AFLP fingerprinting of Kentucky bluegrass (*Poa pratensis* L.) from undisturbed Dutch grasslands: implications for conservation

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Summary

AFLP fingerprinting of Kentucky bluegrass (*Poa pratensis* L.) from undisturbed Dutch grasslands: implications for conservation

Undisturbed grasslands are considered rich sources of promising genotypes for the development of new varieties of Kentucky bluegrass (*Poa pratensis* L.). Grasslands that have not been resown with commercial cultivars nor treated with high doses of nitrogen fertilizer have become rare in the Netherlands. In 1998, a survey among farms still in agricultural use revealed the existence of about 50 such grasslands that were designated "old Dutch grasslands". AFLPs were used to study the genetic diversity among 194 plants from 12 old Dutch grasslands in comparison with 81 plants from undisturbed grasslands from 5 Dutch nature reserves and 220 plants from 11 reference cultivars that played an important role in the development of Dutch grasslands. Of the 275 samples from undisturbed grasslands, 151 plants (55%) displayed genotypes that could be matched with those of reference cultivars or were observed in multiple grasslands, suggesting a widespread occurrence of different genotypes. Based on the observed extent of overlap in genetic diversity and because grasslands from nature reserves are already under protective measures, no specific *in situ* conservation measures were recommended for old Dutch grasslands. However, from the group of grassland plants that could not be matched with the reference cultivars, 46 genotypes unique to single grasslands and 13 genotypes observed in multiple grasslands were maintained for *ex situ* conservation in order to extend the small genebank collection of Kentucky bluegrass in the Netherlands.

Résumé

Empreintes AFLP du paturin (*Poa pratensis* L.) de prairies hollandaises naturelles : implications pour la conservation

Les prairies à l'état naturel sont considérées comme des sources riches en génotypes prometteurs pour le développement de nouvelles variétés de paturin (*Poa pratensis* L.). Les prairies qui n'ont pas été réensemencées avec des cultivars commerciaux ni traitées avec des doses élevées de fertilisants azotés sont devenues rares aux Pays-Bas. En 1998, un recensement dans des exploitations agricoles toujours en activité a révélé l'existence d'environ 50 prairies de ce type qui ont été désignées « vieilles prairies hollandaises ». Des AFLP ont été utilisées pour étudier la diversité génétique parmi 194 plants de 12 vieilles prairies hollandaises. Elles ont été comparées à celles de 81 plants de prairies naturelles situées dans 5 réserves naturelles hollandaises et de 220 plants représentant 11 cultivars de référence qui ont joué un rôle important dans le développement des prairies hollandaises. Sur les 275 échantillons de prairies naturelles, 151 plants (55 %) présentent des génotypes reliés à ceux des cultivars de référence ou ont été observés dans de nombreuses prairies, suggérant une large distribution de génotypes différents. Aucune mesure spécifique de conservation *in situ* n'est préconisée pour les vieilles prairies hollandaises. Cependant, parmi le groupe de plants les plus éloignés des cultivars de référence, 46 génotypes trouvés dans des prairies uniques et 13 génotypes observés dans de nombreuses prairies ont été maintenus en conservation *ex situ* afin d'accroître la petite banque de gènes de *P. pratensis* aux Pays-Bas.

Resumen

Identificación mediante AFLP de poa de los prados (*Poa pratensis* L.) de praderas holandesas: implicaciones para su conservación

Las praderas intactas son ricas fuentes de prometedores genotipos para desarrollar nuevas variedades de poa de los prados (*Poa pratensis* L.). En los Países Bajos son pocas las praderas que no han sido sembradas con cultivares comerciales ni tratadas con dosis elevadas de fertilizantes nitrogenados. En 1998 un examen de granjas que seguían teniendo fines agrícolas reveló la existencia de unas 50 praderas designadas como "antiguas praderas holandesas". Mediante análisis AFLP se comparó la diversidad genética entre 194 plantas de 12 antiguas praderas holandesas, con 81 plantas provenientes de praderas intactas de las reservas naturales y 220 plantas de 11 cultivares de referencia importantes para el desarrollo de las praderas. De las 275 muestras de las praderas intactas, 151 (55%) tenían genotipos equiparables con los de los cultivares de referencia o que aparecían en numerosas praderas, indicando una presencia muy difundida de diferentes genotipos. No se recomendaron medidas específicas de conservación *in situ* para las antiguas praderas holandesas dado el gran asolapado de la diversidad genética y que las praderas de las reservas naturales están ya protegidas. No obstante, del grupo de plantas que no podían equipararse con los cultivares de referencia se conservaron *ex situ* 46 genotipos exclusivos de determinadas praderas y 13 genotipos de praderas múltiples para ampliar la pequeña colección de bancos de genes de poa de los prados en los Países Bajos.

