

Conservation of allelic multiplicity and genotypic frequency by pooling wild populations of perennial ryegrass

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Three experimental breeding populations, created by pooling four to five natural populations of perennial ryegrass, were used to indicate the effect of sampling and pooling on the retention of genetic variation. Conservation of allelic multiplicity and genotypic frequencies was observed using allelic frequencies measured on six isozyme markers. We observed that by bulking four or five natural populations, represented respectively by 25 or 20 plants, in a large polycross design, the allelic and genotypic frequencies of the original mother-plants were conserved. There was no loss of rare alleles ($0.01 < p < 0.10$) in the three experimental populations compared with the original natural populations. Only very rare alleles ($p < 0.01$) were reduced in frequency or lost. The value of such a bulking approach is discussed and presented as a possible alternative to the core collection approach to the management and use of genetic resources of perennial ryegrass.

Keywords: allelic frequency, core collection, gene conservation, isozyme markers, *Lolium perenne*, population structure.

Introduction

The maintenance of a large collection of forage plants with only a limited number of seeds leads to serious problems (see review by Bray, 1983). The concept of the core collection, developed by Frankel (1984), can be an effective way to manage and conserve genetic variation. However, although the constitution of a core collection can improve the management of genetic resources, it may not always provide genetic variability for rapid use in plant breeding programmes.

In the case of a wild cross-pollinated forage species such as perennial ryegrass (*Lolium perenne* L.), genetic variability remains very scattered between and within the populations constituting the core collection (Charmet *et al.*, 1994). Therefore, if the aim is to use genetic resources directly in plant breeding programmes, pooling populations may be an alternative way of providing adapted germplasm for plant breeders. However, it is important to consider the effects of sampling and bulking on the genetic constitution of pooled populations.

In this paper, we will consider the forage grass species, *L. perenne* which is widespread in temperate Europe. Many modern varieties of *L. perenne* have been bred for high production of dry matter under high

levels of nitrogen. However, because of over-production of livestock products within the EC and ecological concerns over intensive agriculture, new varieties are being bred for better adaptation to reduced inputs of fertilizers and herbicides or for sowing on disturbed areas (e.g. motorway verges or ski slopes).

Here, our aim is to determine the most effective way of pooling a finite number of representative plants taken from wild populations, supposed to be of infinite or very large size, in order to create breeding populations which could be useful for achieving new breeding objectives and at the same time minimize loss of alleles.

In the present study we will consider three experimental populations, constituted by pooling four or five natural populations, in order to describe changes in genetic variation due to sampling and the formation of the breeding populations. Conservation of allelic and genotypic frequencies will be studied using six isozyme markers.

Materials and methods

A sample of 550 wild populations of perennial ryegrass was collected as seeds from all over France in 1983–84. This large collection has been evaluated and described (Charmet *et al.*, 1990; Balfourier & Charmet 1991). Several multivariate analyses have been performed on agronomic and ecogeographical data of

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these 550 populations, followed by various clustering methods. This permitted wild populations to be grouped according to their agronomic similarity, as well as their ecological and geographical proximity (Balfourier & Charmet, 1994; Charmet *et al.*, 1994).

For the present study, 14 wild populations were chosen to constitute three experimental populations. Each of these populations was collected as seeds from at least 50 plants taken from an ecologically homogeneous area of 100–1000 m². This was considered to yield a sample of seeds representative of the original panmictic population (Tyler *et al.*, 1984). Table 1 gives the origins and accession numbers of these 14 populations. As described by Balfourier & Charmet (1994), the populations were chosen according to their original habitats and their groupings for agronomic similarity (agronomic cluster).

The first five populations, originating from roadside habitats, were sampled from agronomic cluster no. 9 and were used to form the experimental population named 'BR9'. The next five populations originated from pasture habitats, also from agronomic cluster no. 9, and constituted experimental population 'PP9'. Finally, four populations collected from pathway habitats and sampled from agronomic cluster no. 2 formed experimental population 'CH2'.

In autumn 1990, 20 plants were chosen at random from each of the five original populations constituting BR9. These were planted in an isolated field, in order to allow all 100 plants to intercross panmictically, as perennial ryegrass is a self-incompatible outbreeder. This design was repeated to polycross the five original populations of PP9. However, as breeding population

CH2 consisted of only four original populations, 25 plants per population were chosen randomly to keep the total number of founders to 100.

In summer 1991, seeds produced by outcrossing between the original populations of the three experimental populations were harvested separately from each surviving mother-plant.

The conservation of allelic and genotypic frequencies was assessed using starch gel electrophoresis. Six enzyme systems were examined on slices of a single lithium-borate starch gel: phosphoglucoisomerase (PGI, E.C. 5.3.1.9), acid phosphatase (ACP, E.C. 3.1.3.2), glutamate-oxaloacetate-transaminase (GOT, E.C. 2.6.1.1), superoxide-dismutase (SOD, E.C. 1.15.1.1), peroxidase (PRX, E.C. 1.11.1.7) and isocitrate-dehydrogenase (IDH, E.C. 1.1.1.42). Enzyme extractions, electrophoresis and enzyme assays were carried out according to the procedures of Hayward & McAdam (1977), and of Pollans & Allard (1985) for PRX.

