Theor Appl Genet (2003) 107:1469–1476
DOI 10.1007/s00122-003-1366-1

ORIGINAL PAPER

S. Chebotar · M. S. Röder · V. Korzun · B. Saal · W. E. Weber · A. Börner

Molecular studies on genetic integrity of open-pollinating species rye (*Secale cereale* L.) after long-term genebank maintenance

Received: 2 September 2002 / Accepted: 3 March 2003 / Published online: 30 July 2003

Abstract The genetic integrity of six accessions represented by 14 sub-populations of the open-pollinating species rye (*Secale cereale* L.) was investigated. Seeds available from a herbarium collection (first regeneration) and from the cold store (most recent regeneration) were multiplied two to fourteen times and fingerprinted using microsatellite markers. Four accessions had significantly different allele frequencies. These were multiplied seven to thirteen times. Nearly 50% of the alleles discovered in the original samples were not found in the material present in the cold store. However alleles were detected in the most recently propagated sub-populations, that were not observed in the investigated plants of the original one. The change in allele frequencies is a continuous process. Reasons for the occurrence of genetic changes and consequences for managing open pollinating species maintained in ex situ genebanks are discussed.

Keywords Open-pollinating species · Fingerprinting · Genebank management · Genetic integrity · *Secale cereale* L.

Introduction

With respect to crop plant genetic resources most conservation efforts have concentrated on ex situ conservation. It is estimated that existing ex situ collections contain approximately 6 million accessions world-wide of which over 40%, are cereals (FAO 1998). In the Gatersleben genebank about 150,000 accessions are maintained, including cereals (65,000), legumes (30,000), vegetables (20,000), oil and fibre plants (6,000), medicinal herbs (6,000), forages (10,000) and tubers (6,000) (Börner et al., unpublished).

Depending on the storage conditions and the frequency of providing genebank materials to users, regeneration becomes necessary. Different procedures have to be applied that are determined largely by the pollination system of the particular crop. Open-pollinating species in particular need extended efforts in order to maintain the genetic integrity of the germplasm accessions. However, contamination by foreign pollen or incorrect handling during multiplication may affect the genetic identity of self-pollinating species as well.

Employing molecular markers (microsatellites), Börner et al. (2000a) investigated randomly selected accessions of the self-pollinating species *Triticum aestivum* L. This was possible because in IPK Gatersleben reference (herbarium) collections are maintained as well as the seeds stored in the cold store and originated from the most recent regeneration. Samples of grains and complete spikes from each cereal accession are deposited after they are grown initially. Although the samples are stored at room temperature and, therefore, have lost their germinability, it is still possible to extract DNA for comparative studies. The analyses of the wheat stocks showed a high degree of identity. No contamination due to foreign pollen or incorrect handling during the multiplication cycles was discovered.

In order to obtain some information about the genetic integrity of open-pollinating species maintained in ex situ seedbanks, rye (*Secale cereale* L.) was chosen for a comparable analysis. Rye ($2n = 14$) is a major crop in many areas of northern and eastern Europe. Although on a global scale its production is about 5% of that of wheat or rice, in areas with extreme climatic and poor soil conditions, rye may occupy up to 30% of the acreage (Madej 1996). The main advantages of rye compared to

Communicated by G. Wenzel

S. Chebotar · M. S. Röder · A. Börner (✉)
Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK),
Corrensstrasse 3, 06466 Gatersleben, Germany
e-mail: boerner@ipk-gatersleben.de
Fax: +49-39482-5155

V. Korzun
Lochow-Petkus GmbH, PF 1197, 29296 Bergen, Germany,

B. Saal · W. E. Weber
Institut für Pflanzenzüchtung und Pflanzenschutz,
Martin-Luther-Universität Halle-Wittenberg,
Ludwig-Wucherer-Str. 2, 06108 Halle, Germany

other winter cereals are its excellent tolerance to low temperatures and its ability to realise relatively high grain yields under environmental conditions in which other crops perform poorly. Traditional rye varieties are *panmictic* populations. Random mating is the result of a strong self-incompatibility system and wind pollination. High levels of heterozygosity and heterogeneity are characteristic for open-pollinating species.

