Euphytica (2006) 147: 367–382
DOI: 10.1007/s10681-005-9033-z


# Variation in effective pollination rates in relation to the spatial and temporal distribution of pollen release in rejuvenated perennial ryegrass

R. van Treuren[1,*], P.J. Goossens[1] & M. Ševčíková[2]
[1]*Centre for Genetic Resources, the Netherlands, Wageningen University and Research Centre, P.O. Box 16, 6700 AA Wageningen, the Netherlands;* [2]*OSEVA PRO Ltd. Grassland Research Station Rožnov-Zubří,, Hamerská 698, 756 54 Zubří, Czech Republic (*author for correspondence: e-mail: Robbert.vanTreuren@WUR.NL)*

Received 13 July 2005; accepted 7 October 2005

*Key words:* genetic integrity, paternity analysis, perennial ryegrass, phenology, regeneration, spatial arrangement

**Summary**

Genebank accessions stored as seed populations require periodic rejuvenation in order to maintain sufficient numbers of viable seeds. During rejuvenation the genetic composition of accessions may be altered for a variety of reasons, of which variation in pollination rates between plants is the least understood. In the present study, a paternity exclusion analysis was performed on a rejuvenated accession of perennial ryegrass. In addition, flowering data of the 49 parental plants were collected during the flowering season. The aim of the study was to determine how accurate variation in pollination rates between plants can be predicted from data on the spatial and temporal distribution of pollen release. The parental population and a total of 551 offspring from 12 progeny arrays were genotyped by means of molecular analysis. Using 25 microsatellites, paternity was identified for 81.9% of the offspring, while remaining ambiguities were resolved by AFLP analysis, except in four cases. Within the total sample 9 cases of contamination were observed. Mating within the study population was clearly non-random, as 61.9% of the identified pollen donors were located within 1 m distance from the mother plant. Observed pollination rates were very well described by an inverse quadratic function of inter-plant distance between potential mating pairs. Incorporation of the recorded flowering data in the calculation of expected pollination rates improved the goodness of fit with observed values by only 0.77%. Suggestions to reduce the variance in paternal contributions were presented. However, contamination was considered more threatening to the genetic integrity of perennial ryegrass germplasm than variation in pollination rates between plants, and indicated the need for improved measures to avoid gene flow from other germplasm.

## Introduction

Perennial ryegrass (*Lolium perenne* L.) is considered a major fodder crop and as the major species in cultivated grasslands in temperate climate zones (Holmes, 1980). Perennial ryegrass has its primary centre of diversity in the European-Siberian region (Zeven & de Wet, 1982). However, during the last few decades, optimisation of fodder production has strongly reduced the biodiversity present in temperate grasslands. Therefore, germplasm collections were developed by genebanks in order to conserve genetic resources for present and future use. It has been estimated that about 100,000 accessions of European forage species are conserved in European genebanks, of which 20,000 are currently in urgent need of rejuvenation (Marum et al., 1998).

As a result of loss of germinability through ageing and depletion of stocks through distribution, genetic resources conserved as seed populations require periodic rejuvenation through a cycle of seed multiplication. One of the objectives of genebanks is to maintain the genetic composition of accessions as much as possible. However, each time a heterogeneous accession is rejuvenated the genetic integrity of the accession may be

compromised by genetic drift, inbreeding, selection, and gene flow from other germplasm. The potential for genetic change is particularly relevant to species that have highly heterogeneous populations, such as the wind-pollinated, outcrossing species perennial ryegrass (Forster et al., 2001; Guthridge et al., 2001).

Genetic integrity of accessions may be lost due to variation in seed production between plants. It has been reported that 40% of the plants in a regeneration plot may give rise to 95% of the seeds produced (Sackville Hamilton, 1998). Single-plant harvesting and the composition of balanced bulks have been suggested to avoid loss of genetic integrity through variation in seed production (e.g. Johnson et al., 2002, 2004). However, this procedure does not compensate for loss of genetic integrity through differential pollination rates between plants. Equalising paternal contributions to the seed population would require manual cross-pollination of plants in specific combinations, but the procedures are not considered particularly useful to small-seeded species like perennial ryegrass because of the laborious work involved. Other factors during rejuvenation that may influence the genetic integrity of accessions include mutation, contamination by gene flow from plants that do not belong to the rejuvenated accession and genetic drift and inbreeding due to small numbers of parental plants used.

