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Allozyme variation in rye, *Secale cereale* L.

2. Commercial varieties

D. Adam *, V. Simonsen and V. Loeschcke

Department of Ecology and Genetics, University of Aarhus, Ny Munkegade 550, DK-8000 Aarhus C, Denmark

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Summary. Eleven samples of eight European commercial varieties of winter rye were examined at eight polymorphic enzyme loci. Genotype frequencies fitted Hardy-Weinberg expectations at all loci in all samples studied. Of the total genetic diversity recorded at the 8 loci, only 7% was expressed between varieties. Allele frequency differences between varieties were, however, sufficient to allow a characterization of each variety by a specific set of allele frequencies. Using subsets of the original data, it could be demonstrated that all pairs of varieties but one still showed significant allozyme differences, when only 4 loci were screened in samples half the original size of 200 individuals. Even when only one locus was analyzed, all varieties but two were distinguishable, but this "diagnostic" locus was not identical in all pairwise comparisons.

Key words: Allozyme variation – Commercial rye varieties – Variety identification – Open-pollinated crop species

Introduction

In recent years, the identification of crop cultivars or commercial varieties based on genetic variation at protein marker loci has attracted increasing interest from plant breeders, authorities responsible for vartiety identification and seed testing, and organization involved in bulk-purchasing of agricultural products. In genetically homogeneous crop varieties, seed proteins are mainly being used for variety identification, while enzyme markers play a minor role (see Cooke 1984, for review). In genetically heterogeneous varieties of open-pollinated crop species, enzyme marker loci should be used in variety identification studies (Nielsen 1985), because a straightforward and unambiguous genetic interpretation of seed protein electrophoretic banding patterns often is not available.

Apart from a clear genetic interpretation of their banding patterns, enzyme marker loci suitable for variety identification have to meet four requirements: (1) environmental stability of banding patterns, (2) significant intervarietal variation, (3) minimal intravarietal variation and (4) reproducibility of laboratory results (Bailey 1983). It is the aim of this study to describe the genetic variation in some European commercial varieties of rye at several enzyme marker loci and to see whether this variation could be used for variety identification. The observed intervarietal diversity is compared with the amount of intravarietal diversity registered in some rye varieties previously analysed (Adam and Loeschcke 1987). Two further problems are addressed: (1) the minimal experimental input required for variety identification in rye and (2) the capability of the methods to detect impurities in variety samples.

Materials and methods

Eleven samples of eight common European varieties of winter rye were examined. Of these, all but one sample were taken from populations of certified seed (see Table 1). Samples were obtained from the varieties' breeders/distributors in Denmark, the Federal Republic of Germany and Sweden. Genotype and allele frequencies were scored at eight enzyme marker loci in samples of 200 (German samples) and 150 individuals (other samples).

Electrophoresis was performed on coleoptile and first leaf extracts of seedling plants. Details of the methods for extraction, electrophoretic separation on starch gels and specific staining of enzymes are described elsewhere (Adam and Loeschcke 1987). The following enzyme loci were assayed

* Current address: University of Waikato, Department of Biological Sciences, Private Bag, Hamilton, New Zealand

Table 1. Origin of eight common varieties of rye from the Federal Republic of Germany (D), Poland (PL) and Sweden (S). Samples were taken in Germany, Denmark (DK) and Sweden

Name and origin of variety	Origin of samples
'Carogold' (D)	1982 Certified seed (D)
'Carokurz' (D)	1982 Certified seed (D)
'Dankowskie'	1982 Certified seed (D) ('Danko') [a]
Nove' (PL)	1982 Certified seed (DK) ('Danko') [a]
'Halo' (D)	1982 Certified seed (D)
'Karlshulder Winterroggen' (D)	1981 Certified seed (D)
'Kustro' (D)	1982 Certified seed (D)
	1982 Certified seed (DK) ('Petkus II') [a]
'Kungs II' (S)	1978 Breeder's seed (S)
'Merkator' (D)	1982 Certified seed (D)
	1982 Certified seed (DK)

[a] Names under which varieties are distributed in the countries where they were sampled