Key words: AFLP, conservation, Dutch grasslands, genetic resources, genetic diversity, *Poa pratensis*

Introduction

Kentucky bluegrass (*Poa pratensis* L.) is an important agronomic crop species that is used as a fodder crop and a turf grass. It is a facultative apomictic species that, although the degree of sexuality may vary considerably, reproduces mainly through apomixis (Huff and Bara 1993; Mazzucato 1995b). Consequently, progenies are usually genotypically identical to the parental plant. Local adaptation to environmental variation in combination with the apomictic

breeding behaviour has resulted in the appearance of many unique genotypes (Burt and Christians 1990). Morphological and molecular characterization studies have supported the existence of a wide range of genetic variability within Kentucky bluegrass (Johnson et al. 2002; Curley and Jung 2004). In the development of new varieties, old fields and natural populations have often been rich sources of promising genotypes (Pepin and Funk 1971; Bonos et al. 2000).

The Netherlands is situated in the north-west European part of the European-Siberian region of diversity. A number of temperate grasses and legumes have their centre of diversity within this region, including Kentucky bluegrass (Zeven and de Wet 1982). However, due to the replacement of original diversity with more uniform cultivars and the application of high doses of nitrogen fertilizer, the biodiversity has been strongly reduced within temperate grasslands in the Netherlands. Consequently, undisturbed grasslands have become rare in the Netherlands, but about 50 of such grasslands, designated "old Dutch grasslands", were identified in 1998 during an extensive survey among farms still in agricultural use (van Soest et al. 2005). Due to random processes or adaptation to local environmental conditions, or both, natural populations are expected to show genetic differentiation that will be maintained over time in the absence of gene flow (Allendorf 1983; Loveless and Hamrick 1984). In the present study, the genetic diversity of Kentucky bluegrass from old Dutch grasslands is investigated with AFLPs and compared to that of undisturbed grasslands from nature reserves and widely used cultivars in the Netherlands. The main goal of the study was to support policy decisions concerning the conservation of Kentucky bluegrass from undisturbed grasslands in the Netherlands.

Material and methods

Study material

In a previous study with perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.), population sampling was carried out in 2000 in extensively-managed grasslands, including a group of 16 old Dutch grasslands (denoted A to P) in different geographical areas and with varying soil types in the Netherlands, and a group of 7 nature reserves (denoted R to X) covering a similar geographical range (van Treuren et al. 2005) (Figure 1). In 2005, all 23 grasslands were revisited to collect Kentucky bluegrass plants for the present study. Locations F, H, I and J no longer existed as old Dutch grassland, while no Kentucky bluegrass was observed within the grasslands from nature reserves T and U. Plants collected from the remaining 17 grasslands were potted in separate, medium-sized containers in an outdoor greenhouse, awaiting taxonomic verification and sampling of young leaf tissue for DNA isolation. The aim was to study 20 samples from each grassland, but due to low abundance in some grasslands and incorrect taxonomic status of collected samples, an average of 16.2 Kentucky bluegrass plants were obtained per grassland, resulting in a total of 275 grassland samples.

In addition to the grassland samples, 20 plants from each of 11 reference cultivars were included in the study (Table 1). This reference group comprised 50% of the cultivars that had appeared in the Dutch variety lists since 1935. Of the more 'recent' varieties, no seeds could be obtained of cvs 'Norma' ('Otöfte'), 'Steinacher' and 'Entensa'. The number of years that cultivars were included in Dutch variety lists is indicative of their importance in the cultivation of Dutch grasslands. To date, the cultivars 'Monopoly', 'Julia' and 'Tommy' are still part of the Dutch variety list. Seeds of the reference cultivars were obtained from the collection of grasses of the Centre for Genetic Resources, the Netherlands (CGN). Seedlings from each of the cultivars were subjected to the same procedures as the grassland samples.

Molecular analysis

About 100 mg of leaf material was vacuum freeze-dried and ground into a fine powder, after which total genomic DNA was extracted using a combination of the methods described by Fulton et al. (1995) and the DNeasy 96 Plant Kit (Qiagen, Westburg, the Netherlands). AFLP analyses basically followed the procedures described by Vos et al. (1995). PCR products radio-labelled with P^{33} were separated by polyacrylamide gel-electrophoresis (PAGE). Previous AFLP analysis in Kentucky bluegrass revealed clear and reproducible fingerprinting profiles using *Eco*RI and *Mse*I primer combinations, each having three selective nucleotides (Barcaccia et al. 1998).