In the genebank of the IPK Gatersleben about 2,500 rye accessions are preserved, which represent nearly 10% of the rye collections maintained world-wide (FAO 1998). For the present study, a sample of six accessions regenerated up to 14 times during the last 45 years was randomly selected. DNA, extracted from individual grains of sub-populations of the first (herbarium collection) and most recent (cold store) multiplication, were analysed using rye microsatellite markers.

Materials and methods

Plant materials and DNA isolation

From the Gatersleben rye (*Secale cereale* L. subsp. *cereale*) collection, six rye accessions, regenerated two to fourteen times, were randomly selected for investigation. The geographical origins, the years and frequencies of multiplications and the population sizes of rye accessions analysed are given in Table 1. Thirty-six grains of each accession derived from the herbarium collection and 60 grains from the most recent regeneration cycle were used. For accession R 78, three seed samples originating from three consecutive regenerations (1954, 1956, 1958) were available in the herbarium collection and analysed together with the sample grown in 1993. This gave a total of 14 sub-populations. DNA from individual grains was extracted according to the procedure described by Plaschke et al. (1995).

Microsatellite analysis

Ten rye microsatellite (RMS) markers, developed by the company Lochow Petkus, Bergen, Germany, with different chromosomal locations were chosen. Microsatellite designation, repeat type, fragment size, chromosome location and number of detected alleles of the amplified loci are shown in Table 2. PCR reactions and fragment detection were performed as described by Röder et al. (1995) and Plaschke et al. (1995).

Allele frequencies of microsatellite markers (loci) were calculated for each sub-population. This was done for the first and the most recent generation separately and, in addition, for the two intervening generations of population (accession) R78. First, the homogeneity of allele frequencies among sub-populations of each accession was tested using chi-square statistics for each individual polymorphic microsatellite marker, then a joint test was performed by combining the chi-square values of all polymorphic loci using test procedures described by Everitt (1977) or Sokal and Rolf (1981).

Genetic distances based on allele frequencies (Nei 1973) were calculated for pairwise comparisons between all sub-populations using NTSYS-PC version 1.80. The phenogram was constructed by

Table 1 Origins, years and frequencies of multiplications and population sizes of rye accessions analysed

Catalogue number Gatersleben	Variety	Origin	Years of multiplication	Regeneration frequency	Size of population
R 793	Esto	Germany	1988	2	36
			1995		60
R 784	Landrace	Spain	1986	3	36
			1996		60
R 52	Lungauer Taern	Austria	1963	8	36
			1998		60
R 200	Universal	Germany	1954	12	36
			1993		60
R 78	Waldstauden Roggen	Germany	1954	12	36
			1956		36
			1958		36
			1993		60
R 197	Landrace	Italy	1954	14	36
			1993		60

Table 2 Rang of repeat type, fragment sizes, chromosomal locations and number of detected alleles for the microsatellites used. The average numbers of alleles per locus and variety are given in brackets (sequence information can be obtained on request from Lochow Petkus, Bergen, Germany)

Microsatellites	Repeat type	Fragment size (bp)	Chromosome location	Number of detected alleles
RMS7	$(GT)_{19}$	126–130	4RL	
RMS10	$(CA)_{16}(TA)_6$	142–162	1R	5 (3.3)
RMS12	$(GT)_{27}$	213–224	7RS	28 (12.6)
RMS18	$(GA)_{21}$	111–226	7RL	18 (11.0)
RMS20	$(CA)_{13}$	134–178	7RL	
RMS28	$(GA)_{21}$	234–270	3RL	14 (8.0)
RMS104	$(CA)_{37}$	143–177	6R	18 (10.6)
RMS107	$(CT)_{20}$	64–116	1RL	11 (7.0)
RMS115	$(CT)_{34}$	110–174	5RL	19 (9.6)
RMS121	$(CT)_{20}$	118–194	6RL	18 (10.2)

using the unweighted pair-group method with arithmetic averages (UPGMA).