(EC number in parenthesis): *Aat-3* (2.6.1.1), *Acon-1*, *Acon-2* (4.2.1.3), *Gpi* (5.3.1.9), *Mdh-2* (1.1.1.37), *Pgd-2* (1.1.1.44), *Pgm* (2.7.5.1) and *Tpi-2* (5.3.1.1). The chromosomal location of all these loci except *Tpi-2* has been determined (Chojecki and Gale 1982; Salinas and Benito 1983, 1985; Schmidt et al. 1984; Wehling 1985; Wehling et al. 1985). Mendelian segregation has been confirmed for all loci except *Tpi-2*, which has not yet been studied in this respect (Pérez de la Vega and Allard 1984; Wehling 1985).

As suggested by Nielsen et al. (1985), G-tests (Likelihood-ratio tests) were used to test whether two varieties differ in their allele frequencies at one or several loci. Let x_{ij} be the absolute frequency of the j-th allele in the i-th population ($i = 1, 2$) at a given locus with $j = 1, 2, \ldots, m$ alleles and $n_i = \sum_{j=1}^{m} x_{ij}$. The test value for this locus will then be

$$G^2 = 2 \sum_{i=1}^{2} \sum_{j=1}^{m} x_{ij} \left(\ln \frac{x_{ij}}{n_i} - \ln \frac{x_{1j} + x_{2j}}{n_1 + n_2} \right)$$

which is asymptotically χ^2-distributed with $(m - 1)$ degrees of freedom. For several independent loci the sum of single-locus G^2-values again is asymptotically χ^2-distributed, the number of degrees of freedom being equal to the sum of the single-locus numbers (Nielsen et al. 1985). As with the χ^2-test, the G-test requires that absolute frequencies should be larger than 5 in each observational class. Therefore allele classes were pooled in cases where the absolute frequency of a given allele (x_{ij}) was less than or equal to 5 in at least one of the two populations compared.

This pooling, however, leads to an undue loss in test power when an allele is rare in one and frequent in the other of two populations compared. To avoid this, G-tests were not applied if $0.01 \leqq p_{1j} \leqq 0.05$ and $p_{2j} > 0.05$ or vice versa, p_{1j} and p_{2j} being the relative frequencies of the j-th allele in populations 1 and 2, respectively. Instead, the two populations were considered to be different, if the condition

$$p_{1j} < \left(p_{2j} - 3 \sqrt{\frac{p_{2j} \cdot (1 - p_{2j})}{n_2}} \right),$$

was fulfilled. In other words, differences were assumed to be significant if they were larger than 3 times the standard error of the higher frequency, p_{2j}.

Subpopulation heterozygosity H_s and heterozygosity H_T at each locus were estimated according to Nei (1973 a, 1974). Fixation indices F_{ST} were calculated according to Weir and Cockerham (1984).

Genetic distances between populations were calculated according to Nei (1973 b). A dendrogram based on these genetic distances and constructed using the unweighted pair-group method (Sneath and Sokal 1973) was used to illustrate the genetic relationships among populations of different varieties.

Genotype frequencies were tested for fitting Hardy-Weinberg expectations using $t = F * \sqrt{n}$ as the test statistic; F and n were Wright's fixation index and sample size, respectively. The distribution of t follows the standard normal distribution (Brown 1970).

Results

No significant deviation of genotypic proportions from Hardy-Weinberg expectations was observed. As it is shown in Table 2, significant values of t occurred in 5 out of 83 tests only. In Table 3, allele frequency estimates are given at eight polymorphic enzyme loci. At one locus, *Acon-2*, three alleles were found in several varieties. At the other seven loci, two alleles per locus were found in all samples, except for *Pgm* and *Tpi-2* which were monomorphic in four and one samples, respectively.