Starch gel electrophoresis analysis was carried out at three stages of the breeding experiment: first, samples of 4–6 weeks old plants were taken from each of the 14 original populations and analysed to obtain allelic frequencies and test for Hardy–Weinberg equilibrium; most samples were of 110–150 plants. Secondly, all mother-plants used in the three polycrosses and still growing in the field in Spring 1991, were analysed for the same six isozyme markers. Finally, seeds harvested in Summer 1991 from these mother-plants were used to estimate allelic and genotypic frequencies for the three experimental populations. Two seeds per mother-plant were grown and analysed after 4–6 weeks.

The BIOSYS-1 program (Swofford & Selander, 1981) was used to test Hardy–Weinberg expectations and to compute Wright's *F*-statistics. In order to calculate expected genotypic frequencies for the three experimental populations, we considered perennial ryegrass to be a completely outcrossing species (Cornish *et al.*, 1979).

Results

Preliminary remarks

Each of the six enzyme systems revealed one readable and polymorphic isozyme locus: *Pgi-2*, *Acp-2*, *Got-3*, *Sod-1*, *Prx-1* and *Idh-1*; other loci were observed for PGI, ACP and GOT, but were not used for the present study because of uncertainty in banding pattern interpretation or lack of polymorphism. Allelic frequencies for the six loci, observed in the five natural populations BR9-1, BR9-2, BR9-3, BR9-4 and BR9-5, and also in

Table 1 Accession numbers and origins of the 14 natural populations of perennial ryegrass

Population	Accession number	Region of origin	Original habitat
BR9-1	10875	Franche-Comte	Roadside
BR9-2	11154	Centre	Roadside
BR9-3	11163	Centre	Roadside
BR9-4	11178	Centre	Roadside
BR9-5	11274	Centre	Roadside
PP9-1	10652	Lorraine	Meadow
PP9-2	10656	Lorraine	Meadow
PP9-3	10674	Lorraine	Meadow
PP9-4	11279	Auvergne	Meadow
PP9-5	11280	Auvergne	Meadow
CH2-1	10604	Champagne-Ardenne	Pathway
CH2-2	10607	Champagne-Ardenne	Pathway
CH2-3	10608	Champagne-Ardenne	Pathway
CH2-4	10616	Champagne-Ardenne	Pathway

experimental population BR9, are shown in Table 2; in the same way Tables 3 and 4, respectively, show the results for PP9 and CH2.

For each natural population, allelic frequencies were calculated in two ways: 'n' seedlings were used to estimate allelic frequencies of the original populations and the 'm' mother-plants were also used to calculate allelic frequencies in the sample of plants actually used in the polycross.

The number of seedlings, *n*, mostly ranged from 110 to 150 per population, except for locus *Prx-1* where some difficulties in banding pattern interpretation led us to consider fewer plants. For the mother-plants, polycrosses were made using 5 × 20 (BR9 and PP9) or 4 × 25 (CH2) mother-plants. However, as some of these mother-plants died during winter 1990–91, it was only the 'm' surviving mother-plants (i.e. those which had contributed to pollen and seed production in the polycrosses) which were used to calculate these new allelic frequencies (*m* ranging from 16 to 25).

For each of the experimental populations BR9, PP9 and CH2, three types of allelic frequencies are shown in Tables 2–4: (i) expected frequencies, which were calculated using allelic frequencies of the original populations (column: expected 1), (ii) expected frequencies, predicted by using the exact frequencies of the surviving mother-plants (column: expected 2), (iii) the observed frequencies (column: observed), estimated from 165 to 190 plants per experimental population (two seeds per mother-plant).

Allelic polymorphism

For *Pgi-2*, five alleles were found and denoted *a* to *e*, from the most anodal to the most cathodal. No rare allele faster than *a* was observed. Alleles *a* and *b* were very common; *c* and *d* were observed in some populations as rare alleles ($p < 0.10$), while allele *e* was only noted in one population (BR9-4) with a very low frequency (very rare allele: $p = 0.007$).

Only four active alleles were recorded for *Acp-2* and named *a* to *d*. Alleles *c* and *d* were quite rare. In addition, two extremely rare alleles were recorded in one population each: *a* + ($p = 0.004$) faster than *a* was observed in population BR9-3 and *e* ($p = 0.004$) slower than *d* in population PP9-1.

On analysis of *Got-3*, four alleles were observed and denoted *a* to *d*. Allele *b* was very common whereas *a* and *d* were quite rare.

Only two alleles were revealed for *Sod-1* which is not a very polymorphic locus: rare allele *a* was only observed in a few populations. Similarly, two alleles named *b* and *c* were observed for *Prx-1*, the rarer being *b*.