Results

Out of the ten rye microsatellites chosen for the investigations (Table 2), eight were ultimately used. The microsatellites RMS7 and RMS20 were discarded, because it was to difficult to interpret the banding patterns obtained by electrophoresis. The eight microsatellites were located on five different chromosomes and in total 131 alleles were discovered. The number of alleles per locus ranged from 5 (*Xrms10*) to 28 (*Xrms12*). The average number of alleles per locus and accession ranged from 3.3 to 12.6.

The main objective of our study was a comparison of allele numbers and frequencies detected in the sub-populations obtained from the different multiplication cycles (Table 3).

For accession R 793, microsatellite analysis revealed 43 alleles for seven loci. The analysis of distribution of allele frequencies shows that there were no significant changes (P = 0.05) for any of the seven loci (Table 3) during maintenance of this accession (one multiplication). At loci *Xrms18* and *Xrms104*, four alleles and one allele, respectively, were found in the first sub-population (1988) only. New alleles did not occur in the seed sample harvested in 1995 compared to the 1988 sample (Table 3).

Microsatellite analysis of rye accession R 784 detected 36 alleles at six loci tested. For this accession, multiplied twice after its initial growing in 1986, no significant differences in allele frequencies at loci *Xrms10, Xrms18, Xrms28, Xrms104*, and *Xrms115* were found. A significant difference was detected at locus *Xrms12* (Table 3). Novel alleles were detected at loci *Xrms28* and *Xrms115*. One allele at locus *Xrms18*, which was found in the sub-population from 1986, was not found in the most recent regenerated population (Table 3).

Rye accession R 52, which has been maintained in the genebank since 1963 and multiplied seven times during the period from 1963 to 1998, revealed losses of eight alleles at locus *Xrms12*, nine alleles at *Xrms18*, three alleles at *Xrms28*, three alleles at *Xrms104*, six alleles at *Xrms115* and two alleles at *Xrms121*. On the other hand, several alleles which were not found in the seed sample from 1963 were detected in the sample of 1998—in some cases at high frequencies, as the 186-bp allele at locus *Xrms12* with a frequency of 0.295 or the 128-bp allele at locus *Xrms18* with a frequency of 0.263. As a result, the differences in the allele frequencies were highly significant (Table 3).

In the two sub-populations of rye accession R 200, 53 alleles were detected in the 1954 seed sample of which 26 were present in the material of the recent propagation. At locus *Xrms12*, for example, six alleles discovered in the 1954 seed sample were not revealed in the 1995 sub-population. Two novel alleles of 138 bp at locus *Xrms18* (frequency of 0.133) and 126 bp at locus *Xrms115* (frequency of 0.211) were detected. Analysis of the allele frequency distribution shows that they changed significantly (Table 3).