Of the factors that may affect the genetic integrity of accessions during rejuvenation, variation in pollination rates within populations is the least understood. Spatial proximity, temporal overlap in flowering periods and pollen production may be considered the three primary components affecting pollination rates between plants. Pollination studies in perennial ryegrass have mainly focused on pollen deposition rates in relation to distance, which generally show a leptokurtotic distribution of dispersal (e.g. Giddings et al., 1997a,b; Cunliffe et al., 2004). However, data on variation in pollination rates and on the relative importance of the population characteristics underlying this variation are currently lacking.

Pollination rates in populations can be studied by performing a paternity exclusion analysis using molecular markers, such as microsatellites (e.g. Lexer et al., 1999). Microsatellites are codominant markers that usually display high levels of allelic diversity, which make them particularly useful to population genetic studies (Queller et al., 1993; Jarne & Lagoda, 1996). Drawback of microsatellites is that sequence information is needed for marker development. However, microsatellite markers for perennial ryegrass are already in the public domain (Kubik et al., 1999, 2001, Jones et al., 2001).

In the present study a paternity exclusion analysis using microsatellites was performed on a rejuvenated perennial ryegrass accession. In addition, flowering data of individual plants were collected during the flowering season. The aim of the study was to investigate how accurately variation in pollination rates can be predicted from observations on the spatial and temporal distribution of pollen release.

## Materials and methods

### *Field experiment*

#### *Study population*

In 2001, a population of 49 plants of perennial ryegrass accession BA 11894 (Institute of Grassland & Environmental Research (IGER), Aberystwyth, UK) was established in an experimental field at the Grassland Research Station Rožnov-Zubří (Czech Republic) located at an altitude of 360 m a.s.l. with an average annual rainfall of 903 mm and an annual average temperature of 7.6 °C. This accession had been collected in 1992 from an unimproved grassland located in Bohemia (Czech Republic). The 49 plants were received as vegetative cuttings, propagated from seed by IGER. The plants were randomly arranged in a $7 \times 7$ matrix with 50 cm distance between plants (Figure 1). The distance between different perennial ryegrass populations was at least 30 m, with *xTriticosecale* used as barrier crop. Populations were fenced with chicken wire to avoid damage by hares and deer.

#### *Field data collection*

In 2002, the field study was conducted. Flowering data for each of the 49 plants were collected on a daily basis during the flowering season. For each plant the flowering period was recorded, together with the daily number of inflorescences with open flowers that was used as an estimate for daily pollen production. Meteorological data were recorded on a daily basis during the flowering season in order to obtain an additional set of potential influential parameters that could be included in the modelling of expected variation in pollination rates. These included wind direction and speed during anthesis, which occurred usually between 10.00 a.m. and 5.00 p.m., measured at 5-minute intervals. Seeds were collected from each of the 49 plants from July 2 to August 1, depending on the optimal harvesting date per

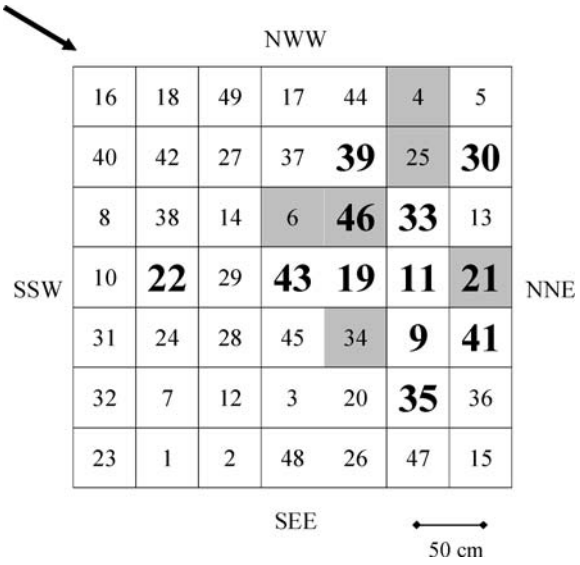



*Figure 1.* Spatial arrangement of the 49 plants used to rejuvenate perennial ryegrass accession BA 11894. Inter-plant distance within rows was 50 cm. The orientation of the plot is indicated at the borders of the figure, together with the prevailing wind direction (SWW, denoted by arrow). Six plants that appeared to be tetraploid are presented in gray shade. The 12 plants whose progeny were used for paternity analysis are indicated in large font.

plant. Seeds were trashed, cleaned and dried according to standard procedures practised at the Grassland Research Station Rožnov-Zubří.

