Application of Molecular Markers in Genetic Resources Management of Perennial Ryegrass

R. van Treuren
Wageningen University and Research Centre, Netherlands

The XX International Grassland Congress took place in Ireland and the UK in June-July 2005. The main congress took place in Dublin from 26 June to 1 July and was followed by post congress satellite workshops in Aberystwyth, Belfast, Cork, Glasgow and Oxford. The meeting was hosted by the Irish Grassland Association and the British Grassland Society.

Proceedings Editor: D. A. McGilloway

Publisher: Wageningen Academic Publishers, The Netherlands

Application of molecular markers in genetic resources management of perennial ryegrass

R. van Treuren
Centre for Genetic Resources, the Netherlands, Wageningen University and Research Centre, P.O. Box 16, 6700 AA Wageningen, the Netherlands, Email: robbert.vantreuren@wur.nl

Key points

1. Molecular markers are effective tools to support traditional approaches in plant genetic resources management.
2. Genetic diversity assessed for perennial ryegrass by AFLP analysis revealed differentiation of populations occurring in traditional Dutch grasslands from commercial varieties, but not from populations occurring in Dutch nature reserves.
3. No specific conservation measures were recommended to maintain the genetic diversity of perennial ryegrass occurring in traditional Dutch grasslands.
4. Pollination rates estimated by microsatellite analysis for a rejuvenated population of a perennial ryegrass genebank accession were very well described by an inverse quadratic function of inter-plant distance between potential mating pairs, while recorded flowering characteristics contributed only to a minor extent.
5. Compared to variation in pollination rates, the genetic integrity of the rejuvenated perennial ryegrass accession was found to be more threatened by contamination, which indicated the need for improved regeneration protocols to prevent gene flow from other germplasm.

Keywords: AFLPs, conservation, microsatellite, perennial ryegrass, seed multiplication

Introduction

Loss of genetic diversity has been widely recognised as a major threat for the maintenance and adaptive potential of species. Therefore, *ex situ* as well as *in situ* strategies have been developed for many plant species to conserve the extant genetic diversity. *Ex situ* conservation of genetic resources includes the storage of species samples in genebanks, which is intended to represent the genetic diversity of a crop as much as possible. It has been estimated that world-wide more than 1300 collections are stored in genebanks, collectively containing more than six million accessions (FAO, 1996). To manage genetic resources effectively the ability to identify genetic variation is indispensable. Characterisation of diversity has long been based on morphological traits mainly. However, morphological variation is often found to be restricted and genotype expression may be affected by environmental conditions, thereby constraining the analysis of genetic variation. Therefore, molecular marker techniques are of increasing interest to genebanks as complementary tools to traditional approaches in the management of plant genetic resources (Bretting & Widrlechner, 1995). Acquisition, maintenance, characterisation and utilisation are considered the four main categories of activities in plant genetic resources management, which may all benefit from the application of molecular genetic markers (Brown & Kresovich, 1996).

During the last few decades a wide variety of techniques to analyse genetic variation have emerged, including Amplified Fragment Length Polymorphisms (AFLPs) and microsatellites (Whitkus *et al*., 1994; Karp *et al*., 1996; Parker *et al*., 1998). AFLPs are DNA fragments obtained from endonuclease restriction of DNA and amplification by the Polymerase Chain Reaction (PCR). The AFLP technique simultaneously generates fragments from multiple genomic sites that are separated by gel-electrophoresis and are generally scored as dominant

markers. Because of the robust and highly informative fingerprinting profiles that are generally obtained, AFLPs can be applied in studies involving genetic identity, parentage, identification of clones and cultivars and gene mapping studies (Vos *et al*., 1995). Microsatellites are DNA fragments consisting of tandem repeat units of very short nucleotide motif. Polymerase slippage during DNA replication, or slipped strand mispairing, is considered to be the main cause of variation in the number of repeat units, resulting in length polymorphisms that can be detected by gel-electrophoresis following amplification of microsatellites by PCR. Due to their robustness, high level of polymorphism and codominant inheritance, microsatellites are informative markers in population genetic and gene mapping studies (Morgante & Olivieri, 1993; Jarne & Lagoda, 1996). In this paper the application of AFLPs and microsatellites in plant genetic resources management is illustrated by two studies in perennial ryegrass (*Lolium perenne* L.).

Perennial ryegrass is a major fodder crop and the main species in cultivated grasslands in temperate climate zones (Holmes, 1980). Apart from being used for forage, perennial ryegrass is also important as a turf species in sports fields, recreational areas and lawns. The primary centre of perennial ryegrass diversity is located in the European-Siberian region of diversity (Zeven & de Wet, 1982). Optimisation of fodder production has strongly reduced the biodiversity present in temperate grasslands during the last few decades. Germplasm collections were therefore developed by genebanks in order to safeguard genetic resources for present and future utilisation. It has been estimated that about 7348 perennial ryegrass accessions are collectively maintained in European genebanks (Sackville Hamilton, 1999).