In Table 4, estimates of the total heterozygosity and of the subpopulation heterozygosity in the eight varieties are presented for each locus, based on one sample of each variety, i.e. not including the samples from Denmark. Fixation indices, F_{ST}, which can be interpreted as the proportion of the total diversity expressed between varieties, are also given. The mean values ($\pm$ SE) of H_T and H_S were 0.295 ($\pm$ 0.060) and 0.274 ($\pm$ 0.065), respectively. The multilocus estimate of the fixation index was rather low (0.068 ± 0.076), i.e. only about 7% of the total genetic variability can be ascribed to intervarietal differences. Yet these differences were large enough to allow a complete differentiation among all the varieties involved. Test values (G^2) for the multilocus comparisons between pairs of variety samples were significant in all cases (Table 5). This was true even for pairs of varieties known to be closely related, e.g. 'Carokurz' and 'Carogold' or 'Kustro' and 'Halo'. These two pairs of varieties, however, showed the smallest intervarietal genetic differences.

In the three cases where two samples of the same variety obtained from different countries were studied, the following G^2-values were obtained for the multilocus pairwise comparisons of these samples: 'Danko' 7.9 ($df = 7$), 'Kustro' 3.7 ($df = 7$), 'Merkator' 27.7 ($df = 6$). Of these, only the last value is significant ($\alpha = 0.01$).

Table 2. Test values t for the test of genotype frequencies fitting Hardy-Weinberg expectations at 8 allozyme loci in 11 samples of 8 European varieties of rye

Locus variety	*Aat-3*	*Acon-1*	*Acon-2*	*Gpi*	*Mdh-2*	*Pgd-2*	*Pgm*	*Tpi-2*
Carogold (D)	− 0.96	− 0.92	0.24	0.86	1.97*	0.07	mono[a]	− 0.14
Carokurz (D)	0.33	1.66	− 1.59	0.46	1.46	0.68	mono	− 1.20
Danko (D)	0.12	2.61*	− 1.28	− 1.27	− 1.71	2.13*	− 0.15	0.24
Danko (DK)	− 0.23	− 3.18*	1.06	1.81	1.43	1.13	− 0.17	0.16
Halo (D)	0.02	0.13	− 1.40	1.61	− 1.36	− 0.63	− 0.22	− 0.22
Karlshulder (D)	1.90	− 0.85	− 0.26	2.12*	0.04	− 0.26	− 0.64	− 0.95
Kustro (D)	0.44	0.48	0.68	1.78	− 0.17	0.51	− 0.14	− 0.44
Petkus II (DK)	0.49	− 0.09	− 1.12	1.91	− 0.89	0.20	− 0.09	− 0.35
Kungs II (S)	− 0.05	− 0.34	− 0.90	− 0.12	1.42	0.71	mono	mono
Merkator (D)	− 0.23	− 0.18	− 0.89	− 0.74	0.73	0.57	− 0.04	− 0.67
Merkator (DK)	− 0.07	− 0.95	− 0.68	− 0.16	0.04	− 1.22	mono	− 0.26

* Significant value ($\alpha = 0.05$)
[a] mono, monomorphic locus

Table 3. Allele frequencies at 8 allozyme loci in 11 samples of 8 European varieties of rye

Locus/allele[a]	*Aat-3*	*Acon-1*	*Acon-2*			*Gpi*	*Mdh-2*	*Pgd-2*	*Pgm*	*Tpi-2*
Variety	p_1	p_1	p_1	p_2	p_3	p_1	p_1	p_1	p_1	p_1
Carogold (D)	0.933	0.769	0	0.684	0.316	0.953	0.826	0.670	0	0.990
Carokurz (D)	0.961	0.821	0	0.540	0.460	0.949	0.700	0.706	0	0.957
Danko (D)	0.795	0.727	0	0.591	0.409	0.889	0.650	0.608	0.010	0.907
Danko (DK)	0.799	0.743	0	0.604	0.369	0.856	0.734	0.614	0.014	0.921
Halo (D)	0.593	0.695	0.005	0.578	0.417	0.940	0.463	0.823	0.015	0.985
Karlshulder (D)	0.754	0.584	0	0.804	0.196	0.867	0.767	0.872	0.085	0.827
Kustro (D)	0.572	0.688	0.005	0.523	0.472	0.923	0.555	0.893	0.010	0.970
Petkus II (DK)	0.634	0.700	0.004	0.518	0.479	0.928	0.564	0.861	0.008	0.971
Kungs II (S)	0.850	0.572	0.348	0.289	0.363	0.990	0.731	0.664	0	1
Merkator (D)	0.567	0.893	0.030	0.673	0.297	0.950	0.537	0.916	0.003	0.955
Merkator (DK)	0.572	0.881	0.006	0.738	0.259	0.988	0.622	0.869	0	0.876