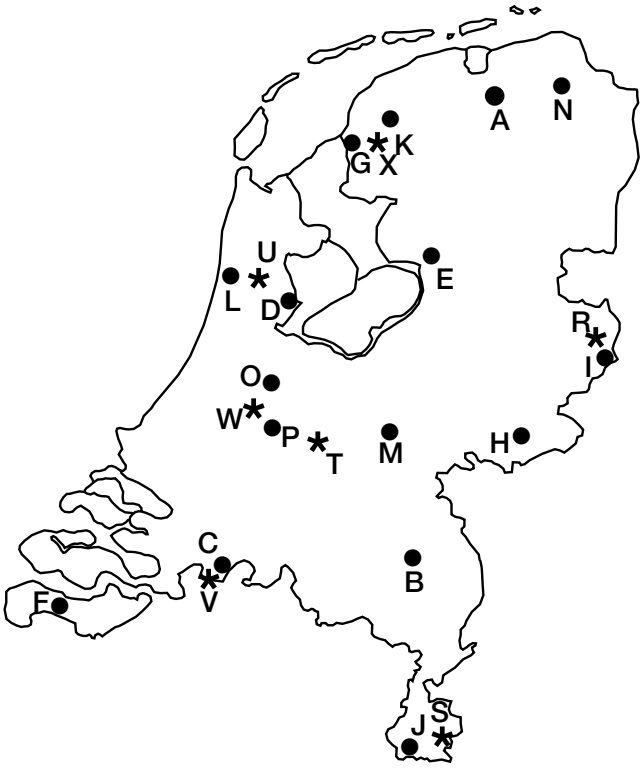

Figure 1. Geographical distribution of the selected old Dutch grasslands (A to P) and nature reserves (R to X). Figure from van Treuren et al. (2005).

Table 1. Reference cultivars used in the present study. In the column 'Period', the years are presented during which the varieties were included in Dutch variety lists.

Variety	Variety name	Breeding company	Period
1	Delft	Cebeco	1958–1987
2	Monopoly	Mommersteeg	1971–present
3	Aquila	van der Have	1976–1989
4	Ampellia	Cebeco	1984–1995
5	Julia	Petersen Saatzucht	1987–present
6	Asset	van der Have	1987–2001
7	Tommy	Green Genetics B.V.	1991–present
8	Barvictor	Barenbrug	1992–2003
9	Prato	van der Have	1964–1973
10	Arista	van Engelen	1965–1973
11	Entopper	van Engelen	1979–1998

In the present study, the reproducibility of AFLP profiles, density of AFLP bands and the ability to score AFLP variation unambiguously was investigated for 20 *Eco*RI/*Mse*I primer pairs, using a test panel of 4 plants (in duplo) from different cultivars. Following this pre-screening, all plants were analysed for *Eco*RI primer E32 (selective nucleotides: AAC) in combination with *Mse*I primer M56 (CGC) and for primer E37 (ACG) combined with M52 (CCC).

Data analysis

Autoradiograms were manually scored for the presence or absence of AFLP fragments in the range of 50–500 base pairs. Jaccard's similarity value was used to express genetic relationships between plants, which were visualized by a cluster analysis using the UPGMA (unweighted pair-group method, arithmetic average) method and by Principal Coordinates (PCO). To quantify genotypic variation, plants showing a similarity value ≥0.95 were considered genotypically similar, corresponding to different marker scores for 0–2 AFLP bands. A threshold value for similarity was used to compensate for a minor level of potential experimental error in discriminating genotypically different samples (*vide* Arens et al. 1998). Data analyses were carried out using the software packages Genstat (release 8.11) and NTSYS-pc (Rolf 1993). Assignment tests following the methods described by Paetkau et al. (1995) were used to investigate to what extent the AFLP data are able to discriminate between populations and between varieties. For this purpose, a custom-designed computer program was used.

Results

Among the total sample of 495 plants, 165 different AFLP bands were scored, of which 156 (95%) were found to be polymorphic. Based on the AFLP fingerprints of the plants, 112 different genotypes (23%) were observed among the total sample. Genotypic variation within the grasslands and varieties varied markedly between the populations, ranging from homogeneity (e.g. reference cvs. 'Ampellia' and 'Julia') to 55% heterogeneity in grassland A (Table 2). No apparent differences in the range of the number of genotypes per population were observed between the group of old Dutch grasslands, nature reserves and reference cultivars. Nearly all populations consisted of low-frequency genotypes and a single or few common genotypes. This was most pronounced for the reference cultivars that all consisted of a single predominant genotype, their frequencies ranging from 0.55 to 1.00 (Table 2). Based on the observed range of similarity values within populations, the genotypes within reference cultivars were generally more closely related to each other than those within grassland populations (Table 2). Lower levels of intra-population variation within the reference cultivars as compared to the grassland populations were also observed for individual AFLP markers as the average fraction of variable AFLP bands per population was 0.39, 0.34 and 0.13 for the old Dutch grasslands, nature reserves and reference cultivars, respectively (results not shown).