*Selection of progenies*

The statistical power of analysing how accurately variation in pollination rates can be predicted from observations on spatial proximity, temporal overlap in flowering periods and pollen production is maximised by selecting mother plants with the greatest expected variance in contributions of potential pollen donors. To determine expected values, proximity data and the recorded field data on flowering were used. First, published data on pollen deposition rates in relation to distance were examined (Giddings et al., 1997a), which revealed a perfect linear relationship between the inverse of pollen deposition and inter-plant distance (Figure 2). This relationship was used to estimate the relative contribution of each potential pollen donor to the pollen cloud around each of the 49 mother plants as a function of spatial arrangement, with the exception that an arbitrary minor pollen contribution by the mother plant was assumed. Second, for each day of the flowering period, the relative contribution of each plant to each of the 49 pollen clouds was estimated based on the recorded number of inflorescences of the pollen donors. Subsequently, these estimates were integrated with those based on spatial proximity in order to estimate the composition of each pollen cloud. Third, it was assumed that pollen deposition to each of the 49 mother plants was a reflection of the composition of the pollen cloud around the mother plant. The number of inflorescences of the mother plant was then used to obtain daily estimates of the contribution of each potential pollen donor to the progeny of each of the 49 mother plants. Fourth, the total contribution of each potential pollen donor to each of the 49 progenies was calculated for the entire flowering period. Finally, the variance in contributions of potential pollen donors was calculated for each mother plant and used as a criterion to select the progenies to be used for paternity analysis. The 12 progenies with the highest variance values were selected and are depicted in Figure 1.

*Genotyping of mother plants*

*DNA extraction*

In April 2002, cuttings were taken from all 49 mother plants, potted in small containers and transferred to a greenhouse for establishment. Subsequently, about 100 mg of tissue material per plant was taken from young fresh leaves, placed in microtubes and frozen in liquid nitrogen. All tubes were stored at –70°C awaiting DNA extraction. Procedures for DNA isolation started with vacuum freeze drying of tissue samples, followed by grinding the dry leaves into a fine powder. Total genomic DNA was then extracted using a combination of the methods described by Fulton et al. (1995) and the DNeasy 96 Plant Kit (Qiagen, Westburg, the Netherlands). DNA yield was estimated on 0.8% (w/v) agarose gels using known concentrations of phage lambda DNA as references.

*PCR procedures*

The 49 mother plants were screened for 38 microsatellites (Table 1). Microsatellites were amplified on Peltier thermo cyclers (PTC-200) using the following thermal profile: 3 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at optimal annealing temperature and 45 s at 72°C. PCR reactions were carried out in 20 $\mu$l volume, consisting of 2 $\mu$l 10x PCR buffer, 2 $\mu$l dNTPs (1 mM), 1 $\mu$l forward primer (2 pmol/$\mu$l), 1 $\mu$l reverse primer