Lastly, four alleles were revealed for *Idh-1* (*a, b, c, d*) but only three banding patterns were scorable on lithium-borate starch gel where the *b* and *c* alleles overlap. Alleles *a* and *d* were observed as rare alleles in some populations.

We observed that common alleles ($p > 0.10$) were correctly represented in the surviving 16–25 mother-plants when sampling 20–25 plants per population (for example alleles *a* and *b* for *Pgi-2* and *Acp-2*, alleles *b* and *c* for *Got-3*, *b* for *Sod-1*, *b* plus *c* for *Idh-1* or *c* for *Prx-1*). In contrast, alleles whose frequencies were < 0.03 in the original populations were mostly lost when sampling only 20–25 plants per population. Thus, rare alleles *Pgi-2d*, *Acp-2d* and *Got-3d* were often lost whereas very rare alleles, for example *Acp-2a* + in BR9-3 and *Pgi-2e* in BR9-4, were nearly always lost. However, when rare alleles were retained in the sample of 20–25 plants, their observed frequency increased because of the relatively low sample size (e.g. allele *Got-3a* for BR9-1, BR9-4, BR9-5 or allele *Idh-1d* for BR9-4, PP9-3 or PP9-5).

Comparisons between allelic frequencies calculated from the original population samples (mostly 110–150 plants) and those from the 16–25 living mother-plants were in agreement with the expectations of Bray (1983). If the frequency of allele *A* is *p* and that of *a* is *q*, then the probability of losing allele *a*, taking *n* seeds, is $(p^2 + Fpq)^n$, where *F* is the average inbreeding coefficient. The value of this coefficient has been estimated at 0.04 by Charmet *et al.* (1993) based on 60 populations of *L. perenne*, which is the same value as that reported by Hayward & McAdam (1977).

Thus, when we take 16–25 seeds, it is likely, as observed, that some rare alleles whose frequency *q* is < 0.03 will be lost, as for $q = 0.03$, the probability of loss ranges from 0.38 (for 16 plants) to 0.23 (for 25 plants). However, the probability of saving at least one copy of each very rare allele is $1 - (0.38)^5 = 0.992$ when we use five original populations (if this allele has the same frequency, $q = 0.03$, in all five populations) and $1 - (0.23)^4 = 0.997$ for four original populations.

Results concerning experimental populations BR9, PP9 and CH2 are also given in Table 2–4, respectively. The allelic frequencies among the mother-plants of each polycross permitted prediction of expected frequencies (column 2) for each experimental population. All χ^2 tests, calculated from observed and expected frequencies (column 2), were not significant, whatever the number of plants sampled in each population: 4 × 25 or 5 × 20. Also, except for the *Acp-2* locus of BR9, all χ^2 tests between observed and expected frequencies (column 1) (i.e. calculated with the original population allelic frequencies) were not significant. Only very rare alleles ($p < 0.01$) were lost (e.g. alleles *Pgi-2e*, *Acp-2a* + and *Got-3d* in BR9,

Table 2 Allelic frequencies, at six loci, for populations BR9-1, BR9-2, BB9-3, BR9-4, BR9-5 and experimental population BR9