An assignment test carried out within the total sample showed that the percentage of plants correctly assigned to the population of origin was 64% for plants from old Dutch grasslands and 46% for plants from nature reserves, indicating low genetic differentiation between the grassland populations. For plants from the reference cultivars this figure was 98%, while all 220 samples were correctly assigned to their own population when the test was restricted to the group of reference cultivars. The high genetic identity of the reference cultivars was also revealed by a UPGMA cluster analysis, grouping all samples from each cultivar together in 11 separate clusters (Figure 2). No clear separation of populations was observed when a UPGMA cluster analysis was performed for the grassland samples (results not shown).

The lack of differentiation between the grassland populations was in line with the considerable degree of overlap in genotypic variation that was observed both among the grasslands and between the grasslands and reference cultivars (Table 3). Of the 275 grassland plants, 34

Table 2. Genotypic variation observed within the old Dutch grasslands (A–P), nature reserves (R–X) and reference cultivars.

Sample	Sample size	Number of genotypes	Frequency of the common genotype	Range of Jaccard similarity values
A	20	11	0.20	0.39–1.00
B	18	3	0.44	0.41–1.00
C	20	7	0.60	0.41–1.00
D	19	4	0.47	0.46–1.00
E	19	8	0.42	0.39–1.00
G	19	5	0.68	0.48–1.00
K	20	3	0.55	0.54–1.00
L	20	6	0.45	0.47–1.00
M	20	10	0.20	0.38–1.00
N	14	7	0.29	0.50–1.00
O	2	1	1.00	1.00
P	3	2	0.67	0.63–1.00
R	18	3	0.83	0.61–1.00
S	20	3	0.75	0.35–1.00
V	20	6	0.45	0.41–1.00
W	3	1	1.00	1.00
X	20	9	0.25	0.45–1.00
Delft	20	8	0.65	0.53–1.00
Monopoly	20	4	0.85	0.69–1.00
Aquila	20	6	0.75	0.65–1.00
Ampellia	20	1	1.00	0.95–1.00
Julia	20	1	1.00	0.98–1.00
Asset	20	10	0.55	0.58–1.00
Tommy	20	3	0.90	0.67–1.00
Barvictor	20	10	0.55	0.56–1.00
Prato	20	3	0.90	0.73–1.00
Arista	20	3	0.90	0.61–1.00
Entopper	20	4	0.80	0.86–1.00

samples (12%) could be ascribed to five different reference cultivars. In all these cases, the predominant genotype of the cultivars was involved. Cv. 'Monopoly' was even observed in six different grasslands. Overlap in genotypic constitution between grasslands also occurred without the presence of reference cultivars, as in the case of a genotype that was identified in grasslands A, D, G and O involving a total of 25 plants (Table 3). No apparent relationship with population characteristics or geographic distribution was observed for the grasslands that shared genotypes. Identical genotypes were even observed between grasslands that are separated by more than 200 km, as in the case of grasslands C and X, located in respectively the south and north of the Netherlands (Figure 1). The 18 shared genotypes constituted 28% of the 64 different genotypes observed within the grasslands, together comprising 151 (55%) of the 275 grassland samples (Table 3). Of the 59 different grassland genotypes that did not match any of the reference cultivars, 38 from old Dutch grasslands and 8 from nature reserves were unique to a single grassland, while 13 were found in multiple grasslands. The grassland of nature reserve S was found to be a single population consisting of genotypes that were absent from both the reference cultivars and other grasslands.

As could be expected, considering the observed overlap in genotypic variation between the samples, PCO analysis of the total sample did not indicate any distinction between the gene pools of the three groups investigated (results not shown). A similar result was obtained when the analysis was restricted to the samples from the reference cultivars and the samples that were unique to a single grassland (Figure 3). Only 20% of the total observed variation was explained by the first two principal axes, indicating that many independent markers contribute to the genetic structure among the populations and that no clear population differentiation can be observed from the data. Apart from the reference cultivars, no clustering of genotypes from the same population was observed, nor was