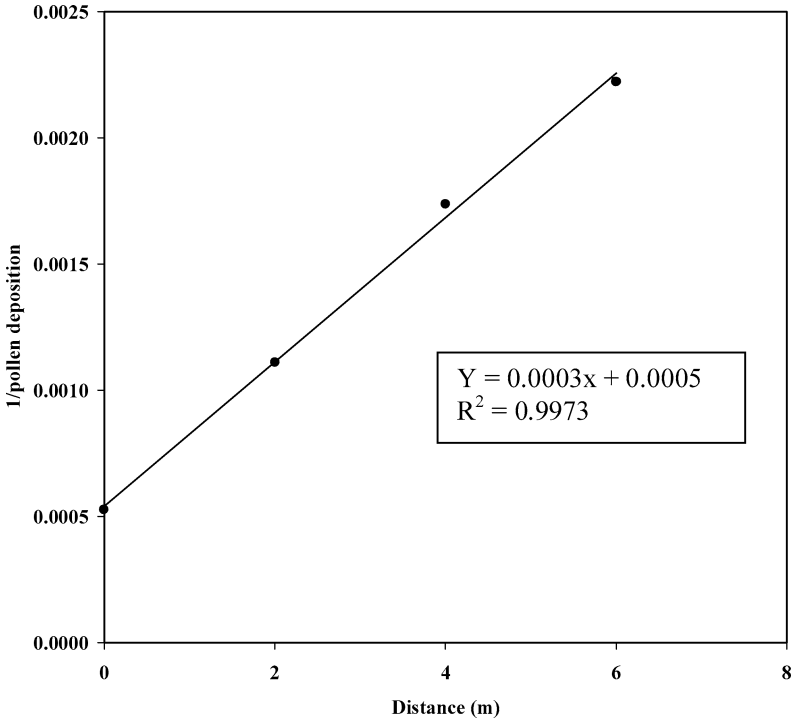



*Figure 2*. Relationship between the inverse of pollen deposition and inter-plant distance in perennial ryegrass. Figure drawn from data of Giddings et al. (1997a).

(2 pmol/$\mu$l), 0.04 $\mu$l Goldstar *Taq* DNA polymerase (5 units/$\mu$l), 8 $\mu$l DNA (2 ng/$\mu$l), 1.2–3.2 $\mu$l $MgCl_2$ (25 mM) and 2.76–4.76 $\mu$l MQ $H_2O$. Optimal annealing temperatures, ranging from 40–65°C and $MgCl_2$ concentrations, ranging from 1.5–4.0 mM depended on the microsatellite examined. For some microsatellites thermo profiles and PCR reactions were slightly modified to obtain better amplification results. Details are available upon request. Microsatellite primers were obtained from Applied Biosystems (Warrington, UK). Forward primers were fluorescently labelled at the 5'end with either NED (yellow), HEX (green) or 6-FAM (blue). To facilitate accurate genotyping, reverse primers were extended with a pigtail (GTTT) at the 5'end (Brownstein et al., 1996). Amplification products (5 $\mu$l) were loaded on 1.5% (w/v) agarose gels to verify successful amplification and to quantify the PCR product. A negative control, consisting of the PCR mixture without DNA template, was included for each microsatellite.

*Microsatellite analysis*

Amplified microsatellites were purified by transferring PCR products to a 96-well Millipore multiscreen filter plate containing Sephadex G50 fine (Sigma). In this purification step, three microsatellites labelled with different fluorescent dyes were multiplexed into a single sample of approximately 15 $\mu$l total volume. Based on the estimated quantity of PCR products, microsatellites were combined in such ratios that the fluorescent signals were expected to have approximately the same intensity. In determining these ratios it was taken into account that the intrinsic intensity of the NED-labelling is about half the value of the HEX and 6-FAM labelling. One to three $\mu$l of multiplexed PCR product, corresponding to approximately 6 ng DNA, were added to 10 $\mu$l of loading mix, consisting of 0.3–0.4 $\mu$l sizer mix per 10 $\mu$l ultra pure formamide, depending on the strength of the fluorescent signal. The sizer mix was obtained by amplification of the plasmid vector $SK^+$, using a ROX (red) labelled reverse primer in combination with each of ten pigtailed forward primers, yielding size references in the range of 80-400 basepairs. Microsatellite products were separated by capillary electrophoresis using an ABI Prism 3700 DNA Analyzer, and sized using the program GeneScan. Generated output was analyzed with the software package Genotyper, version 3.5 NT (Perkin Elmer).

*Table 1*. Perennial ryegrass microsatellites investigated in the present study, with the information source denoted by a-e. The range of observed allele sizes of the informative microsatellites is presented in number of base pairs, with the number of observed alleles given between parentheses and the occurrence of null-alleles denoted by *. The estimated resolving power for paternity identification is expressed as the mean number of potential pollen donors that can be excluded, considering all possible matings within the study population. Non-informative microsatellites were due to either PCR amplification problems, interpretation difficulties of the peak patterns or monomorphism