Locus	Allele	Natural populations												Experimental population BR9		
		BR9-1		BR9-2		BR9-3		BR9-4		BR9-5		Expected 1 (Orig)	Expected 2 (Mo-th-pl)	Observed		
		Orig <i>n</i>	Mo-th-pl <i>m</i>	Orig <i>n</i>	Mo-th-pl <i>m</i>	Orig <i>n</i>	Mo-th-pl <i>m</i>	Orig <i>n</i>	Mo-th-pl <i>m</i>	Orig <i>n</i>	Mo-th-pl <i>m</i>					
<i>Pgi-2</i>		178	19	111	20	122	19	136	20	118	20	0.447	0.485	183		
	<i>a</i>	0.511	0.553	0.392	0.400	0.389	0.368	0.445	0.500	0.500	0.600	0.447	0.485	0.519		
	<i>b</i>	0.452	0.547	0.532	0.500	0.561	0.553	0.482	0.425	0.468	0.375	0.499	0.459	0.413		
	<i>c</i>	0.028	0.000	0.036	0.025	0.037	0.079	0.051	0.050	0.013	0.000	0.033	0.031	0.036		
	<i>d</i>	0.008	0.000	0.041	0.075	0.012	0.000	0.015	0.025	0.021	0.025	0.020	0.025	0.033		
<i>Acp-2</i>		177	19	109	20	121	19	136	20	119	20	<0.001	0.000	180		
	<i>a+</i>	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.000	0.000	0.000	<0.001	0.000	0.000		
	<i>a</i>	0.376	0.447	0.541	0.775	0.364	0.421	0.320	0.375	0.555	0.579	0.431	0.521	0.478		
	<i>b</i>	0.565	0.500	0.376	0.150	0.434	0.395	0.529	0.450	0.319	0.237	0.445	0.345	0.350		
	<i>c+d</i>	0.060	0.053	0.082	0.075	0.199	0.184	0.151	0.175	0.126	0.184	0.124	0.134	0.172		
<i>Gor-3</i>		91	19	107	20	118	19	130	20	119	20	0.022	0.036	187		
	<i>a</i>	0.011	0.026	0.000	0.000	0.064	0.053	0.015	0.050	0.021	0.050	0.022	0.036	0.025		
	<i>b</i>	0.813	0.737	0.841	0.850	0.814	0.895	0.942	0.850	0.739	0.775	0.830	0.821	0.846		
	<i>c</i>	0.170	0.237	0.159	0.150	0.123	0.053	0.042	0.100	0.239	0.175	0.147	0.143	0.129		
	<i>d</i>	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000		
<i>Sod-1</i>		179	19	111	20	122	19	125	20	120	20	0.018	0.005	188		
	<i>a</i>	0.000	0.000	0.009	0.000	0.004	0.000	0.076	0.025	0.000	0.000	0.018	0.005	0.011		
	<i>b</i>	1.000	1.000	0.991	1.000	0.996	1.000	0.924	0.975	1.000	1.000	0.982	0.995	0.989		
<i>Prx-1</i>		91	19	64	20	102	19	75	20	106	20	0.003	0.000	188		
	<i>b</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.013	0.000	0.000	0.000	0.003	0.000	0.000		
	<i>c</i>	1.000	1.000	1.000	1.000	1.000	1.000	0.987	1.000	1.000	1.000	0.997	1.000	1.000		
<i>Ndh-1</i>		91	19	111	20	122	19	136	20	120	20	0.005	0.000	188		
	<i>a</i>	0.000	0.000	0.000	0.000	0.016	0.000	0.011	0.000	0.000	0.000	0.005	0.000	0.000		
	<i>b+c</i>	1.000	1.000	0.973	0.975	0.959	0.974	0.967	0.950	0.908	0.900	0.961	0.959	0.952		
	<i>d</i>	0.000	0.000	0.027	0.025	0.025	0.026	0.022	0.050	0.092	0.100	0.034	0.041	0.048		

Orig: original population sample; Mo-th-pl: mother plant.

Table 3 Allelic frequencies, at six loci, for populations PP9-1, PP9-2, PP9-3, PP9-4, PP9-5 and experimental population PP9

Locus	Allele	Natural populations										Experimental population PP9		
		PP9-1		PP9-2		PP9-3		PP9-4		PP9-5		Expected 1 (Orig)	Expected 2 (Moth-pl)	Observed
		Orig n	Moth-pl m	Orig n	Moth-pl m	Orig n	Moth-pl m	Orig n	Moth-pl m	Orig n	Moth-pl m			
<i>Pgi-2</i>	<i>a</i>	117	19	117	20	13	16	127	18	114	19	0.416	0.423	171
	<i>b</i>	0.368	0.395	0.299	0.250	0.504	0.313	0.429	0.667	0.478	0.500	0.463	0.467	0.421
	<i>c</i>	0.483	0.474	0.615	0.700	0.447	0.469	0.480	0.167	0.289	0.500	0.098	0.077	0.480
	<i>d</i>	0.137	0.132	0.085	0.050	0.049	0.219	0.000	0.000	0.219	0.000	0.023	0.033	0.088
	<i>e</i>	0.013	0.000	0.000	0.000	0.000	0.000	0.091	0.167	0.013	0.000	0.000	0.000	0.012
<i>Acp-2</i>	<i>a+</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	<i>a</i>	118	19	118	118	20	112	16	123	18	106	19	0.000	0.000
	<i>b</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	<i>c</i>	0.678	0.684	0.517	0.650	0.723	0.750	0.520	0.594	0.354	0.237	0.559	0.574	0.582
	<i>d</i>	0.254	0.184	0.309	0.225	0.205	0.214	0.386	0.406	0.552	0.711	0.341	0.352	0.342
<i>Got-3</i>	<i>a</i>	0.034	0.079	0.110	0.125	0.040	0.036	0.093	0.000	0.024	0.026	0.060	0.057	0.053
	<i>b</i>	0.030	0.026	0.064	0.000	0.031	0.000	0.000	0.000	0.071	0.026	0.039	0.011	0.012
	<i>c</i>	0.004	0.026	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	<0.001	0.006	0.012
	<i>d</i>	117	19	113	20	116	16	128	18	110	19	0.054	0.076	165
	<i>e</i>	0.009	0.000	0.093	0.125	0.000	0.000	0.047	0.111	0.123	0.132	0.721	0.690	0.067
<i>Sod-1</i>	<i>a</i>	0.739	0.737	0.575	0.500	0.750	0.656	0.785	0.806	0.755	0.763	0.197	0.207	0.676
	<i>b</i>	0.205	0.211	0.301	0.375	0.241	0.344	0.125	0.000	0.114	0.105	0.028	0.027	0.221
	<i>c</i>	0.047	0.053	0.031	0.000	0.009	0.000	0.043	0.083	0.009	0.000	0.000	0.000	0.036
	<i>d</i>	118	19	118	20	116	16	128	18	114	19	0.015	0.016	173
<i>Prx-1</i>	<i>a</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.031	0.028	0.044	0.053	0.985	0.984	0.012
	<i>b</i>	1.000	1.000	1.000	1.000	1.000	1.000	0.969	0.972	0.956	0.947	0.038	0.021	0.023
	<i>c</i>	0.970	0.921	0.991	1.000	0.996	1.000	0.866	1.000	0.985	1.000	0.962	0.979	0.977
<i>Idh-1</i>	<i>a</i>	118	19	114	20	116	16	112	18	68	19	0.004	0.000	172
	<i>b</i>	0.030	0.079	0.009	0.000	0.004	0.000	0.134	0.000	0.015	0.000	0.004	0.000	0.000
	<i>c</i>	0.970	0.921	0.991	1.000	0.996	1.000	0.866	1.000	0.985	1.000	0.963	0.944	0.977
	<i>d</i>	118	19	118	20	114	16	128	18	116	19	0.033	0.056	0.023