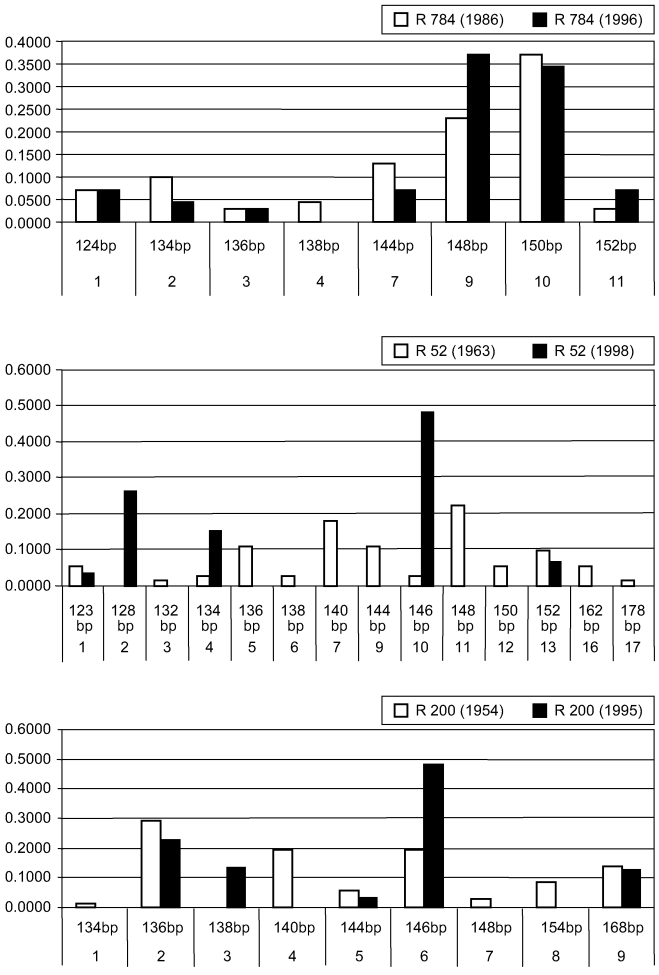

Fig. 1 Allele frequencies at locus *Xrms18* based on an analysis of seed samples of rye accessions R 784, R 52 and R 200 originating from the first (white columns) and most recent (black columns) regeneration

Analysis of rye accession R 197, which was represented in our investigation by two sub-populations harvested in 1953 and 1995, has also shown rapid allele dynamics at microsatellite loci. At locus *Xrms12*, ten alleles, which were detected in the sub-population of 1953 with an average frequency of 0.083, were not found in the sub-population grown recently; at locus *Xrms28*, seven alleles with frequencies ranging from 0.016 to 0.219 were not detected in 1995. On the other hand, different alleles with high frequencies were discovered in the 1995 material. For *Xrms12*, a 178-bp allele appeared at a frequency of 0.350. Alleles with sizes of 231 bp and 215 bp and frequencies of 0.316 and 0.194, respectively, were found for *Xrms10*. The differences in distribution of the allele frequencies at the loci tested were again highly significant (Table 3). As an example, the distribution of allele frequencies at locus *Xrms18* for the sub-populations of three accessions (R 784, R 52, R 200) is given in Fig. 1.

Table 3 Number of alleles and common alleles, chi-square homogeneity tests of rye accessions having altered field multiplications. (*na* not available)