Perennial ryegrass is one of the most important species of Dutch grasslands. However, during recent decades the original diversity of many grasslands has been replaced through the widespread use of more uniform commercial cultivars, adapted to the application of high doses of nitrogenous fertiliser. Therefore, grasslands that have not been resown and that have been treated with no or only limited amounts of nitrogen fertiliser have become scarce in the Netherlands. The Centre for Genetic Resources, the Netherlands (CGN) managed to identify about 50 such grasslands in 1998 by an inventory of Dutch farms still in agricultural use. Because it was unknown whether unique diversity still existed among these traditional grasslands, a diversity study using AFLPs was undertaken for perennial ryegrass to support conservation policies.

Genetic resources conserved as seed populations need periodic rejuvenation by means of seed multiplication because of decreased germinability of seeds by ageing and reduced seed supplies due to distribution. During seed multiplication, genebanks aim to avoid loss of genetic integrity of accessions as much as possible. The potential for genetic change particularly applies to species that are characterised by highly heterogeneous populations, including the outbreeding, wind-pollinated, perennial ryegrass (Forster *et al*., 2001; Guthridge *et al*., 2001). For forage species conserved in European genebanks it has been estimated that about 20% of the accessions are in desperate need of rejuvenation (Marum *et al*., 1998). Both from a genetic and economic viewpoint, optimisation of the regeneration protocols is therefore indispensable, but the necessary empirical data are largely absent. Loss of genetic integrity may result from a variety of reasons, including contamination, variation in pollination rates and differential seed production among plants. Microsatellites were used to analyse mating patterns in a regenerated perennial ryegrass accession and to investigate how well observed pollination rates could be predicted from data on the spatial and temporal distribution of pollen release.

Materials and methods

Assessment of diversity in traditional Dutch grasslands

From the 50 traditional grasslands identified by CGN in 1998, 16 were selected for the present study based on variation in geographic location and variation in soil type. In order to enable comparison of the diversity observed within the traditional grasslands with the diversity present in varieties, eight cultivars were selected that have played a major role in Dutch grassland cultivation during the last 50 years. These cultivars were 'Perma', 'Lamora', 'Barenza', 'Pelo', 'Vigor', 'Barmaco', 'Tresor' and 'Semperweide'. In addition, six nature reserves were selected to enable comparison with the genetic diversity of grasslands that are already conserved *in situ*. These grasslands were also managed extensively and displayed a similar geographic range as the traditional grasslands. From each grassland and cultivar, 36 plants per population were investigated by AFLP analysis.

DNA extractions were carried out using about 100 mg of freeze dried tissue material taken from young fresh leaves and largely followed the procedures described by Fulton *et al*. (1995). AFLP procedures basically followed the protocol of Vos *et al*. (1995) using about 300 ng of total genomic DNA and restriction enzymes *Eco*RI and *Mse*I. PCR reactions were carried out on a PE 9600 thermo cycler. Amplified fragments using ^{33}P were separated on 6% denaturing polyacrylamide gels. All samples were screened for the primer combinations E32/M51 and E32/M54. Details about experimental procedures are presented by Treuren *et al*. (in press).

AFLP fragments on the autoradiograms were scored manually for presence or absence in the range of approximately 50-500 bp. Band frequencies of AFLP markers were calculated in each of the populations. Genetic relationships among populations were investigated by an UPGMA cluster analysis (e.g. Nei, 1987) using the software package TFPGA (Miller, 1997). To determine to what extent the AFLP data were able to distinguish between populations, an assignment test was performed using the procedures of Paetkau *et al.* (1995). These analyses were carried out by a custom-designed computer programme.

Analysis of mating patterns during regeneration

Perennial ryegrass accession BA 11894 (Institute of Grassland & Environmental Research, Aberystwyth, UK) was regenerated in an experimental field at the Grassland Research Station Rožnov-Zubří (Czech Republic). The 49 plants used as parents for the rejuvenation were arranged in a 7x7 matrix with 50 cm distance between plants. During the flowering season, the daily number of inflorescences with open flowers was recorded for each of the 49 plants. From these data, the flowering period of each plant could be determined and estimates of pollen production could be derived. For the regenerated accession, a paternity exclusion analysis was carried out by microsatellite analysis of the 49 parental plants and 46 offspring from each of 12 different progenies.

DNA extraction procedures followed those described for the AFLP study, with the exception that a combination of the protocol of Fulton *et al*. (1995) and the DNeasy 96 Plant Kit was used. The parental plants were screened for 38 microsatellites, of which 25 appeared to be useful. Details for the majority of the microsatellites used are presented by Kubik *et al*. (1999; 2001) and Jones *et al*. (2001). Microsatellites were amplified on Peltier thermo cyclers (PTC-200) and PCR products were separated by capillary electrophoresis using an ABI Prism 3700

DNA Analyzer. Microsatellite alleles were sized using the program GeneScan and the resulting output was analysed with the software package Genotyper, version 3.5 NT (Perkin Elmer). All offspring samples were analysed for an initial set of 12 informative microsatellites. Samples were not investigated any further in case of identified paternity, otherwise samples were screened for additional microsatellites. In case of remaining ambiguities, offspring were screened by AFLP analysis, together with their maternal plant and the remaining potential pollen donors.