Orig: original population sample; Moth-pl: mother plant.

Table 4 Allelic frequencies, at six loci, for populations CH2-1, CH2-2, CH2-3, CH2-4, and experimental population CH2

Locus	Allele	Natural populations														
		CH2-1			CH2-2			CH3-3			CH2-4			Experimental population CH2		
		Orig <i>n</i>	Moth-pl <i>m</i>	Orig <i>n</i>	Moth-pl <i>m</i>	Orig <i>n</i>	Moth-pl <i>m</i>	Orig <i>n</i>	Moth-pl <i>m</i>	Orig <i>n</i>	Moth-pl <i>m</i>	Expected 1 (Orig)	Expected 2 (Moth-pl)	Observed		
<i>Pgi-2</i>	<i>a</i>	104	25	157	24	122	25	110	24	0.469	0.421	187				
	<i>b</i>	0.563	0.520	0.535	0.479	0.365	0.280	0.414	0.405	0.435	0.474	0.409				
	<i>c</i>	0.409	0.440	0.392	0.396	0.434	0.600	0.505	0.452	0.062	0.089	0.508				
	<i>d</i>	0.019	0.020	0.070	0.125	0.102	0.080	0.055	0.143	0.034	0.016	0.067				
	<i>e</i>	0.010	0.020	0.003	0.000	0.098	0.040	0.027	0.000	0.000	0.000	0.000				
<i>Acp-2</i>	<i>a+</i>	105	25	151	24	121	25	112	24	0.000	0.000	190				
	<i>a</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000				
	<i>b</i>	0.386	0.540	0.474	0.438	0.413	0.420	0.576	0.609	0.462	0.500	0.484				
	<i>c</i>	0.495	0.420	0.437	0.396	0.525	0.580	0.339	0.348	0.449	0.438	0.447				
	<i>d</i>	0.090	0.040	0.036	0.146	0.033	0.000	0.058	0.043	0.054	0.057	0.032				
<i>Got-3</i>	<i>a</i>	100	25	158	24	118	25	112	24	0.036	0.015	189				
	<i>b</i>	0.065	0.020	0.025	0.000	0.038	0.020	0.018	0.021	0.736	0.760	0.016				
	<i>c</i>	0.675	0.740	0.801	0.771	0.631	0.700	0.835	0.833	0.203	0.199	0.783				
	<i>d</i>	0.245	0.200	0.133	0.229	0.301	0.220	0.134	0.146	0.025	0.026	0.188				
	<i>e</i>	0.015	0.040	0.041	0.000	0.030	0.060	0.013	0.000	0.000	0.000	0.013				
<i>Sod-1</i>	<i>a</i>	105	25	158	24	123	25	113	24	0.006	0.010	191				
	<i>b</i>	0.000	0.000	0.000	0.000	0.016	0.040	0.009	0.000	0.994	0.990	0.008				
<i>Prx-1</i>	<i>b</i>	1.000	1.000	1.000	1.000	0.984	0.960	0.991	1.000	0.010	0.000	0.000				
	<i>c</i>	93	25	158	24	123	25	113	24	0.990	1.000	0.992				
	<i>d</i>	0.005	0.000	0.006	0.000	0.012	0.000	0.018	0.000	0.010	0.000	0.000				
<i>Idh-1</i>	<i>a</i>	0.995	1.000	0.994	1.000	0.988	1.000	0.982	0.000	0.990	1.000	1.000				
	<i>b+c</i>	105	25	64	24	123	25	113	24	0.012	0.005	191				
	<i>d</i>	0.000	0.000	0.016	0.021	0.033	0.000	0.000	0.000	0.012	0.005	0.003				
		1.000	1.000	0.977	0.979	0.963	1.000	0.982	0.979	0.981	0.990	0.994				
	0.000	0.000	0.008	0.000	0.004	0.000	0.018	0.021	0.007	0.005	0.003					

Orig: original population sample; Moth-pl: mother plant.