Accession	R 52 (1963 vs. 1998)					R 78 (1954 vs. 1993)					R 197 (1953 vs. 1995)				
Progenies after first multiplication	7					11					13				
Years with winter damage[a]	1971^{+++}, 1979^{+++}, 1983^{+++}					1958^{+}, 1962^{++}					1957^{+++}, 1976^{+}				
Marker	Number of alleles					Number of alleles					Number of alleles				
	1963	1963 ∩ 1998[b]	1998 not 1963	Chi-square	*P* value	1954	1954 ∩ 1993	1993 not 1954	Chi-square	*P* value	1953	1953 ∩ 1995	1995 not 1953	Chi-square	*P* value
Xrms10	4	4	0	16.27	<0.001	3	3	1	16.27	<0.001	1	1	2	13.82	<0.001
Xrms12	12	4	3	36.10	<0.001	15	6	0	36.10	<0.001	14	4	4	40.80	<0.001
Xrms18	13	4	1	34.50	<0.001	10	2	2	31.30	<0.001	na	na	na		
Xrms28	6	3	2	24.32	<0.001	9	6	0	24.18	0.002	9	2	1	27.88	<0.001
Xrms104	13	10	0	32.90	<0.001	12	5	4	37.70	<0.001	8	8	2	27.90	<0.001
Xrms107	na	na	na			8	4	3	29.60	<0.001	na	na	na		
Xrms115	10	4	3	32.90	<0.001	11	8	1	31.30	<0.001	9	3	3	31.30	<0.001
Xrms121	10	8	1	29.60	<0.001	12	9	0	31.30	<0.001	na	na	na		
Sum	68	37	10	206.59	<0.001	80	43	11	237.75	<0.001	41	18	12	141.70	<0.001
Accession	R 200 (1954 vs. 1993)					R 784 (1986 vs. 1996)					R 793 (1988 vs. 1995)				
Progenies after first multiplication	11					2					1				
Years with winter damage[a]	1957^{++}, 1972^{+}, 1976^{+}, 1977^{+}					1988^{+}									
Marker	Number of alleles					Number of alleles					Number of alleles				
	1954	1954 ∩ 1993	1993 not 1954	Chi-square	*P* value	1986	1986 ∩ 1996	1996 not 1986	Chi-square	*P* value	1988	1988 ∩ 1995	1995 not 1988	Chi-square	*P* value
Xrms10	3	3	0	9.84	0.007	2	2	0	0.08	0.780	3	3	0	4.86	0.087
Xrms12	10	4	0	27.88	<0.001	6	5	1	14.36	0.010	3	3	0	2.04	0.360
Xrms18	8	4	1	26.13	<0.001	8	7	0	9.83	0.020	11	7	0	16.97	0.080
Xrms28	6	3	0	20.52	<0.001	6	6	0	2.56	0.770	6	6	0	3.68	0.600
Xrms104	6	5	0	14.15	0.015	7	7	0	9.48	0.150	9	8	0	9.15	0.330
Xrms107	3	3	0	9.12	0.010	na	na	na			na	na	na		
Xrms115	7	1	1	24.32	<0.001	5	5	2	11.64	0.070	3	3	0	2.49	0.29
Xrms121	10	3	0	27.88	<0.001	na	na	na			8	8	0	10.34	0.170
Sum	53	26	2	159.84	<0.001	34	32	3	47.95	0.020	43	38	0	49.54	0.066

[a] +, About 50% lost; ++, about 75% lost; +++, about 90% lost
[b] ∩, Number of alleles common to both generations

Table 4 Detailed information for accession R 78; number of alleles and common alleles and chi-square homogeneity tests for five microsatellite loci between subsequent generations of accession R 78

Accession	R 78 (1954 vs. 1956)					R 78 (1956 vs. 1958)				R 78 (1958 vs. 1993)			
Marker	Number of alleles			Chi-square	*P* value	Number of alleles		Chi-square	*P* value	Number of alleles		Chi-square	*P* value
	1954	1954 ∩ 1956[a]	1956 not 1954			1956 ∩ 1958	1958 not 1956			1958 ∩ 1993	1993 not 1958		
Xrms10	3	3	1	5.80	0.121	3	1	21.01	<0.001	4	0	27.29	<0.001
Xrms12	15	10	1	19.64	0.186	6	1	21.63	0.027	4	2	38.77	<0.001
Xrms18	10	8	1	15.09	0.128	9	1	6.63	0.675	4	0	68.51	<0.001
Xrms28	9	7	1	13.69	0.134	7	0	22.80	0.002	5	1	50.63	<0.001
Xrms115	11	8	4	26.91	0.020	7	2	21.39	0.065	7	3	22.01	0.024
Sum	48	36	8	81.13	0.005	32	5	93.46	<0.001	24	6	207.19	<0.001