To relate observed pollination rates to data on the spatial and temporal distribution of pollen release, first several functions were considered that may describe the effect of inter-plant distance on pollen deposition rates between pairs of plants, including functions that assume an exponential reduction in pollen deposition rates with increasing inter-plant distance. From these functions the relative paternal contribution of plants to other plants was estimated, assuming an effect of distance only. Estimates were compared to observed data to evaluate the goodness of fit of the examined functions. The recorded numbers of inflorescences per plant were then used to estimate the contribution of each plant to the total pollen pool and to estimate the paternal contribution of each plant to the progenies of other plants on each day of the flowering season. Subsequently, paternal contributions of plants to progenies were calculated for the entire flowering season in order to estimate relative contributions of plants to individual total progenies. Estimates of relative paternal contributions of plants to progenies based on inter-plant distance and flowering data were then combined and compared to observed pollination rates. Self-fertilisation was disregarded in all estimation procedures. Microsoft Excel and custom-designed computer programs were used for data analyses.

Results

Assessment of diversity in traditional Dutch grasslands

Within the total sample, 101 variable bands (88.6%) were scored. The average fraction of polymorphic loci was lower for the cultivars (0.582) than for the traditional grasslands (0.682) and the grasslands from nature reserves (0.703), indicating lower levels of variability within the cultivars. Within the group of traditional grasslands 95 polymorphic bands were observed, of which 94.7% and 93.7% were also found polymorphic in the group of cultivars and the group of nature reserves, respectively. Polymorphisms absent from the latter two groups involved low-frequency alleles (<10%) in the group of traditional grasslands in all cases, indicating that the common alleles are present in all three groups. Differential fixation of alleles between populations was observed for none of the investigated markers. However, band frequencies of markers could differ substantially between populations, particularly for the reference cultivars.

Assignment tests using the observed band frequencies showed low fractions of genotypes correctly assigned to their population of origin for both the traditional grasslands (range: 0.0% to 27.8%) and the nature reserves (range: 6.3% to 50.5%), indicating low population differentiation among the investigated grasslands (Table 1). Better results were found for the reference cultivars (range: 61.1% to 94.4%), indicating a clearer identity among this material. When separate analyses were performed in each of the three groups, some of the cultivars even showed 100% correctly assigned genotypes.

Table 1 Percentage of genotypes assigned correctly to the population of origin based on the assessed AFLP variation. Analyses were carried out for the entire set of populations (total sample), and separately (own group) for the traditional grasslands (A), the grasslands from nature reserves (B) and the reference cultivars (C).

Population	Total sample	Own group	Population	Total sample	Own group
Traditional grasslands			*Nature reserves*		
Grijpskerk	13.9	19.4	Oldenzaal	8.6	28.6
Milheeze	2.9	11.4	Schin op Geul	38.7	58.1
Zundert	0.0	0.0	Culemborg	6.3	37.5
Warder	11.4	17.1	Achtmaal	30.4	56.5
SintJansklooster	8.3	13.9	Zegveld	22.9	31.4
Driewegen	5.7	17.1	Oostereind	50.5	67.9
Idsegahuizum	16.7	19.4			
Halle	11.1	19.4	*C. Reference cultivars*		
Losser	8.6	11.4	Perma	83.3	100.0
Eckelrade	8.6	11.4	Semperweide	72.2	97.2
Britswerd	20.6	20.6	Lamora	83.3	88.9
Castricum	19.4	30.6	Barenza	61.1	75.0
Wageningen	27.8	30.6	Pelo	63.9	75.0
Thesinge	8.3	25.0	Vigor	63.9	91.2
Mijdrecht	13.9	19.4	Barmaco	94.4	100.0
Oudewater	17.6	23.5	Tresor	72.2	86.1

Genetic relationships between populations were represented by a dendrogram based on Nei's unbiased estimate of standard genetic distance (Figure 1). Genetic distances among the grassland populations appeared small, all clustering together within a single group. As could be expected based on the results of the assignment tests, larger genetic distances were found among the reference cultivars and between the reference cultivars and the grassland populations. Genetic relationships between populations were also investigated by Principal Component Analysis (results not shown), which were in line with the results of the UPGMA cluster analysis. PCA plots supported the observed distinction between the reference cultivars and the grassland populations and the finding that the traditional grasslands and the grasslands from nature reserves basically cover the same range of genetic diversity.

