk. This departure from Hardy-Weinberg equilibrium was due to locus *F-Est* in both populations as well as locus *C-Est* in Tobolsk, but no departure was detected at other variable loci. So, in general, populations were in Hardy-Weinberg equilibrium.

Table 2 Genetic diversity parameters based on 17 allozyme loci. H_{exp} is the expected heterozygosity, H_{obs} is the observed heterozygosity, *P*(0.95) is the percent of polymorphic loci (the most frequent allele has a frequency less than 0.95) and A is the average number of alleles per locus

Item	Hexp.	Hobs.	*P*(0.95)	*P*(0.99)	A
Permsky-c	0.1922	0.1670	0.5294	0.5882	2.2353
s.d.	0.2105	0.1843			
K-ufim-c	0.1700	0.1600	0.4118	0.5882	2.1176
s.d.	0.2054	0.1892			
Duvan-c	0.1648	0.1675	0.4706	0.5294	2.0000
s.d.	0.1951	0.2014			
Kurgan-c	0.1815	0.1816	0.4706	0.6471	2.2941
s.d.	0.2035	0.2088			
Kyshtim-c	0.1958	0.1988	0.5294	0.7647	2.4706
s.d.	0.2045	0.2168			
Urdv-c	0.1809	0.1740	0.4118	0.7059	2.2941
s.d.	0.2051	0.2033			
Memmos-c	0.1547	0.1706	0.4706	0.5294	1.7647
s.d.	0.1891	0.2181			
Medium-c	0.1616	0.1567	0.5294	0.5882	1.7059
s.d.	0.1861	0.1791			
Serov-c	0.1659	0.1629	0.5294	0.5294	1.8824
s.d.	0.1865	0.1867			
Intzer-n	0.1926	0.1836	0.4706	0.5882	2.1176
s.d.	0.2077	0.1973			
Djabyk-n	0.1277	0.1506	0.4118	0.4706	1.4706
s.d.	0.1903	0.2398			
N.Sergi-n	0.1681	0.1555	0.4706	0.5294	1.7647
s.d.	0.2199	0.2040			
Kytlim-n	0.1593	0.1421	0.4706	0.5882	1.8235
s.d.	0.1967	0.1801			
Tobolsk-n	0.1628	0.1634	0.4706	0.6471	2.0000
s.d.	0.1935	0.2044			
Belsky-n	0.1658	0.1622	0.5294	0.5882	1.8235
s.d.	0.1886	0.1886			
Ivdel-n	0.1653	0.1689	0.4706	0.5882	1.7647
s.d.	0.1902	0.1976			

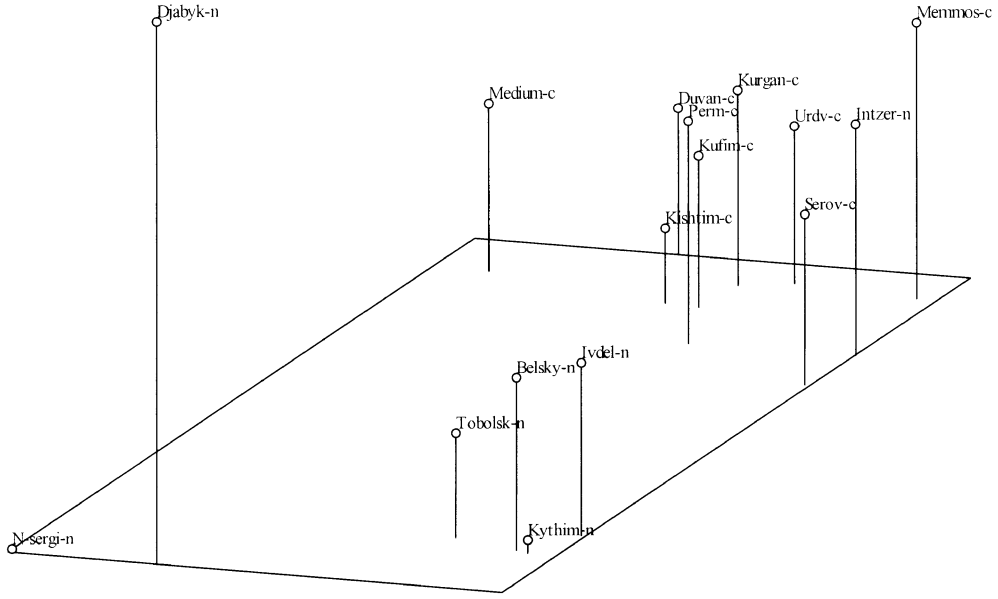

Fig. 2 Three-dimensional representation of the principal component analysis based on allele frequencies at the 17 loci. The three axes explain 89% of the total variation