[a] ∩, Number of alleles common to both generations

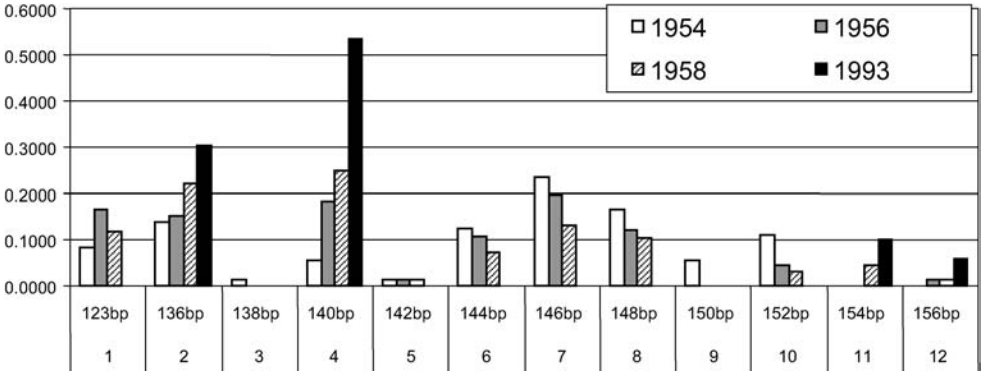

Fig. 2 Dynamics of allele frequencies at locus *Xrms18* in seed samples of rye accession R78 harvested in 1954, 1956, 1958 and 1993

For rye accession R 78, four sub-populations harvested in 1954 (36 grains), 1956 (36 grains), 1958 (36 grains) and 1993 (60 grains) were available and analysed. In the 1954 sub-population, we revealed 80 alleles at eight microsatellite loci that had been reduced to only 55 in 1993. The differences between the frequencies of both years were significant. Comparing the allele frequencies of the consecutive years it was shown, that for the sub-populations from 1954 and 1956 no significant differences were detected for loci *Xrms10, Xrms12* and *Xrms18*, whereas for *Xrms115* the differences became significant (Table 3). Comparison of the distributions of allele frequencies in the 1956 and 1958 sub-populations revealed changes at loci *Xrms10* (disappearance and appearance of one allele, respectively), *Xrms12* (disappearance of five and appearance of one allele), *Xrms18* (appearance of one allele), *Xrms28* (disappearance of one allele) and *Xrms115* (disappearance of five alleles and appearance of two alleles). The differences were significant for *Xrms10, Xrms12* and *Xrms28* (Table 4). The dynamics of the changeability of allele frequencies at the locus *Xrms18* in the seed samples harvested in 1954, 1956, 1958 and 1993, respectively, is shown in Fig. 2.

With the purpose of determining just how far the sub-populations investigated diverged from each other during maintenance in a genebank, we constructed a phenogram of genetic distances of the analysed sub-populations (Fig. 3). Several clusters were found. One was created by the four rye sub-populations of accession R 78, which originated from Germany. Closest to this cluster were the two sub-populations of a second German accession, R 200. The two sub-populations of accession R 197 clustered together with a genetic distance of 0.59, whereas the sub-populations of R 52 fell into two branches without clustering. Very small genetic distances were obtained for R 793 (0.03) and R 784 (0.055), the two accessions having the lowest regeneration frequencies.

Discussion

Most of the previous studies on the variability and relationships among populations of rye used allozymes (Perez de la Vega and Allard 1984; Ramirez et al. 1985; Adam et al. 1987; Carnide et al. 1997) or RAPD and ISSR markers (Matos et al. 2001; Persson et al. 2001). For allozyme studies, however, only material can be used which shows enzyme activity. Analyses of 30- to 50-year-old seeds stored in the Gatersleben herbarium collection at room temperature have revealed that enzyme activity gets lost (unpublished data). On the other hand, most RAPD and ISSR markers do not detect heterozygous genotypes.