[a] Alleles are numbered according to the electrophoretic mobility of the enzymes they code for, the allele coding for the 'fast' enzyme being designated 1. Apart from *Acon-2*, only two alleles per locus were found, and only the frequency of the 'fast' allele is given

As mentioned, each variety can be characterized by its multilocus allele frequency pattern. The ability of single loci to differentiate the varieties can be expressed in terms of the relative number of significant pairwise comparisons observed between varieties (Table 6). As expected from the allele frequency data, considerable variation in the discriminative power of different loci was detected. For five loci, more than two thirds of all comparisons were significant, as were about 50% for two others. *Pgm* had the lowest discriminatory powers: it was monomorphic in 3 out of the 8 varieties and had a very skewed allele frequency distribution in the others.

These results indicated that an identification of the varieties studied should still be possible, if subsets of the original data were used, comprising both fewer than 8 loci and fewer than 200 individuals per sample. When we re-analyzed our original data in this respect, it turned out that all varieties still could be identified with certainty ($\alpha = 0.01$), when only 4 loci (*Acon-1, Acon-2, Mdh-2, Pgd-2*) instead of the full set of eight enzyme loci were assayed, keeping the sample size at $n = 200$. When the sample size was reduced to $n = 100$, all pairwise comparisons between varieties still were significant at the 1% level except for one, namely 'Halo' and 'Kustro'. These results hold both for four (same as above) and eight loci assayed. Furthermore, at $n = 100$, significant single-locus differences were found for all pairs of varieties except 'Halo' and 'Kustro' at one of the four loci mentioned above.

The genetic relationships between the eight rye varieties were examined on the basis of Nei's genetic distance data (c.f. Table 5). The dendrogram presented in Fig. 1 illustrates the relationships between these

varieties. Included in this data were the samples of 'Danko', 'Kustro' (= 'Petkus II') and 'Merkator' purchased in Denmark.

Discussion

Genetic diversity in rye compared to other species

Out of 21 enzyme loci assayed, 9 proved to be polymorphic in different commercial varieties of winter rye (Adam and Loeschcke 1987), 8 of which are studied here. In comparing the genetic diversity found at these loci with the results of other studies, Nei's H-statistics rather than his D-statistic (genetic distance) were used (c.f. Brown 1979). In rye, Pérez de la Vega and Allard (1984) obtained diversity estimates somewhat lower than ours from a study of two Spanish, one North-American and one Japanese variety. Average total heterozygosity was 0.191 ± 0.011 and the multilocus estimate of F_{ST} was 0.027 ± 0.051, compared to the value of 0.068 obtained in our study. Both these results indicate a rather low degree of between-variety allozyme variation in rye. For the varieties we examined, this genetic similarity can partly be explained by, as far as is known to us, some of these varieties actually being related (see Fig. 2).

Table 4. Total heterozygosity H_T, subpopulation heterozygosity H_S and fixation index (F_{ST}) for eight allozyme loci in eight European varieties of rye

	H_T	H_S	F_{ST}
Locus			
Aat-3	0.372	0.327	0.128
Acon-1	0.404	0.383	0.065
Acon-2	0.521	0.481	0.067
Gpi	0.126	0.123	0.021
Mdh-2	0.453	0.425	0.050
Pgd-2	0.355	0.330	0.057
Pgm	0.030	0.029	0.049
Tpi-2	0.097	0.091	0.050

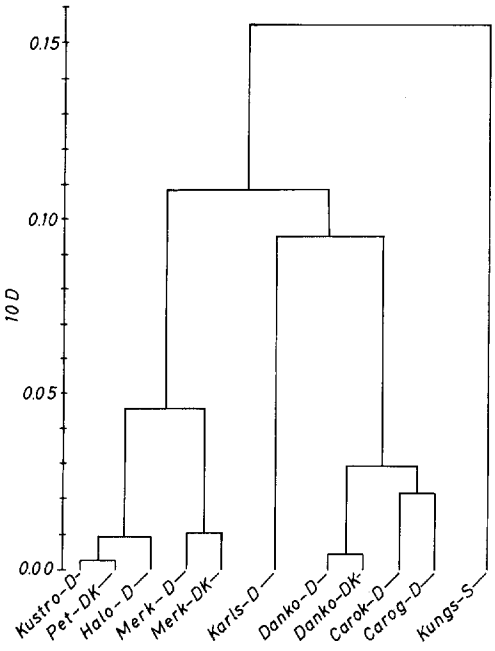

Fig. 1. Phenogram showing the observed genetic relationships between eight European varieties of rye