Analysis of mating patterns during regeneration

Flowering was observed for all 49 plants of the regeneration plot and none of the plants showed non-overlapping flowering periods with other plants. Daily registration of the number of inflorescences with open flowers was performed over a 50-day period. Microsatellite data of the parental plants indicated tetraploidy instead of diploidy in six cases based on the observation of three or four alleles for the majority of microsatellites investigated. Tetraploidy of the six plants was supported by morphological examination of the plants and confirmed by flow cytometry.

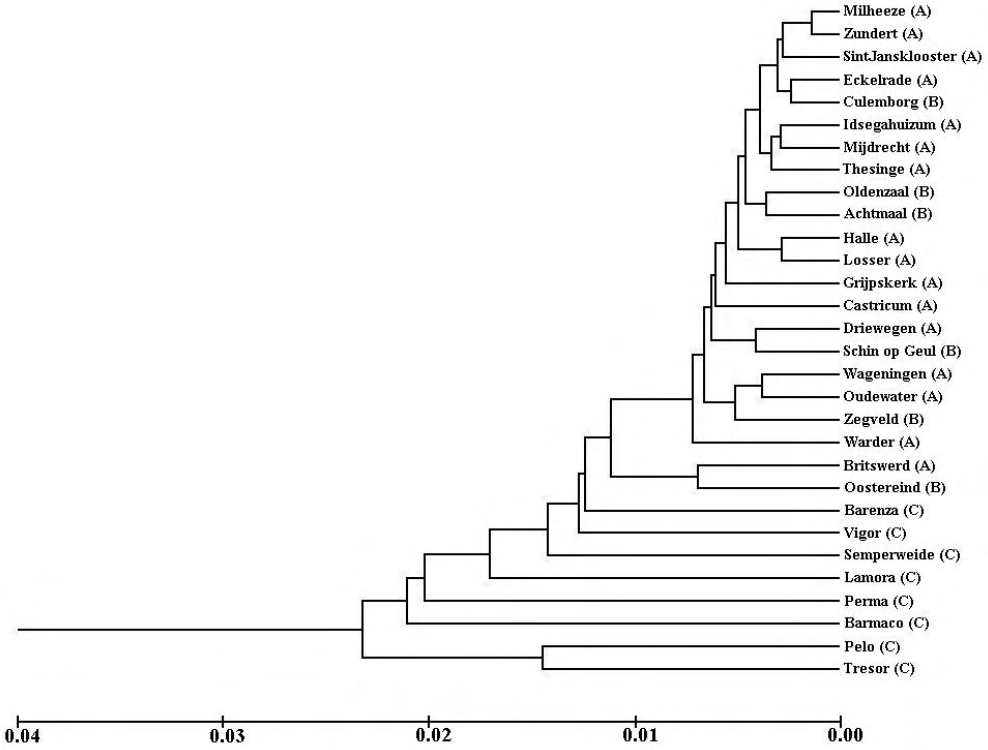

Figure 1 UPGMA cluster analysis of perennial ryegrass populations based on Nei's unbiased estimate of standard genetic distance derived from AFLP data. Traditional grasslands are denoted by (A), grasslands from nature reserves by (B) and reference cultivars by (C).

Out of the 551 offspring analysed, 451 actual pollen donors (81.9%) were identified based on the microsatellite data. Remaining ambiguities could be resolved with additional AFLP analysis, except in four cases (Table 2). For nine offspring, microsatellites alleles were observed at multiple loci that were unknown to the parental population. These included five cases of matching alleles with the maternal plant that were considered pollen contaminants and four cases of mismatches with the mother that were regarded seed contaminants. Despite the fact that selfing rates are generally assumed to be low in perennial ryegrass because of a self-incompatibility system, 19 cases of self-fertilisation were observed. Self-fertilisation was most pronounced for the tetraploid plant 21 (Table 2). Based on the molecular data, no cross-pollination between plants of different ploidy level was observed in the study plot. Consequently the two groups of different ploidy level were treated as two reproductively isolated populations in further analysis of the data.

Out of the total number of identified paternal plants, 61.9% were located within 1 m of the maternal plant, indicating that inter-plant distance had a large effect on pollination probabilities between plants (Table 2). High pollination rates observed within progenies involved neighbouring plants in nearly all cases, as is shown for progeny 22 in Figure 2. In contrast to the impact of inter-plant distance on pollination rates, no clear effect was observed of pollen production and extent of temporal overlap in flowering.

Table 2 Paternity data obtained from microsatellite and AFLP analysis after the regeneration of perennial ryegrass accession BA 11894 using 49 parental plants. The 12 analysed progenies are denoted by the code of the maternal plant (9, 11, …, 46), plants 21 and 46 having a tetraploid genome. Paternity data are classified by self-fertilisation, inter-plant distance in cm between the parental pair, absence of matching with any of the 49 parental plants and remaining ambiguous paternity.