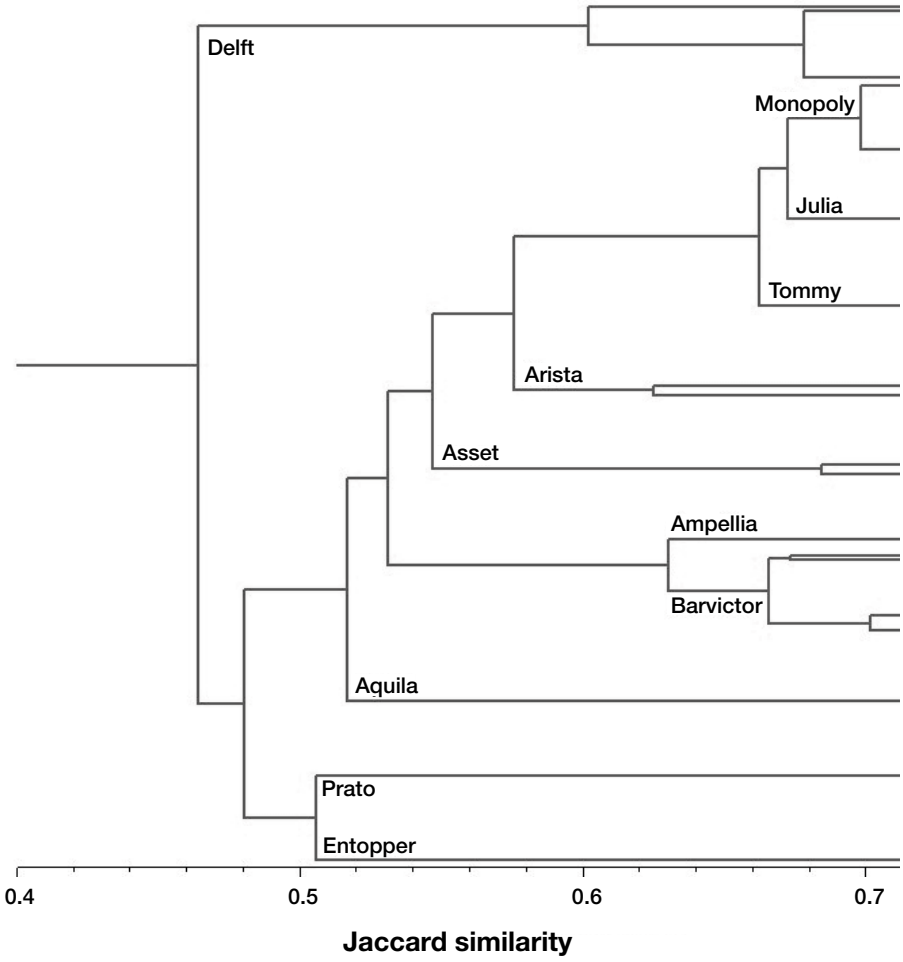

Figure 2. UPGMA cluster analysis of the 220 samples from the 11 reference cultivars. Because of the large sample size, the dendrogram is cut off at Jaccard similarity value 0.72. Eleven clusters can be distinguished that fully corresponded to the 11 cultivars involved. The names of the cultivars are denoted at the branching point of the clusters.

Table 3. Overview of the 18 genotypes that were observed in multiple populations. Each table row contains a single genotype. The grasslands in which the genotypes were observed are denoted by the sample code. The number of plants involved is given between parentheses.

Old Dutch grasslands		Nature reserves		Reference cultivars		Total number of plants involved
B	(6)	V	(1)	Entopper	(16)	23
E, G, L, M	(8)	V, X	(13)	Monopoly	(17)	38
B, L	(5)	R, V	(17)			22
C	(12)	V	(1)			13
C	(1)	X	(2)			3
D, G, P	(5)	W	(3)			8
E	(3)	X	(5)			8
K	(11)	X	(2)			13
K	(4)	X	(2)			6
M	(1)	X	(1)			2
E	(3)			Delft	(12)	15
A, D, G, O	(25)					25
A, N	(4)					4
B, M	(9)					9
E, L	(2)					2
L, N	(2)					2
		V	(1)	Aquila	(15)	16
		R	(2)	Julia	(20)	22
	(101)		(50)		(80)	231