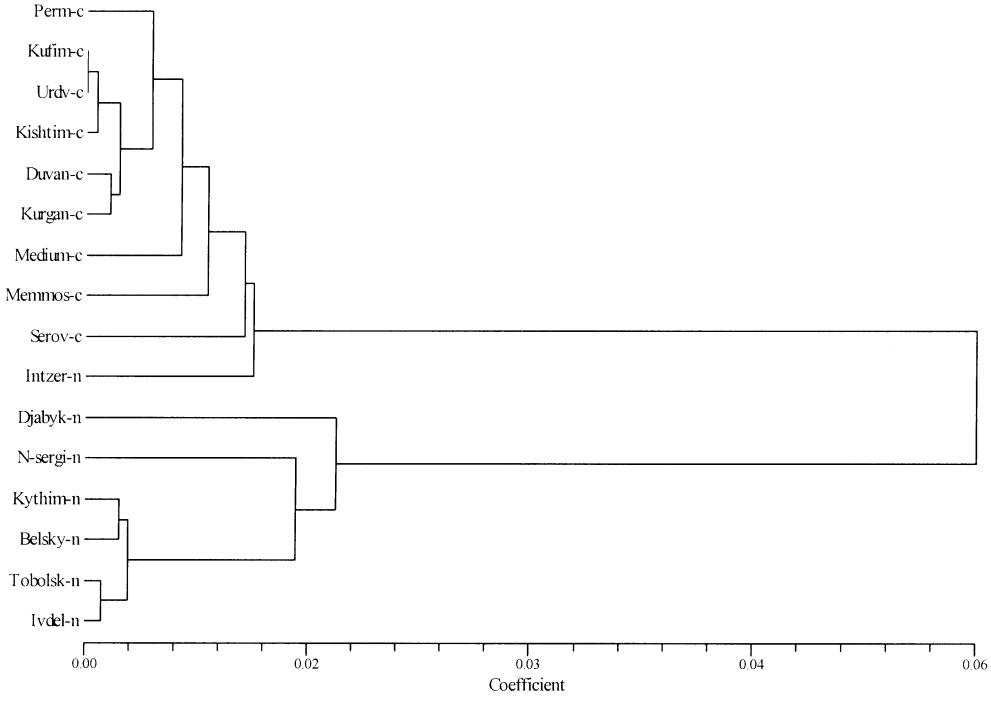

Fig. 3 UPGMA dendrogram based on Nei's distance

Statistical independence among loci

Significant linkage disequilibrium was observed in cultivars Medium and Serov among a limited number of loci (four and three pairs of loci, respectively; data not shown). Otherwise, the Fisher exact test indicated independence between loci in all other populations. Globally only the pair *Pgi-B-Adh* showed a significant linkage disequilibrium: this was mostly due to non-independence between the two loci in the cultivar Medium.

Population differentiation and introgression

Principal component analysis of the allele frequency matrix shows a clear differentiation of the wild populations and the cultivars, with the exception of population Intzer that clustered with the cultivars (Fig. 2). The three axes explain 89% of the total variation. Four of the wild populations (Bielsky, Tobolsk, Ivdel and Kytlim) clustered together, populations Djabyk and N-Sergi being clearly apart. UPGMA phenograms based on Nei's genetic distance basically led to the same clustering (Fig. 3). Wright's F_{ST} fixation indices are given in Table 3. F_{ST}

Table 3 Wright fixation indices calculated over all populations

Locus	F_{IS}	F_{ST}	F_{IT}
Pgi-B	–0.0435	0.0009	–0.0425
Got-A	0.1525	0.4798	0.5592
Got-B	–0.0318	0.0196	–0.0116
Got-C	–0.0148	0.0182	0.0037
Lap	–0.0038	0.0561	0.0525
Skdh	–0.0139	0.0995	0.0870
Pgd-A	0.0146	0.0879	0.1012
Pgd-B	–0.0675	0.0303	–0.0351
F-Est	0.1192	0.0153	0.1328
C-Est	0.0984	0.0360	0.1309
Sod-A	0.0656	0.2024	0.2548
Pgi-A	–	–	–
Dia-A	–	–	–
Dia-B	–	–	–
G-6-p	–0.0133	0.0063	–0.0070
Adh	–0.0371	0.0335	–0.0023
Sod-B	–	–	–
All:	0.0334	0.1184	0.1478
99% CI	–0.030	0.027	0.020
	0.097	0.304	0.358

values were generally low except for loci *Got-A* (0.47) and *Sod-A* (0.20). In both cases the high F_{ST} values resulted from the near fixation of an allele in the group of wild populations (*Got-A*) or the group of cultivars (*Sod-A*). The differentiation among wild populations was low (F_{ST} = 0.0371).

Isolation by distance in wild populations

Isolation by distance was assessed using both Rousset's method (Rousset 1997) as implemented by Genepop (procedure Isolde), and Hardy's method (Hardy 2000) as implemented in AutocorG. None of them allowed the detection of a significant pattern of isolation by distance even when populations Intzer, that clearly is of cultivated origin, and Djabyk, that is located far from the other populations, were ignored.