		Inter-plant distance						
Progeny	Selfing	1-100	101-200	201-300	301-400	No match	Ambiguity	Total
9	0	27	11	6	0	0	1	45
11	0	32	12	1	-	1	0	46
19	1	24	16	4	-	1	0	46
22	0	38	7	1	-	0	0	46
30	3	15	16	7	2	2	1	46
33	0	28	13	4	0	1	0	46
35	0	31	9	5	0	0	1	46
39	0	32	9	4	0	0	1	46
41	0	23	13	7	2	1	0	46
43	0	19	24	1	-	2	0	46
21 (4n)	15	-	30	-	-	1	0	46
46 (4n)	0	45	1	-	-	0	0	46
Total	19	314	161	40	4	9	4	551

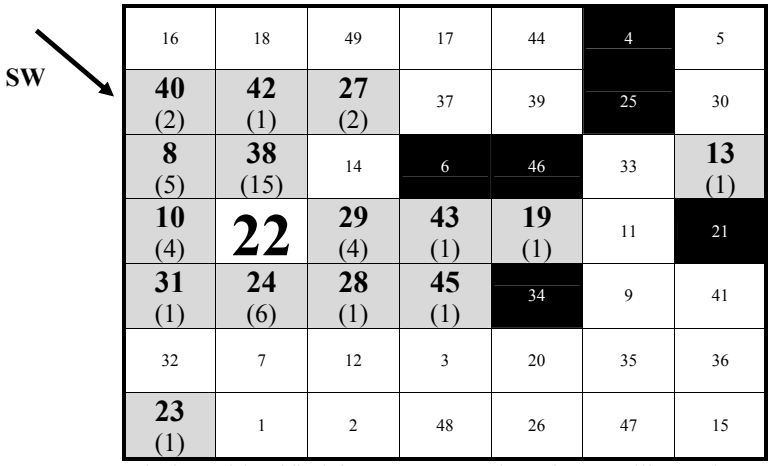

Figure 2 Paternal plants identified for progeny 22 by microsatellite and AFLP analysis after the regeneration of perennial ryegrass accession BA 11894. The regeneration plot consisted of 49 plants arranged in a 7x7 matrix with 50 cm inter-plant distance. Paternal plants are indicated by the grey shade of the cells within the matrix with the number of observed matings given between brackets. Six plants of the regeneration population appeared to be tetraploid and are indicated by black shading. The prevailing wind direction during the flowering period (SWW) is denoted by an arrow.

To analyse the effects of inter-plant distance and flowering characteristics in closer detail, pollination data of different progenies were combined and classified into observed distance categories. Because inter-plant distances within the plot showed a discrete distribution, pollination rates as a function of inter-plant distance were presented in a cumulative manner. Subsequently, observed cumulative pollination rates were compared to expected values based on the progeny samples sizes and various functions relating pollen deposition rates to inter-plant distance. These analyses showed that expected values based solely on an inverse quadratic function of inter-plant distance already fitted the observed pollination rates very well (Figure 3). Using the sum of squared differences between observed and expected values of all 430 potential mating pairs as a measure of goodness of fit, the inverse quadratic function displayed a 51.0% better fit than a random mating model, a 28.4% better fit than an inverse distance function and a 9.5 % better fit than an inverse third power distance function. The fit of the inverse quadratic function improved with only 0.77% when the flowering data were included in the calculation of expected values (Figure 3), indicating that spatial proximity was the main cause of variation in pollination rates within the study plot.

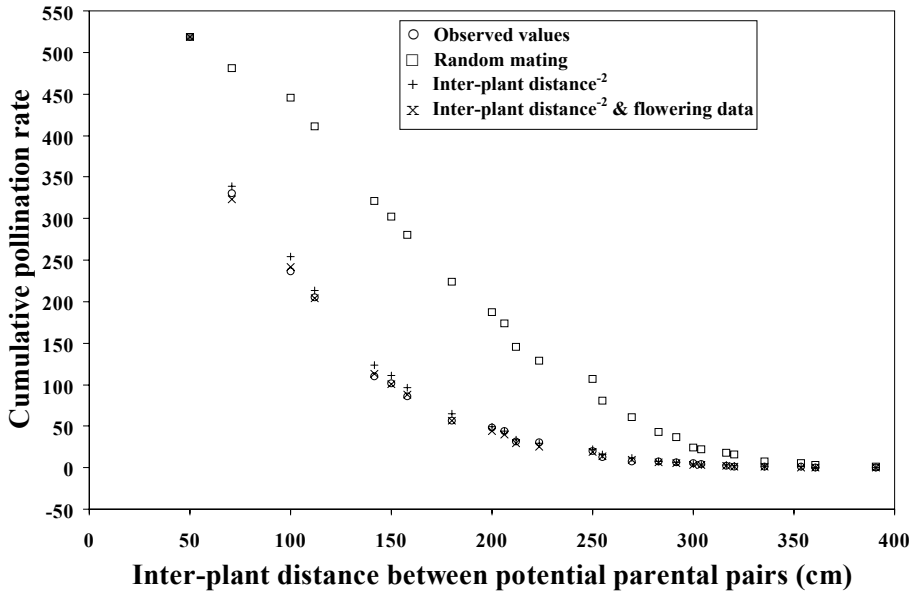

Figure 3 Cumulative number of pollinations in relation to inter-plant distance between pairs of plants presented for the observed data, estimates based on random mating and estimates based on an inverse quadratic function of inter-plant distance. For the latter function, estimates were derived both excluding and including the recorded flowering data.