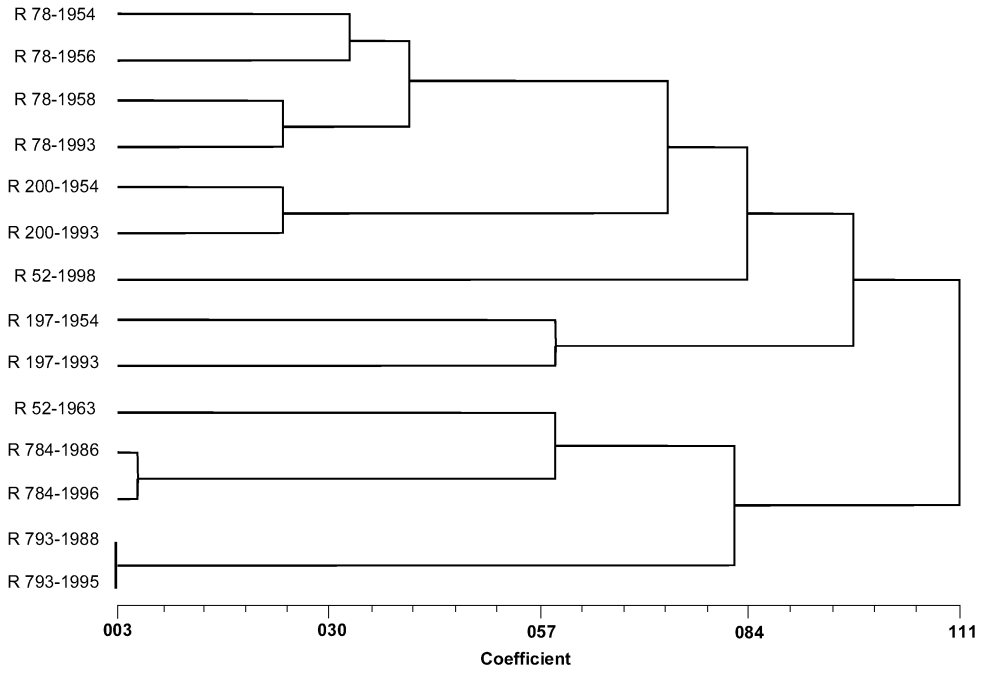

Fig. 3 UPGMA phenogram describing the relationships among rye sub-populations based on Nei's genetic distance

Therefore, we decided to use simple sequence repeats (SSRs; *syn*: microsatellites). Comparative studies in crop plants, including wheat, have shown that these are more variable than most other molecular markers (Powell et al. 1996; Taramino and Tingey 1996; Pejic et al. 1998) and provide a powerful method for discriminating genotypes (Yang et al. 1996; Russell et al. 1997; Bredemejer et al. 1998). They have been shown to be highly suitable as genetic markers in crops for the mapping of major genes (Korzun et al. 1997a, 1998; Börner et al. 2000b) or quantitative trait loci (Prasad et al. 1999; Varshney et al. 2000; Khlestkina et al. 2002), studying the genetic diversity of germplasm (Plaschke et al. 1995; Fahima et al. 1998; Donini et al. 1998; Davierwala et al. 2000; Huang et al. 2002) or verifying the identity of cytogenetic stocks (Korzun et al. 1997b; Pestsova et al. 2001; Salina et al. 2002) and genebank accessions (Börner et al. 2000a).

The RMS markers used were shown to be highly polymorphic, detecting on average 16.4 alleles per locus. This is comparable to the data described by Huang et al. (2002) in which 18.1 alleles per locus were described when 998 wheat accessions of the Gatersleben genebank were analysed. Investigating one of the ancestors of bread wheat, the wild relative *Aegilops tauschii* (Pestsova et al. 2000), found on average 18.8 alleles per locus. In other studies, however, lower allele frequencies of about five were described when *S. cereale* (Saal and Wricke 1999), *T. aestivum* (Plaschke et al. 1995) or *T. aestivum* and *T. durum* (Ben Amer et al. 2000) were analysed.

Here for the first time microsatellite markers were used to study the genetic integrity of an open pollinating species maintained for up to 45 years in an ex situ genebank. Four out of the six accessions investigated showed significantly different allele frequencies after having been multiplied thirteen (R 197), eleven (R 78, R 200) or seven (R 52) times. From the 242 alleles discovered in the original samples, 118 (nearly 50%) were not found in the material present in the cold store. On the other hand, 26 alleles were detected in the sub-populations regenerated recently, that were not observed in the investigated plants of the first harvest. For accessions having only one (R 793) or two (R 748) multiplication cycles, the differences in allele frequencies between the sub-populations were not significant for most of the loci tested. Of the 76 alleles found in total for accessions R 784 and R 793 in the original samples, 70 were detected in the sub-populations after multiplications (>90%).