Table 5. Genetic distances (above diagonal) and G-test values (degrees of freedom) for pairwise comparisons (below diagonal) among populations representing eight European varieties of rye. All G-test values are significant ($\alpha = 0.05$)

Varieties	Carogold	Carokurz	Danko	Halo	Karls-hulder	Kustro	Kungs II	Merkator
Carogold (D)	–	0.0022	0.0038	0.0152	0.0087	0.0151	0.0103	0.0156
Carokurz (D)	44.6(6)	–	0.0028	0.0115	0.0121	0.0116	0.0092	0.0130
Danko (D)	89.3(6)	101.8(7)	–	0.0071	0.0089	0.0081	0.0088	0.0108
Halo (D)	287.2(7)	277.6(7)	147.9(7)	–	0.0118	0.0009	0.0153	0.0035
Karlshulder (D)	192.6(6)	368.3(7)	194.5(7)	302.7(8)	–	0.0106	0.0170	0.0123
Kustro (D)	302.2(6)	293.3(7)	167.5(7)	20.8(7)	239.1(7)	–	0.0146	0.0037
Kungs II (S)	163.7(5)	133.8(5)	96.3(5)	201.5(5)	296.7(5)	160.0(5)	–	0.0222
Merkator (D)	308.4(6)	299.8(7)	225.9(7)	84.3(7)	291.3(7)	76.8(7)	430.1(6)	–

Table 6. Percentage of pairwise comparisons between varieties showing significant allele frequency differences ($\alpha = 0.01$) at single enzyme marker loci

Locus	*Aat-3*	*Acon-1*	*Acon-2*	*Gpi*	*Mdh-2*	*Pgd-2*	*Pgm*	*Tpi-2*
%	79	75	71	54	68	68	25	54

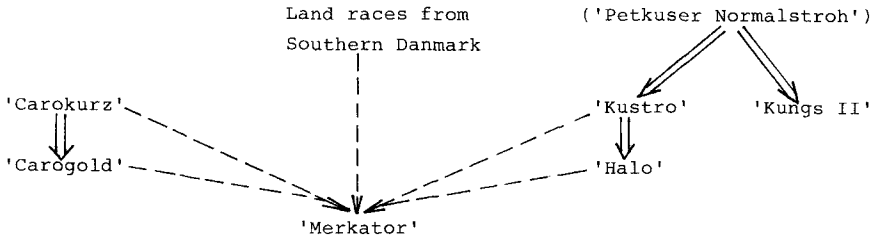

Fig. 2. Relationships between some of the rye varieties studied (c.f. Plarre 1985). ⇒: selected from; - - →: lines selected from

In other outbreeding crop species, considerably higher amounts of inter-population diversity have been reported. In radish (*Raphanus sativus*), Ellstrand and Marshall (1985) analyzed small samples of 16 commercial varieties from the USA. Their estimate of F_{ST} was 0.31. In maize, Dobley et al. (1985) analyzed allozyme variation in a large number of samples of land races collected in Mexico. Their estimate of the total heterozygosity, H_T, was close to the value found in the present study. The proportion of H_T that was expressed between collections, however, was about 4 times as high as the estimate of F_{ST} obtained by us. In commercial varieties of ryegrass (*Lolium* sp.), Nielsen et al. (1985) found values of $H_T = 0.47$ and $F_{ST} = 0.054$ for perennial rye grass (*L. perenne*) and of $H_T = 0.28$ and $F_{ST} = 0.030$ for Italian ryegrass (*L. multiflorum*).