Discussion

Assessment of diversity in traditional Dutch grasslands

High levels of variation were found within the grassland populations, which were in line with the results from other studies in perennial ryegrass (e.g. Roldán-Ruiz *et al.*, 2000; Creswell *et*

al., 2001; Guthridge *et al*., 2001). Compared to the grassland populations, lower levels of diversity were observed within the reference cultivars, which were mainly the result of the absence of rare alleles occurring in grasslands. These results suggested that diversity has been sampled thoroughly from grasslands at the onset of modern plant breeding.

Despite the fact that the traditional grasslands, reference cultivars and grasslands from nature reserves shared the major part of the AFLP variation, band frequencies of AFLP markers could vary considerably between populations. These results were in accordance with the results of an AFLP study of perennial ryegrass populations from Portugal that did not reveal diagnostic bands but showed that the total AFLP profile of a genotype was a reliable indicator of the population of origin (Cresswell *et al*., 2001). In the present study this was observed to some extent only for the reference cultivars. These results indicated distinction between the reference cultivars and the grassland populations and low levels of population differentiation within the latter group. This was supported by cluster analyses of the investigated material, grouping the traditional grasslands and grasslands from nature reserves together and separating these populations from the reference cultivars. Genetic relationships among the investigated material were in agreement with a morphological study carried out on the same genotypes from the traditional grasslands and reference cultivars (Treuren *et al*., in press).

The low levels of population differentiation among the grasslands suggested that in the past the perennial ryegrass populations have not gone through severe bottlenecks, nor have experienced restricted gene flow. Based on the distinction between the grasslands and the reference cultivars, the grassland populations do not seem to have experienced substantial introgression from cultivated material (see also Loos, 1994). The finding of large differences in marker frequencies for subsets of the markers may indicate linkage to genes under selection. Differences in frequencies for these markers observed between some traditional grasslands were accompanied by strong differences in agronomic characters, notably heading date (Treuren *et al*., in press). It has been suggested that alleles that are common only in certain populations may indicate adaptive significance and, therefore, that conservation should focus on such populations (Marshal & Brown, 1975; Allard, 1992). This could apply to some of the traditional grasslands. However, the fact that the traditional grasslands and grasslands from nature reserves covered basically the same range of variation did not support specific conservation measures for traditional grasslands. Proper *in situ* conservation of the nature reserves is considered sufficient to maintain the genetic diversity of perennial ryegrass occurring in traditional grasslands. A similar conclusion was reached from an accompanying study in white clover carried out for the same traditional grasslands and nature reserves (Treuren *et al*., in press). Whether this conclusion also will apply to species that usually display higher levels of population differentiation, such as less outcrossing species, is still an open question. Moreover, in the present study only genetic diversity within species was considered. However, conservation issues may also include other aspects of biodiversity, such as species diversity.

Analysis of mating patterns during regeneration

A large effect of inter-plant distance on pollination rates was observed for the study plot, which was in accordance with the generally observed leptokurtotic distribution of pollen dispersal in perennial ryegrass (Giddings *et al*., 1997a; 1997b; Cunliffe *et al*., 2004). Pollination rates were best described by an inverse quadratic function of inter-plant distance, which corresponds to a pollen cloud rapidly diluting with increasing distance in two dimensions. Flowering characteristics appeared to have only a minor effect on pollination

rates. However, the magnitude of this effect strongly depends on the variation in flowering characteristics among plants and, therefore, may be higher for populations displaying larger variance values. Curve fitting was performed on cumulative data obtained from the combined results of different progenies. Analysis of individual progenies suggested that incompatibility for specific parental combinations was at least one additional factor affecting pollination rates. Other influencing factors may include disturbance of natural pollination rates by daily visit of the plot to record flowering data. Other studies in perennial ryegrass have suggested an effect of wind parameters on pollen dispersal (Giddings *et al*., 1997a; 1997b), but no clear relationship was found in the present study.