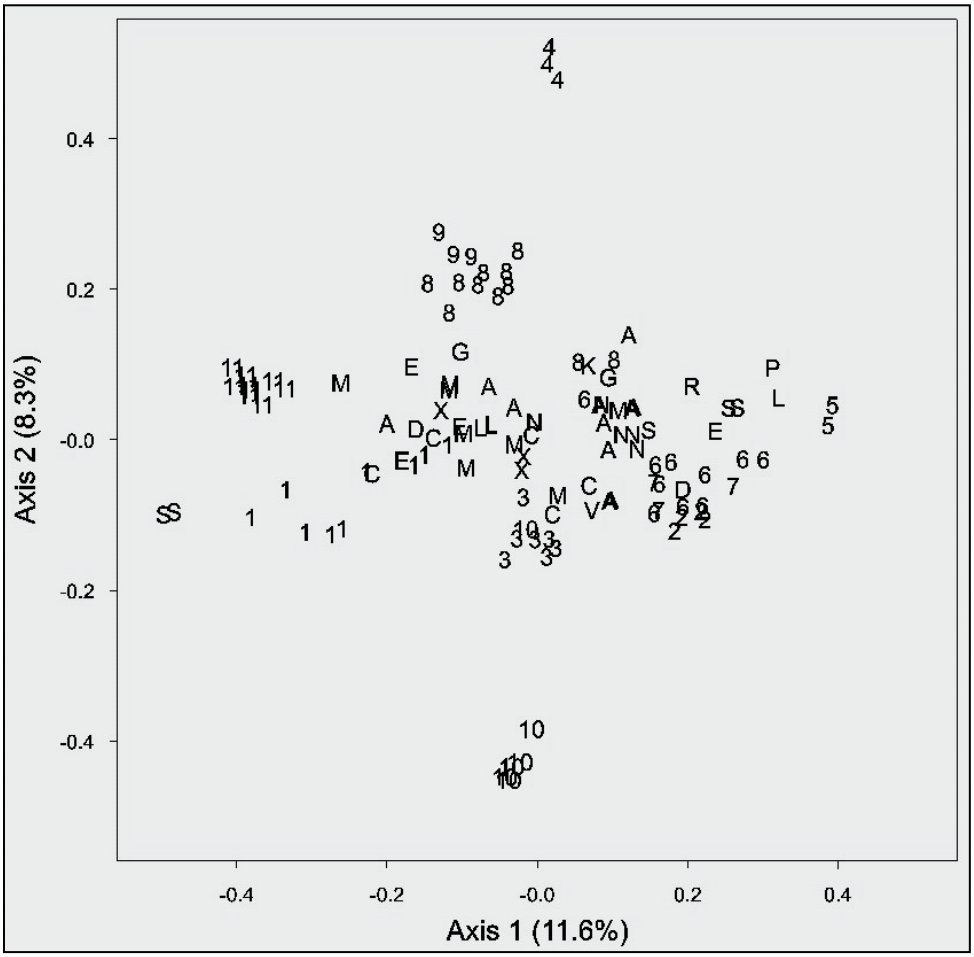

Figure 3. Principal coordinate plot of the samples, excluding grassland genotypes that could be matched with reference cultivars or were observed in multiple grasslands. Sample from old Dutch grasslands are denoted by A–P, those from the nature reserves by R–X and those from the reference cultivars by 1–11. The percentage of variation explained by the axes is presented in parentheses in the axis legend.

any relationship revealed between the clustering pattern and geographical distribution. Thus, even after removing the obvious overlap between populations, still no separation between the gene pools of the three groups was observed, nor did any of the old Dutch grasslands show highly differentiated genotypes.

Discussion

Diversity within varieties varied considerably between the reference cultivars, ranging from a single to ten different genotypes among the 20 samples studied per cultivar. In quantifying genotypic variation, plants showing a similarity value ≥0.95 were considered genotypically similar, corresponding to only 0–2 polymorphisms. Such small differences between genotypes were occasionally observed both in reference cultivars and in grasslands, and all these cases concerned minor deviations from the predominant genotype. These minor differences may have resulted from experimental error or mutations. In the latter case, the genotypes have to be regarded as strongly related rather than similar. Sexuality probably underlaid the larger genotypic differences observed within the reference cultivars. The proportion of apomictic seeds produced is known to vary considerably between different cultivars of Kentucky bluegrass (Mazzucato 1995b; Porceddu et al. 2002). High levels of sexual reproduction were also considered a probable cause of the high within-cultivar genetic variability based on RAPD analysis of Kentucky bluegrass varieties (Curley and Jung 2004). Other causes of within-cultivar diversity may include gene flow between populations during the simultaneous regeneration of genebank accessions (Sackville Hamilton and Chorlton 1997; van Treuren et al. 2006). The fact that sexuality in Kentucky bluegrass may include both self- and cross-fertilization (Huff and Bara 1993) and the fact that at CGN no specific protective measures are practised to avoid gene flow between Kentucky bluegrass populations during regeneration potentially offer the opportunity for contamination. Although the possibility of contamination cannot be ruled out completely, the high genetic identity observed for the reference cultivars does not seem to support the presence of contaminants within the reference cultivars.

When an assignment test was performed with the 220 cultivar samples, all plants were correctly assigned to the population of origin. Therefore, AFLP fingerprinting may offer a potential value for variety discrimination and registration (e.g. Lombard et al. 2000). RAPD markers have also been found useful to discriminate between multiple Kentucky bluegrass cultivars (Huff 2001). RAPD analysis is quite easy and quick to perform, with relatively low cost,

whereas AFLP analysis is more laborious and hence more costly. However, AFLPs offer the advantage of generating considerably more polymorphic bands per assay and do not face the reproducibility problems often encountered with fingerprinting profiles based on RAPDs (Jones et al. 1997).