Several reasons may account for the differences in allele frequencies observed in the sub-populations investigated. The detection of rare alleles depends very much on the sample size used for the analysis. Whereas enough seed was available from the sub-populations taken from the cold store, the number of grains in the herbarium collection was limited. Since only between 100 and 500 grains are stored, only 36 grains were assayed. The low sample numbers bear the risk that rare alleles will be not detected eventhough they are still present in the population. A re-discovery may be possible by analysing large sample sizes. However, people ordering genebank material usually receive only small samples (</=100 seeds) for their investigations, which definitely will not cover the diversity of the original sample. Because of that, one should also reconsider the evaluation data obtained from different sub-samples over the years.

When the frequencies obtained were compared, highly significant differences were discovered, which should be due to the selection pressure that occurred during the regeneration cycles. After an inspection of the field books used during the regenerations, it became clear that there

was strong winter damage in particular years which seriously decreased the sizes of the populations grown. In Table 3, years with winter damage are given. For rye accession R 52, for example, there was strong winter damage in 1971, 1979 and 1983. In each of these 3 years 90% of the plants were lost. From the 150 to 200 plants usually grown per plot only about 30–40 survived. This strong reduction in the population size is a bottle neck, coupled with a loss of alleles. Also, biotic factors like diseases may affect the sizes of the populations and their integrity. Especially true for R 52: it is clearly shown that the two sub-populations are different, falling into two separate branches of the phenogram. It became clear that for open-pollinating species each regeneration bears the risk of changing the genetic integrity of an accession. For R 78, it was demonstrated that the change of allele frequencies is a continuous process.

It should be noted that a shift in allele frequencies was observed that was independent of the geographical origin of the rye accessions. Even the German varieties, expected to be more adapted to the climatic conditions of the area where the multiplication takes place, were strongly affected. Although only six accessions were investigated, it may be concluded that genetic changes occurred in other rye accessions as well and, most probably, also in the accessions of other open pollinating species maintained in ex situ genebanks. In order to minimise the changes in genetic integrity of that species, the number of multiplications necessary should be as low as possible. Therefore, compared to self-pollinating crops, more efforts are necessary for the long-term storage of seeds. For rye, it was shown that a high germinability can be maintained for nearly 20 years if the seeds are stored at a low temperature of –15 °C (Specht and Börner 1998).

If regeneration becomes necessary it must be guaranteed that the size of the plots will be large enough for growing a sufficient number of individual plants covering the whole diversity of the populations. In the case of serious decreases in the total number of individuals during the regeneration cycles, due to abiotic (frost, drought, lodging) or biotic (pests, diseases) stresses, the harvest should be omitted. A repeated sowing in the coming season should be favoured instead, provided that a certain amount of seeds of the particular accession was kept. Another important point is the distance between the regeneration plots, which should be maximised. This is necessary to prevent cross-pollination, which can never be omitted completely and was, most probably, the reason for the appearance of new alleles at high frequencies.

Finally, it should be stated that in ex situ genebanks open-pollinating species need to be stored both as base and active collections. The seeds of the base collection will represent the most original sample saved under long-term storage conditions. Sub-samples of this stock will be used to produce seeds maintained in the active collection. The latter provides the material for a third party on request. When the amount of seeds of the present active collection is minimised, another sub-sample of the base collection will be grown to produce a new supply. Compared to the system of consecutive regeneration cycles, the division of the resources into base and active collections reduces the risk of genetic changes occurring during ex situ maintenance.

Acknowledgements We thank Dr. Anatoly Voylokov for doing the isozyme studies and his fruitful comments, Dr. A. Potokina for discussing the genetic distances calculations by using NTSYS-PC, Michael Grau for providing the field management data and Marina Schäfer for supplying the seed samples.

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