| Informative microsatellites | Range of observed allele sizes | Estimated resolving power | Non-informative Microsatellites |
|---|---|---|---|
| M4-136[a] | 188–213 (5) | 2.7 | M2–148[a] |
| M15–185[a] | 166–202 (10) | 7.4 | M12–52[a] |
| M16-B[a] | 124–165 (8) | 1.9 | LP204[b] |
| LP8[b] | 97–120 (7)* | 2.0 | M144[b] |
| LP20[b] | 87–106 (6)* | 16.4 | PR39[b] |
| LP165[b] | 102–108 (3) | 2.5 | LPSSRH01A10[c] |
| M10138[b] | 144–160 (4) | 1.4 | LPSSRH01D09[c] |
| PR3[b] | 140–175 (8)* | 19.9 | LPSSRH01F02[c] |
| PR8[b] | 104–143 (9) | 3.1 | LPSSRK01A03[c] |
| PR14[b] | 134–154 (4) | 3.8 | LPACA32G1[d] |
| PR24[b] | 137–167 (6)* | 25.3 | LPHCA18A7[d] |
| PR25[b] | 99–152 (11)* | 25.5 | RYE-12[e] |
| PR37[b] | 105–132 (11) | 0.5 | RYE-40[e] |
| PRE[b] | 102–144 (13) | 6.8 | |
| PRG[b] | 120–139 (4) | 4.1 | |
| LPSSRH01A02[c] | 128–166 (7)* | 11.5 | |
| LPSSRH01A07[c] | 72–85 (3) | 0.6 | |
| LPSSRH01E10[c] | 114–116 (2) | 2.5 | |
| LPSSRH01H06[c] | 149–176 (4) | 0.9 | |
| LPSSRH02C11[c] | 173–204 (4)* | 10.6 | |
| LPACA8A8a[d] | 124–135 (3)* | 7.5 | |
| LPACT26C11[d] | 167–199 (8)* | 4.8 | |
| LPACT43B11[d] | 168–196 (5)* | 16.4 | |
| LPHCA17F4[d] | 157–163 (3)* | 0.4 | |
| RYE-14[e] | 209–253 (8) | 2.2 | |

[a]Kubik et al. (1999).
[b]Kubik et al. (2001).
[c]Jones et al. (2001).
[d]Institute of Grassland & Environmental Research, Aberystwyth, UK.
[e]Plant Research International, Wageningen, the Netherlands.

*Resolving power of the analyzed microsatellites*

To support the choice of markers to be used for progeny analysis, a simulation program was developed to estimate the resolving power of the analysed microsatellites for paternity identification. This simulation program followed the rationale that no pollen donors can be ruled out as actual father with absolute certainty if they may give rise to a same genotype in the progeny of a particular mother plant. First, mother plant 1 was hypothetically selfed, and for the first microsatellite the resulting genotypes were determined. It was then investigated whether crossing mother plant 1 with plant 2 could result in identical genotypes as obtained by selfing. If not, plant 2 was excluded as possible pollen donor, given that the offspring genotypes were due to selfing. Subsequently, the procedure was repeated with all other pollen donors, given the hypothetical mating. It was then determined for mother plant 1 and microsatellite 1 how many potential pollen donors could be excluded given the hypothetical mating. Second, step 1 was repeated for each of the other 48 hypothetical matings with mother plant 1. The average number of excluded pollen donors was then determined in order to obtain an estimate of the resolving power of the microsatellite for the mother plant considered. Third, steps 1 and 2 were repeated for each of the other 48 plants assumed to be mother plants. Using the 49 estimates of the mother plants, the average number of excluded pollen donors was then determined in order to obtain an estimate of the resolving power of the microsatellite for paternity identification in the study population (Table 1). Fourth, the whole procedure was repeated for the other microsatellites and all possible combinations of multiple microsatellites. For simplicity reasons, the occurrence of null-alleles (allelic variants that do not result in an amplification product) was disregarded in the simulation program, which means that a homozygous genotype was assumed when only a single allele was observed, and that no paternity was excluded using a particular microsatellite when no alleles were observed for either the mother plant or potential pollen donor.