To maximize the probability of sampling genetic diversity as widely as possible, the grasslands investigated were selected based on a wide geographical distribution and a large variation in soil type (van Treuren et al. 2005). In spite of this effort, a large degree of overlap in genetic diversity was observed between the grassland populations, and in many cases grassland plants could be matched with reference cultivars. Considering the information obtained from the managers or owners of the grasslands, namely that no re-sowing with commercial varieties had occurred for at least the last 40 years, the presence of cultivars in the grasslands may either indicate that these cultivars may once have been collected from Dutch grasslands as promising genotypes for variety development, or that unconscious gene flow has occurred. Apart from the cultivars 'Delft' (selection from a Danish variety), 'Julia' (German ecotype) and 'Asset' (American origin), the reference cultivars investigated were Dutch ecotypes. For a long time, ecotype breeding has been the predominant method of variety development in Kentucky bluegrass. It has been argued that genotypes with desired characteristics are widely distributed in Europe and North America because they appeared regularly in ecotype collections (Duyvendak and Luesink 1979). For example, the cultivar 'Parade' has been collected as an ecotype from a grassland located in the Dutch province Zeeuws-Vlaanderen. Before its release as a registered cultivar, ecotypes that could not be distinguished from 'Parade' were collected by several breeding companies from various geographical regions in the Netherlands (Arnd-Jan van Wijk, pers. comm.). Given the apomictic reproductive behaviour of Kentucky bluegrass, the persistence of specific genotypes in natural populations is not unlikely. However, it cannot be ruled out completely that unconscious gene flow from cultivated pastures may have contributed to the observation of reference cultivars in grasslands. All old Dutch grasslands investigated have cultivated pastures in their close vicinity, which offers the opportunity for seed transport between these sites. Plant introductions by human activities have been considered an important factor affecting the distribution of Kentucky bluegrass and compromising origin data (e.g. Johnson et al. 2002). Gene flow is known to have a strong homogenizing effect on genetic diversity between populations (Falconer 1981).

Implications for conservation

Compared to the storage of seed samples in genebanks, *in situ* conservation offers the advantage that basically the entire grassland ecosystem is conserved, including the sometimes complex interactions among the constituent species. Furthermore, genetic diversity may be maintained due to specific management regimes. Investigation of the prospects for Dutch grasslands indicated that their maintenance is highly threatened in the event of the farm being sold or if the owners are succeeded by relatives (Janssens et al. 2002). This was confirmed by the present study, as between 2000 and 2005, 4 out of 16 old Dutch grasslands were given another use by the new owners. Two locations were now used for growing maize, while in two cases the grassland was ploughed for unknown purposes. However, considering the observed overlap in genetic diversity between undisturbed grasslands and the fact that grasslands from nature reserves are already under protective measures, no specific *in situ* conservation measures were recommended for old Dutch grasslands. A similar conclusion was reached in a previous study with the outcrossing species perennial ryegrass and white clover (van Treuren et al. 2005). Since grasslands in nature reserves are also managed extensively and because only a subset of the Dutch grassland reserves were investigated, the major part of the diversity observed in old Dutch grasslands is expected to be maintained in Dutch grassland reserves.

Ex situ conservation offers the advantage that genetic resources are more accessible to the user community. In the present study, 59 genotypes could not be matched with reference cultivars, of which 46 were unique to a single grassland. Thus far, these genotypes have not been evaluated for agro-morphological characteristics, but genetic diversity estimated by marker analysis has been shown to correlate well with morphology-based variation patterns in Kentucky bluegrass (Mazzucato 1995a; Huff 2001; Johnson et al. 2002; Curley and Jung 2004). Because of current under-representation, it has been suggested to extend genebank collections of Kentucky bluegrass with material from wild or naturalized populations (Johnson et al. 2002). Also CGN's small collection of 52 Kentucky bluegrass accessions currently comprises only cultivated material. Therefore, 59 grassland genotypes were maintained for *ex situ* conservation. Whether these genotypes represent existing varieties not included in the reference panel or novel material with a potential use value remains to be determined.

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References

Allendorf FW. 1983. Isolation, gene flow, and genetic differentiation among populations. *In:* CS Schonewald-Cox, SM Chambers, B MacBryde and L Thomas (editors). Genetics and Conservation: a Reference for Managing Wild Animal and Plant Populations. Benjamin-Cummings, London, UK. pp. 51–65.

Arens P, Coops H, Jansen J, Vosman B. 1998. Molecular genetic analysis of black poplar (*Populus nigra* L.) along Dutch rivers. Molecular Ecology 7: 11–18.

Barcaccia G, Mazzucato A, Albertini E, Zethof J, Gerats A, Pezotti M, Falcinelli M. 1998. Inheritance of parthenogenesis in *Poa pratensis* L.: auxin test and AFLP linkage analyses support monogenic control. Theoretical and Applied Genetics 97: 74–82.

Bonos SA, Meyer WA, Murphy JA. 2000. Classification of Kentcky bluegrass genotypes grown as spaced-plants. American Society for Horticultural Science 35: 910–913.

Burt MG, Christians NE. 1990. Morphological and growth characteristics of low- and high-maintenance Kentucky bluegrass cultivars. Crop Science 30: 1239–1243.