Differential pollination rates between plants may cause unequal contributions of plants to the next generation. Skewed contributions by plants will reduce the effective population size and, therefore, increase the inbreeding coefficient (Falconer, 1981). This will compromise the objective of genebanks to maintain the genetic integrity of accessions as much as possible. However, if the spatial arrangement of plants is the main factor influencing pollination rates, paternal contributions and the variance therein between plants can be estimated from the inverse quadratic function of inter-plant distance. Modifications of the regeneration protocol that minimise unequal contributions by plants will reduce loss of genetic integrity. At least two modifications could be envisaged. First, arranging the plants into a more linear plot design will reduce the variance in paternal contributions. For example, a single row of 49 plants will reduce the variance by 72% compared to a 7x7 matrix, while the variance can be reduced to zero using a single circle of 49 plants. However, it can be questioned whether the observed distance function will hold for such plot designs. Moreover, apart from practical disadvantages, a linear or circular plot design will make the pollination of plants more dependent on its direct neighbours, while overall pollination rates will be strongly affected by prevailing wind directions. Second, unequal paternal contributions could be compensated by harvesting differential numbers of seeds from individual plants. Seed numbers required per plant can easily be determined by a simple computer program, minimising the variance in the sum of maternal and paternal contributions between plants. Differential seed harvesting may be relevant to genebanks that already practise 'balanced bulking' to correct for differences in seed production between plants (e.g. Johnson *et al.,* 2002; 2004). Implementation of this modification could be supported by validation of the general application of the distance function.

A total of nine contaminants were detected among the 551 offspring analysed (1.63%), which at first sight may seem only a minor contamination rate. However, based on this contamination level, the probability of selecting at least one contaminant as parent for a next regeneration with 49 plants is 57%. Thus, even 'minor' contamination levels may cause persistence of foreign germplasm in accessions. This will even be more severe in case the original germplasm has a selective disadvantage compared to the contaminants. In particular, the six tetraploid plants detected among the parental plants were worrisome because investigation of the original seed lot of the accession by flow cytometry did not indicate that they originated from the accession. Additional data sources showed that contamination must have occurred in the preparatory phase of the regeneration, somewhere in between seed sowing and establishment of the study plot. Although no genetic data from other populations were available for confirmation, pollen contaminations were likely the result of pollen flow from other regeneration plots, while seed contaminants were probably due to post-harvest seed handling. Thus, the present study indicated that contamination may occur at various stages of the regeneration process, and can be considered more threatening to the genetic integrity of perennial ryegrass accessions than variation in pollination rates between plants.

Therefore, apart from considering modifications to reduce the variance in contributions by plants, regeneration procedures need to be carefully re-examined and extended with safety precautions to avoid mix-up of different germplasm.

Acknowledgements

The project on genetic diversity in traditional Dutch grasslands was carried out within the framework of the research program Agrobiodiversity, financed by the Netherlands Ministry of Agriculture, Nature and Food Quality. The study of mating patterns was carried out within the framework of a research project aiming at the optimisation of regeneration protocols for perennial European forage species (ICONFORS), which was funded by the European Union (QLRT-1999-30621).

References

Allard, R.W. (1992). Predictive methods for germplasm identification. In: H.T. Stalker & J.P. Murphy (eds.) Plant Breeding in the 1990s. CAB International, Wallingford, UK, 119-146.

Bretting, P.K. & M.P. Widrlechner (1995). Genetic markers and plant genetic resource management. *Plant Breeding Reviews*, 31, 11-86.

Brown, S.M. & S. Kresovich (1996). Molecular characterization for plant genetic resources conservation. In: H. Paterson (ed.) Genome Mapping of Plants. Academic Press, San Diego, 85-93.

Cresswell, A., N.R. Sackville Hamilton, A.K. Roy & B.M.F. Viegas (2001). Use of amplified fragment length polymorphism markers to assess genetic diversity of *Lolium* species from Portugal. *Molecular Ecology*, 10, 229-241.

Cunliffe, K.V., A.C. Vecchies, E.S. Jones, G.A. Kearney, J.W. Forster, G.C. Spangenberg & K.F. Smith (2004). Assessment of gene flow using tetraploid genotypes of perennial ryegrass (*Lolium perenne* L.). Australian Journal of Agricultural Research, 55, 389-396.

FAO (1996). FAO State of the World's Plant Genetic Resources for Food and Agriculture. Food and Agriculture Organisation of the United Nations, Rome.

Falconer, D.S. (1981). Introduction to Quantitative Genetics. Longman, London.

Forster, J.W., E.S. Jones, R. Kölliker, *et al*. (2001). DNA profiling in outbreeding forage species. In: R. Henry (ed.) Plant Genotyping – the DNA Fingerprinting of Plants. CABI Press, 299-320.

Fulton, T.M., J. Chunwongse, S.D. Tanksley (1995). Microprep protocol for extraction of DNA from tomato and other herbaceous plants. *Plant Molecular Biology Reporter*, 13, 207-209.

Giddings, G.D., N.R. Sackville Hamilton & M.D. Hayward (1997a). The release of genetically modified grasses. 1: Pollen dispersal to traps in *Lolium perenne*. *Theoretical and Applied Genetics*, 94, 1000-1006.

Giddings, G.D., N.R. Sackville Hamilton & M.D. Hayward (1997b). The release of genetically modified grasses. 2: The influence of wind direction on pollen dispersal. *Theoretical and Applied Genetics*, 94, 1007-1014.