Phylogenetic trees and genetic relationships

Phylogenetic trees are generally used for studying kinship. They might, however, also be used for elucidating the genetic relationship and thereby the origin of commercial varieties. The tree presented in Fig. 1 was constructed according to the unweighted pair group method (UPGMA) described by Sneath and Sokal (1973). For comparison we have constructed trees following a method proposed by Fitch and Margoliash (1967) and a maximum likelihood procedure given by Felsenstein (1981), using his "PHYLIB" programme package.

The overall pattern of relationships between varieties obtained by the different methods is quite similar, apart from some differences for the more remote relationships. With the UPGMA method, two almost equally likely groupings were obtained. Of those, we chose the grouping that was most consistent with the groupings suggested by the maximum likelihood and the Fitch-Margoliash methods.

The genetic relationships indicated in this grouping are in good agreement with the known relationships between the varieties and samples analyzed (c.f. Figs. 1 and 2). The rather large genetic differences between 'Kustro' and 'Kungs II' may be due to a founder effect, because the original population of 'Kungs II' was based on offspring from very few individuals of 'Petkuser Normalstroh', the common ancestor of both varieties.

Varieties identification on the basis of allozyme polymorphism

One goal of our study was to investigate whether allozyme variation can be used for the identification of commercial varieties of rye. Identification of commercial varieties here means both the characterization of a variety by its allozyme frequencies to facilitate its registrtion and the use of the characteristic allozyme frequency pattern of a variety to confirm its identity, e.g. in seed certification.

The first requirement for variety identification is the presence of sufficient genetic diversity between varieties at enzyme marker loci. In our study we were able to show that, with eight polymorphic loci and a sample size of 200, each of the eight rye varieties could be characterized by a specific set of allele frequencies. However, the ability to detect allozyme differences between varieties depends upon the genetic stability of the individual varieties. The definition of "genetically stable", i.e. the amount of genetic variation between samples of one variety that is tolerable, depends on the amount of variation found between the varieties. An example of insufficient varietal stability was described by Nielsen et al. (1985). In their case, allozyme differences between different seed lots of the same variety were larger than the between-variety differences in several varieties of perennial ryegrass (*Lolium perenne*) and Italian ryegrass (*L. multiflorum*).

In rye, Adam and Loeschcke (1987) studied a number of samples of certified seed and other seed multiplication populations of the varieties 'Carokurz' and 'Karlshulder'. Although single cases of significant allele frequency differences between seed lots of the same variety occurred in these investigations, deviating samples always showed far more genetic resemblance to the variety they were supposed to belong to than to seed lots of other varieties. In the present study, two samples of 'Merkator' showed significant allele frequency differences. This may be due to 'Merkator' being a synthetic variety, composed of lines selected

from three genetically different populations (c.f. Fig. 2). Again, however, these two samples of 'Merkator' resembled another much more than each resembled seed lots of any other variety.

Another indirect way of testing the genetic stability of a variety is to compare single-locus and two-locus genotype frequencies with those expected at genetic equilibrium (Nielsen et al. 1985). Apart from random deviations, single-locus genotypic proportions were in agreement with Hardy-Weinberg expectations in all varieties studied here, and no systematic deviations from linkage equilibrium were found (Adam, unpublished data).

Assaying a sample of 200 individuals at eight enzyme loci is a rather laborious and expensive procedure. Therefore we performed G-tests on subsets of the allele frequency data recorded. It could be shown that apart from the closely related varieties 'Kustro' and 'Halo', it is sufficient to assay 100 individuals at 4 loci to identify a sample of an unknown variety. Moreover, the three enzymes to which these four loci belong can be run on a single starch gel.

In conclusion, allele frequency differences at enzyme marker loci between eight European varieties of rye were found to be rather small. This can, in part, be explained by the known genetic relationships between these varieties. Nevertheless, a characteristic allele frequency pattern could be found for each variety. An identification of varieties on the basis of their allozyme frequencies alone is thus possible in rye. Provided that suitable enzyme marker loci are found and a reasonable genetic stability of individual varieties can be assumed, allozyme electrophoresis combined with a suitable statistical test of allele or genotype frequency differences can be expected to be a useful tool in the identification of varieties of other open-pollinated crop species, too.

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