Discussion

Level of genetic variation

Genetic diversity in both cultivars and wild populations was of the same magnitude as that observed on average in other plant species (H = 0.150, P = 50.5 and A = 2.90, cited by Hagen and Hamrick 1998). Diversity estimates were however lower than those observed in nine naturalized populations of red clover from southeastern United States (Hagen and Hamrick 1998). The level of genetic variation was also somewhat higher in cultivars than in natural populations. There are many possible and non-exclusive explanations to this pattern. First, it probably reflects the fact that Russian cultivars and naturalized populations originate from one of the centres of diversity of the species, in this case probably the Eastern Mediterranean (Zohary 1972), whereas the Urals populations are close to the northeastern limit of its natural range (Govorukhin 1937). A lower genetic diversity in marginal populations than in former glacial refugia has generally been observed in other plant species. Second, it also suggests that breeding in red clover has had a limited impact on genetic variation at allozyme loci. As already pointed out by Hagen and Hamrick (1998) high levels of genetic diversity might have been deliberately maintained in red clover. Third, and related to the latter, some of the cultivars, for instance cultivar Urdv ("Uralskii Dvuukosnii") in the present study, were created by using source plants from different cultivars (three different cultivars in the case of Urdv, Voitehova 1987). Finally, it is also possible that a few local alleles were integrated in the cultivar gene pool as it spread eastward. This would have effectively countered the effect of drift that one could have expected to occur through repeated "bottlenecks". The presence at low frequency of allele 2 at locus *Sod-A* in cultivars "Kurgan" and "Kyshtim" is consistent with this hypothesis: this allele was absent in all other cultivars but present in all natural populations, sometimes at high frequency (for instance in population Djabyck).

Origin of cultivars and gene flow between cultivars and wild populations

Apart from population Intzer, which obviously is a naturalized cultivar or hybrid population, other wild populations were clearly differentiated from the cultivars (Fig. 2). This indicates that wild populations are autochtonous populations and not naturalized cultivars. It also supports the hypothesis that all cultivars, including the two American ones, are of the same origin, probably from western Europe. Hence the scenario proposed by Lisitsin (1947) seems to be the correct one: Russian cultivars were derived from western ones; Russian names, names of local localities as in the present case, being given to them later on. Seeds of local origin might have been added but, by and large, the cultivar integrity has been fairly well preserved. There are some naturalized populations, for instance population Intzer, but this does not seem to have been the rule, although it should be stressed that we minimized the likelihood of detection by selecting wild populations that apparently differed morphologically from cultivars. On the other hand, retaining populations that were morphologically well differentiated from cultivars allows us to conclude that gene introgression from cultivars has not been pervading: had this been the case those populations would no longer exist and would have been genetically similar to the cultivars at the protein loci. This is clearly not the case.

Differentiation between natural populations

As in the study by Hagen and Hamrick (1998) the level of population differentiation was low. Our estimate of

F_{ST}, with a value of 0.0371, was an order of magnitude below the average value observed for geographically widely dispersed, outcrossing and animal-pollinated species (Hamrick and Godt 1989). Red clover populations from the Urals are far from putative glacial refuges, and thus one would *a priori* have expected a more-pronounced genetic differentiation. Austerlitz et al. (2000) showed that successive founder events occurring during recolonization yield a strong differentiation and a low within-population divergence, unless characteristics of the life cycle limit the founder effects. For instance, the high genetic variation within, and the low differentiation between, forest tree populations can be explained by a reasonably high amount of gene flow and long juvenile phases that diminish the foundation effects. In the case of red clover, a high rate of gene flow together with very rapid population growth might be sufficient to make the founder effect minimal, and actually even lead to lower F_{ST} values during the recolonization process than in the refuges (Austerlitz et al. 2000). However, if gene flow among populations is important one would have expected convergence between the introduced varieties and the natural populations. This is not the case. The limited gene flow between cultivars and natural populations may be caused by: (1) a rapid turn-over of the cultivars, (2) different flowering time between cultivars and wild populations, and (3) our sampling of the natural populations that avoided plants whose morphology was close from the cultivated types. Finally, population Djabyck, which is located in a dry steppe zone, was clearly differentiated from the other populations that all come from the taiga. This was primarily due to allele frequency differences at the *Sod-A* locus. The locus S*od-A* is diallelic and almost fixed for allele 1 in all populations except Djabyck where the two alleles are approximately in equal frequency. In *Drosophila melanogaster* balancing selection was suggested at the SOD locus, though this was recently questioned by Hudson et al. (1997). In red clover, variation at the *Sod-A* locus could be associated with the resistance to drought. However, if that were the case one might have expected a significant departure from Hardy-Weinberg equilibrium, but no departure was detected. Hence the difference in frequency between Djabyck and the other populations at that locus might instead be of demographic origin.

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