Guthridge, K.M., M.P. Dupal, R. Kölliker, E.S. Jones, K.F. Smith & J.W. Forster (2001). AFLP analysis of genetic diversity within and between populations of perennial ryegrass (*Lolium perenne* L.). *Euphytica*, 122, 191-201.

Holmes, W. (1980). Grass, its Production and Utilization. Blackwell scientific publications, Oxford, UK.

Jarne, P. & P.J.L. Lagoda (1996). Microsatellites, from molecules to populations and back. *Trends in Ecology and Evolution*, 11, 424-429.

Johnson, R.C., V.L. Bradley & M.A. Evans (2002). Effective population size during grass germplasm seed regeneration. *Crop Science*, 42, 286-290.

Johnson, R.C., V.L. Bradley & M.A. Evans (2004). Inflorescence sampling improves effective population size of grasses. *Crop Science*, 44, 1450-1455.

Jones, E.S., M.P. Dupal, R. Kölliker, M.C. Drayton & J.W. Forster (2001). Development and characterisation of simple sequence repeat (SSR) markers for perennial ryegrass (*Lolium perenne* L.). *Theoretical and Applied Genetics*, 102, 405-415.

Karp, A., O. Seberg & M. Buiatti (1996). Molecular techniques in the assessment of botanical diversity. *Annals of Botany*, 78, 143-149.

Kubik, C., W.A. Meyer & B.S. Gaut (1999). Assessing the abundance and polymorphism of simple sequence repeats in perennial ryegrass. *Crop Science*, 39, 1136-1141.

Kubik, C., M. Sawkins, W.A. Meyer & B.S. Gaut (2001). Genetic diversity in seven perennial ryegrass (*lolium perenne* L.) cultivars based on SSR markers. *Crop Science*, 41, 1565-1572.

Loos, B.P. (1994). Morphological variation in Dutch perennial ryegrass (*Lolium perenne* L.) populations, in relation to environmental factors. *Euphytica*, 74, 97-107.
Marshall, D.R. & A.H.D. Brown (1975). Optimum sampling strategies in genetic conservation. In: O.H. Frankel & J.G. Hawkes (eds.) Genetic Resources for Today and Tomorrow. Cambridge University Press, Cambridge, UK.
Marum, P., I.D. Thomas & M. Veteläinen (1998). Summary of germplasm holdings. In: L. Maggioni, P. Marum, N.R. Sackville Hamilton, I. Thomas, T. Gass & E. Lipman (eds.) Report of a Working Group on Forages (Sixth Meeting, 6-8 March 1997, Beitostølen, Norway). European Co-operative Programme for Crop Genetic Resources Networks (ECP/GR). International Plant Genetic Resources Institute, Rome, Italy, 184-190.
Miller, M.P. (1997). Tools for population genetic analyses (TFPGA) 1.3: A Windows program for the analysis of allozym and molecular population genetic data. Computer software distributed by author.
Morgante, M. & A.M. Olivieri (1993). PCR-amplified microsatellites as markers in plant genetics. *The Plant Journal*, 3, 175-182.
Paetkau D, W. Calvert, I. Sterling, & C. Strobeck (1995). Microsatellite analysis of population structure in Canadian polar bears. *Molecular Ecology*, 4, 347-354.
Parker, P.G., A.A. Snow, M.D. Schug, G.C. Booton & P.A. Fuerst (1998). What molecules can tell us about populations: choosing and using a molecular marker. *Ecology*, 79, 361-382.
Roldán-Ruiz, I., J. Dendauw, E. van Bockstaele, A. Depicker & M. de Loose (2000). AFLP markers reveal high polymorphic rates in ryegrass (*Lolium* spp.). *Molecular Breeding*, 6, 125-134.
Sackville Hamilton, N.R. (1999). European *Lolium* and *Trifolium repens* Databases. In: L. Maggioni, P. Marum, N.R. Sackville Hamilton, M. Hulden & E. Lipman (eds.) Report of a Working Group on Forages (Seventh Meeting, 18-20 November 1999, Elvas, Portugal). European Co-operative Programme for Crop Genetic Resources Networks (ECP/GR). International Plant Genetic Resources Institute, Rome, Italy, 42-46.
Treuren, R. van, N. Bas, P.J. Goossens, H. Jansen & L.J.M. van Soest (in press). Genetic diversity in perennial ryegrass and white clover among old Dutch grasslands as compared to cultivars and nature reserves. *Molecular Ecology*.
Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper & M. Zabeau (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acid Research*, 23, 4407-4414.
Whitkus, R., J. Doebley & J.F. Wendel (1994). Nuclear DNA markers in systematics and evolution. In: R.L. Phillips & I.K. Vasil (eds.) DNA-based Markers in Plants (Advances in Cellular and Molecular Biology of Plants, Vol. 1). Kluwer Academic Publishers, Dordrecht, the Netherlands, 116-141.
Zeven, A.C., J.M.J. de Wet (1982). Dictionary of Cultivated Plants and their Regions of Diversity. Pudoc, Wageningen, the Netherlands.