Prx-1b in BR9 and CH2, *Idh-1a* in PP9) and this did not affect the value of the χ^2 statistics.

Genetic variability

Table 5 summarizes genetic variability for the six loci in the 14 natural populations and the three experimental populations. Natural populations show mean numbers of alleles per locus ranging from 2.5 to 3.2 and high percentages of polymorphic loci. Furthermore, comparisons between the mean heterozygosities per locus by direct counting and the mean heterozygosities expected assuming Hardy-Weinberg distributions (Nei, 1978) showed no significant deviations (standard errors are given in brackets). Furthermore, comparisons between the mean heterozygosities per locus by direct counting and the mean heterozygosities expected assuming Hardy-Weinberg distributions (Nei, 1978) showed no significant deviations (standard errors are given in brackets). Nevertheless, for all 14 populations the expected heterozygosity was greater than that observed and, therefore, there may be a slight deficiency of heterozygotes in the original populations.

We observed decreases in the number of alleles and the percentage of polymorphic loci in mother-plants compared with the original natural populations. However, the experimental populations contained mean numbers of alleles per locus similar to the natural populations. Values for heterozygosity indicated that satisfactory panmictic conditions had occurred in all polycrosses (standard errors are given in brackets).

Table 6 shows Wright's F_{IS} statistics for four loci in the 14 natural populations; 95 per cent confidence intervals, calculated by the jackknife method (Efron, 1982), are given in brackets. Results are not given for loci *Sod-1* and *Prx-1* because the lack of heterozygosity may arise from sampling errors and the uncertainty of rare allele detection (*Sod-1a* and *Prx-1b*). The genotypic distributions were found on average to be in Hardy-Weinberg proportions at the *Pgi-2*, *Got-3* and *Idh-1* loci ($F_{IS} < 0.08$). In contrast, F_{IS} values were larger for *Acp-2*.

Table 7 gives expected and observed sizes of the different classes of genotypes for the *Pgi-2*, *Acp-2* and *Got-3* loci in the three experimental populations; χ^2 tests indicate no significant differences, with the exception of the *Acp-2* locus in experimental population BR9.

Discussion

Polymorphism within populations of *Lolium perenne*

Allelic frequencies, mean numbers of alleles and mean heterozygosities were consistent with the results found

for the same species by different authors (Hayward & McAdam, 1977; Ostergaard *et al.*, 1985; Lallemand *et al.*, 1991). In contrast, mean numbers of alleles and percentages of polymorphic loci were greater for *L. perenne* than for another forage species *Festuca ovina* (Weibull *et al.*, 1986). The F_{IS} values indicate very low levels of inbreeding, probably caused by mating between related plants. The higher values for *Acp-2* in each population might result from the presence of a silent allele (Ostergaard *et al.*, 1985) or from misinterpretation when using lithium-borate gels which did not allow discrimination between the *b* and *c* alleles.

These different results indicate that on average the 14 natural populations were in panmictic equilibrium. We might be surprised by the similarity of the populations in their allelic frequencies despite the fact that they have been allocated to different groups by ecogeographical origin and cluster analysis on agronomic traits. In fact, for an allogamous species like *L. perenne*, there is little differentiation between wild populations, most of the differentiation being within populations (Charmet *et al.*, 1993). A practical consequence of this low differentiation between populations could be that a small number of populations would be sufficient to preserve most of the genetic diversity. However, this result only applies to isozyme variation and cannot, at present, be generalized to other agronomic traits which are polygenic and subjected to various selection pressures. Work is progressing to compare the agronomic value of natural populations with the experimental populations originated by pooling.

Conservation of polymorphisms after sampling and bulking populations

Many studies have been carried out concerning the loss of alleles as a result of random drift or founder effect (bottleneck effect) caused by a reduction of population size. However, most are theoretical studies, such as Kimura & Crow (1964), Nei *et al.* (1975), Sirkkomaa (1983) or Maruyama & Fuerst (1985). Some experimental studies have been made mainly on animal species, such as *Drosophila* (Wright & Kerr, 1954; Buri, 1956), elephant seal (Bonnell & Selander, 1974) and more recently a snail (Johnson, 1988) and pacific salmon (Waples, 1990) in which base populations are generally small.

In comparing wild populations of barley and maize with modern cultivars, Allard (1992) has suggested that it is the most frequent alleles in the wild populations which are the most potentially useful for breeding and are, therefore, preserved in modern varieties.