Curley J, Jung G. 2004. RAPD-based genetic relationships in Kentuckey bluegrass: comparison of cultivars, interspecific hybrids and plant introductions. Crop Science 44: 1299–1306.

Duyvendak R, Luesink B. 1979. Preservation of genetic resources in grasses. *In:* AC Zeven and AM van Harten (editors). Proceedings of the Conference Broadening the Genetic Base of Crops. Pudoc, Wageningen, the Netherlands. pp. 67–73.

Falconer DS. 1981. Introduction to Quantitative Genetics. (2nd edition). Longman, London, UK.

Fulton TM, Chunwongse J, Tanksley SD. 1995. Microprep protocol for extraction of DNA from tomato and other herbaceous plants. Plant Molecular Biology Reporter 13: 207–209.

Huff DR, Bara JM. 1993. Determining genetic origins of aberrant progeny from facultative apomictic Kentucky bluegrass using a combination of flow cytometry and silver-stained RAPD markers. Theoretical and Applied Genetics 87: 201–208.

Huff DR. 2001. Characterization of Kentucky bluegrass cultivars using RAPD markers. International Turfgrass Society Research Journal 9: 169–175.

Janssens B, de Savornin Lohman AF, van Soest LJM, de Zwijger-de Brabander C. 2002. Oude Graslanden in Nederland: Verkenning naar Motieven, Bedrijfsvoering en Perspectieven voor *in situ* Beheer. Report 3.02.04. LEI, Den Haag, The Netherlands.

Johnson RC, Johnston WJ, Golob CT, Nelson MC, Soreng RJ. 2002. Characterization of the USDA *Poa pratensis* collection using RAPD markers and agronomic descriptors. Genetic Resources and Crop Evolution 49: 349–361.

Jones CJ, Edwards KJ, Castaglione S, Winfield MO, Sala F, van de Wiel C, et al. 1997. Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. Molecular Breeding 3: 381–390.

Lombard V, Baril CP, Dubreuil P, Blouet F, Zhang D. 2000. Genetic relationships and fingerprinting of rapeseed cultivars by AFLP: consequences for varietal registration. Crop Science 40: 1417–1425.

Loveless MD, Hamrick JL. 1984. Ecological determinants of genetic structure in plant populations. Annual Review of Ecology and Systematics 15: 65–95.

Mazzucato A. 1995a. Italian germplasm of *Poa pratensis* L. I. Variability and mode of reproduction. Journal of Genetics and Breeding 49: 111–118.

Mazzucato A. 1995b. Italian germplasm of *Poa pratensis* L. II. Isozyme progeny test to characterize genotypes for their mode of reproduction. Journal of Genetics and Breeding 49: 119–126.

Paetkau D, Calvert W, Sterling I, Strobeck C. 1995. Microsatellite analysis of population structure in Canadian polar bears. Molecular Ecology 4: 347–354.

Pepin GW, Funk CR. 1971. Intraspecific hybridization as a method of breeding Kentucky bluegrass *Poa pratensis* for turf. Crop Science 11: 445–448.

Porceddu A, Albertini E, Barcaccia G, Falistocco E, Falcinelli M. 2002. Linkage mapping in apomictic and sexual Kentucky bluegrass (*Poa pratensis* L.) genotypes using a two-way pseudo-testcross strategy based on AFLP and SAMPL markers. Theoretical and Applied Genetics 104: 273–280.

Rolf FJ. 1993. NTSYS-pc Numerical Taxonomy and Multivariate Analysis System, Version 1.8. Exeter Software, Setauket, New York, USA.

Sackville Hamilton NR, Chorlton KH. 1997. Regeneration of accessions in seed collections: a decision guide. Handbook for Genebanks No. 5. International Plant Genetic Resources Institute, Rome, Italy.

van Soest LJM, Bas N, van Treuren R. 2005. Genetic resources activities on forages in the Netherlands. *In:* B Boller, E Willner, L Maggioni and E Lipman (editors). Report of a Working Group on Forages. Eighth meeting. Linz, Austria, 10–12 April 2003. International Plant Genetic Resources Institute, Rome, Italy. pp. 70–74.

van Treuren R, Bas N, Goossens PJ, Jansen H, van Soest LJM. 2005. Genetic diversity in perennial ryegrass and white clover among old Dutch grasslands as compared to cultivars and nature reserves. Molecular Ecology 14: 39–52.

van Treuren R, Goossens PJ, Ševčíková M. 2006. Variation in effective pollination rates in relation to the spatial and temporal distribution of pollen release in rejuvenated perennial ryegrass. Euphytica 147: 367–382.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, et al. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23: 4407–4414.

Zeven AC, de Wet JMJ. 1982. Dictionary of Cultivated Plants and their Regions of Diversity. Pudoc, Wageningen, the Netherlands.