As stated previously, each of the 14 original populations was collected as seeds from at least 50 plants in a restricted area. According to Yonezawa (1985), we can

Table 5 Summary of genetic variability calculated on the natural and the experimental populations (standard errors are given in brackets)

Populations	Natural populations				Mother-plants			
	Mean no. of alleles per locus	Percentage of loci polymorphic	Mean heterozygosity		Mean no. of alleles per locus	Percentage of loci polymorphic	Mean heterozygosity	
			(H_o)	(H_s)			(H_o)	(H_s)
BR9-1	2.5	50.0	0.191 (0.093)	0.231 (0.109)	1.8	50.0	0.149 (0.074)	0.247 (0.112)
BR9-2	2.5	83.3	0.201 (0.095)	0.244 (0.108)	2.2	66.7	0.192 (0.103)	0.215 (0.099)
BR9-3	3.0	83.3	0.237 (0.101)	0.267 (0.116)	2.2	66.7	0.211 (0.103)	0.245 (0.119)
BR9-4	3.2	100.0	0.207 (0.081)	0.253 (0.107)	2.5	83.3	0.208 (0.093)	0.274 (0.113)
BR9-5	2.5	66.7	0.275 (0.107)	0.281 (0.107)	2.2	66.7	0.297 (0.121)	0.277 (0.104)
PP9-1	3.0	83.3	0.247 (0.110)	0.263 (0.110)	2.5	66.7	0.263 (0.120)	0.282 (0.109)
PP9-2	2.7	83.3	0.287 (0.120)	0.301 (0.124)	2.0	50.0	0.199 (0.096)	0.265 (0.120)
PP9-3	2.5	83.3	0.232 (0.084)	0.261 (0.093)	2.0	66.7	0.311 (0.107)	0.312 (0.107)
PP9-4	2.8	100.0	0.282 (0.097)	0.309 (0.097)	2.2	83.3	0.269 (0.117)	0.244 (0.096)
PP9-5	3.0	100.0	0.287 (0.113)	0.292 (0.115)	2.3	83.3	0.295 (0.103)	0.269 (0.086)
CH2-1	2.7	66.7	0.228 (0.102)	0.269 (0.120)	2.3	50.0	0.207 (0.095)	0.251 (0.114)
CH2-2	3.0	83.3	0.219 (0.092)	0.256 (0.112)	2.2	66.7	0.229 (0.101)	0.276 (0.124)
CH2-3	3.2	100.0	0.256 (0.105)	0.309 (0.121)	2.3	66.7	0.259 (0.118)	0.268 (0.110)
CH2-4	3.0	100.0	0.222 (0.099)	0.249 (0.107)	2.2	66.7	0.216 (0.100)	0.246 (0.113)
Experimental population				BR9	2.7	83.3	0.244 (0.102)	0.262 (0.113)
				PP9	3.2	100.0	0.261 (0.102)	0.289 (0.113)
				CH2	3.2	100.0	0.244 (0.109)	0.255 (0.113)

H_o : observed by direct counting; H_s : expected assuming Hardy-Weinberg distribution.

assume that this sampling technique is effective in collecting the majority of genetic variability in each population. By using mostly 110–150 plants per natural population for the electrophoretic survey, we ensure, as reported by Gregorius (1980), that all alleles with frequency >0.04 are detected with a probability of 95 per cent. If we define an allele to be rare if it never occurs with frequency >0.10 (Brown, 1978), we can assume that rare alleles, with a frequency between 0.10 and 0.04, are detected with a probability of 95 per

cent. In our case, only the very rare alleles ($p < 0.01$) were lost in the experimental populations.

Our results indicate that the establishment of polycrosses, when using 16–25 mother-plants per population, can result in the loss of rare alleles. However, except for very rare alleles, such as *Acp-2a +* or *Pgi-2e*, the loss of an allele in some populations is compensated by a randomly increased frequency in other populations of the same group (BR9, PP9 or CH2) which produces a buffering effect (e.g. allele *Idh-1d* in

Table 6 Wright's F_{IS} statistics, for four loci, in the 14 natural populations

Population	Mean sample size per locus	<i>Pgi-2</i>	<i>Acp-2</i>	<i>Got-3</i>	<i>Idh-1</i>
BR9-1	134.5	0.031	0.263	0.255	*
BR9-2	102.2	-0.028	0.346	0.231	-0.028
BR9-3	117.8	-0.002	0.235	-0.010	0.277
BR9-4	123.0	0.144	0.291	-0.049	0.202
BR9-5	117.0	-0.083	0.048	0.065	0.099
PP9-1	117.7	-0.074	0.177	0.081	-0.009
PP9-2	116.3	0.104	0.181	-0.166	-0.031
PP9-3	114.5	0.056	0.091	0.318	-0.123
PP9-4	124.3	-0.092	0.161	0.098	-0.021
PP9-5	104.7	-0.029	0.064	0.029	-0.013
CH2-1	102.0	0.013	0.218	0.208	*
CH2-2	141.0	0.117	0.259	0.010	-0.019
CH2-3	121.7	0.091	0.356	0.050	0.196
CH2-4	112.2	-0.067	0.353	-0.004	-0.018
Weighted average		0.012 (-0.128-0.154)	0.218 (0.034-0.400)	0.076 (-0.154-0.314)	0.041 (-0.175-0.260)

*Absence of heterozygotes; 95 per cent confidence intervals are given in brackets.

PP9). This phenomenon is very well explained by Pollans & Allard (1989) who showed that the variance of allelic frequencies increased when population size decreased.

Furthermore, our results showed that allelic loss occurs more rapidly than loss of genetic heterozygosity and, as reported by Maruyama & Fuerst (1985), the deficit in allele number arises principally from a lack of those alleles that are expected to appear only once or twice in the sample.

In order to estimate accurately the probability of finding expected allelic frequencies for rare alleles in the three experimental populations, we analysed, as recommended by Gregorius (1980), 165-190 plants chosen by sampling two seeds per mother-plant. In this way we revealed very rare alleles, the expected frequencies of which were about 0.01, and we observed a very good fit between expected and observed allelic frequencies. All χ^2 tests (data not shown) were not significant.

We have seen that the number of founders involved in the PP9 experimental population, for instance, allowed the transfer of all alleles of a 5-allelic locus (e.g. *Acp-2*) to the next generation. These results are in agreement with those reported by Sirkkoma (1983). In the same way, rare alleles, such as *Pgi-2c* and *d*, *Got-3d*, *Sod-1a*, *Prx-1b*, *Idh-1a* and *d* were well conserved by pooling populations with such a number of founders.

Also, genotypic frequencies were maintained after pooling populations; indeed, the observed and expected sizes of the different genotypic classes were similar, except for the *Acp-2* locus in BR9 probably because of uncertainty and confusion in reading *b* and *c* alleles. On average, no linkage disequilibrium was apparent.

These results lead to the conclusion that bulking allows allelic and genotypic frequencies of original populations to be retained in most cases. Bearing in mind that the original populations had rather similar allelic compositions, no allelic loss was observed for alleles with $p > 0.01$, and the original polymorphisms seemed to be conserved in the experimental populations. In fact, this study shows that the sampling effects (founder effect/bottleneck effect), which occur when sampling several single natural populations, can be offset by pooling these same populations. Thus, pooling accessions may have some advantages over taking a representative sample of single populations, which is the core collection concept. Although bulking could be an alternative way to manage and use genetic resources of perennial ryegrass, these results have been obtained by studying presumed neutral traits (isozymes). It would be judicious to check whether agronomic traits, expressed in the original populations, were retained in the bulked populations. Also, it would be important to study the influence of bulking on genetic parameters

Table 7 Observed and expected sizes of genotypic classes, at three loci, for the three experimental populations

Locus	Experimental population	Genotype															χ^2	d.f.					
		aa	ab	bb	bc	ac	ad	bd	cc	cd	dd	ae	be	ce	de	ee							
<i>Pgi-2</i>	BR9	Observed	48	82	29	7	4	8	4	8	4	1	0	0					183	5.50	5		
		Expected	43.01	81.43	38.61	5.12	5.49	4.39	4.21	0.18	0.29	0.11											
	PP9	Observed	34	61	45	12	12	3	1	3	0	0							171	4.87	5		
		Expected	30.61	67.54	37.28	12.31	11.11	4.11	5.30	1.03	0.86	0.17											
	CH2	Observed	28	82	49	10	11	4	0	1	2	0							187	5.41	5		
		Expected	33.10	74.61	42.08	15.71	14.03	2.43	2.81	1.49	0.56	0.04											
<i>Acp-2</i>	BR9	Observed	51	41	33	7	12	17	12	1	1	5							180	15.33**	4		
		Expected	40.95	60.10	22.05	7.68	10.47	19.06	13.98	0.67	2.43	2.22											
	PP9	Observed	60	60	26	1	16	2	2	0	0	0	1	2	1	0	0	0	171	9.99	5		
		Expected	56.34	69.10	21.20	6.86	11.18	1.03	1.37	0.55	0.21	0.02	0.68	1.18	0.12	0.02	0	0					
	CH2	Observed	51	71	42	4	8	3	11	0	0	0							190	6.65	4		
		Expected	47.50	83.22	36.48	9.50	10.83	0.95	0.76	0.62	0.11	0											
<i>Got-3</i>	BR9	Observed	0	9	132	44	0	0	0	2	0	0							187	3.31	3		
		Expected	0.19	11.03	126.04	43.95	1.87	0	0	3.93	0	0											
	PP9	Observed	2	16	78	43	2	0	8	12	4	0							165	7.48	5		
		Expected	0.99	17.32	78.54	47.19	5.11	0.66	6.11	7.09	1.81	0.17											
	CH2	Observed	0	5	117	53	1	0	4	8	1	0							189	2.56	4		
		Expected	0.04	4.35	109.24	57.08	1.13	0.15	7.56	7.56	1.89	0.19											

** $P < 0.01$.

and on the expected response to selection. These problems are under investigation.

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