*Progeny genotyping*

In 2003, tissue samples were collected from about 4-week-old seedlings, raised in a greenhouse from 100 randomly chosen seeds per selected progeny. Protocols for DNA extraction, PCR procedures and microsatellite analysis followed those described for the mother plants. Microsatellite analyses were performed on 46 individuals per progeny, except for progeny 9 for which one sample was lost during experimentation. DNA from the mother plant was included in each progeny analysis for verification, together with a negative control. All samples of progeny 9, 11, 19, 22,

30 and 33 were analysed for microsatellites M4-136, M15-185, M16-B, LP20, LP165, PR3, PR14, PR24, PR25, PRE, PRG and LPSSRH02C11. This set of microsatellites was selected for initial analysis based on a combination of estimated resolving power, potential occurrence of null-alleles and type of fluorescent labelling. During the data analysis of the progenies it appeared that the variation of microsatellites PR3 and PR24 was not independent. Therefore, microsatellite PR24 was replaced by LPSSRH01A02 for the initial analyses of all samples of progenies 21, 35, 39, 41, 43 and 46. Based on the results of the simulation program, the mean expected number of excluded pollen donors was 43.2 and 42.6, respectively, for the two marker sets. In case paternity identification was unambiguous, samples were not examined any further, otherwise samples were analysed for additional informative microsatellites until either the actual pollen donor was identified or all informative microsatellites had been used (Table 1). In the latter case, offspring were subjected to AFLP analysis, together with their mother plant and potential pollen donors remaining after microsatellite analysis. AFLP analysis basically followed the procedures of Vos et al. (1995), using the *Eco*RI primer E32 (selective nucleotides: AAC) in combination with the *Mse*I primer M54 (selective nucleotides: CCT). Ambiguities still remaining after this primer combination were further analyzed with *Eco*RI primer E32 in combination with *Mse*I primer M51 (selective nucleotides: CCA) and with *Eco*RI primer E38 (selective nucleotides: ACT) in combination with *Mse*I primer M54. The *Eco*RI primer was end-labelled with $^{33}$P before selective amplification. Amplified fragments were separated on 6% denaturing polyacrylamide gels. Further details about the AFLP procedures are presented by Van Treuren et al. (2005).

*Data analysis*

*Paternity assignment*

Because of the potential occurrence of null-alleles, paternity exclusion was started by focussing on microsatellite alleles that were observed in the offspring but were absent from the mother plant. This straightforward approach, which does not require any assumptions about the presence of null-alleles, followed the rationale that potential pollen donors cannot be the actual father of an offspring if that offspring carries an allele that is lacking in both the mother plant and the potential pollen donor. In case the combined data of all 49 parental plants and all analysed offspring suggested absence of null-alleles for a microsatellite, genotypic data of that microsatellite were also taken into account in paternity exclusion. This means that plants showing a single allele were considered homozygotes and that absence of offspring alleles in the mother was no longer required in excluding potential fathers, thereby enhancing the level of information derived from the obtained microsatellite data. Because of dominant inheritance, paternity analysis using AFLP markers necessarily concentrated on fragments observed in the offspring that were absent from the mother plant.

*Paternity in relation to field data*

The effect of inter-plant distance on pollen deposition rates between pairs of plants was estimated by several functions, including the one derived from the data of Giddings et al. (1997a) that was used in the selection of progenies, and functions assuming an exponential reduction in pollen deposition rates with increasing inter-plant distance. These functions were used to estimate the relative contributions of potential pollen donors to the progenies of analysed mother plants, assuming that pollination rates were a function of distance only. The estimated variation in pollination rates was then used to examine which function best fitted the observed data. Subsequently, the recorded flowering data were included in estimating pollination rates. First, for each day of the flowering period, the composition of the pollen cloud around each analysed mother plant was estimated based on the recorded number of inflorescences of the potential pollen donors. No contribution to the pollen cloud was assumed from the mother plant, which excluded the occurrence of self-fertilisation. Second, it was assumed that fertilisation occurred according to the estimated composition of the pollen cloud, thereby taking into account the number of inflorescences of the mother plant. This resulted in an estimate of the contribution of each potential pollen donor to the progeny of each analysed mother plant on each day of the flowering period. Third, total contributions of potential pollen donors to each analysed mother plant were calculated for the entire flowering period, resulting in estimates of the relative contribution of potential pollen donors to the analysed progenies, assuming that flowering characteristics were the only parameters influencing this distribution. Estimated relative contributions based on inter-plant distance and flower characteristics were then combined to obtain expected pollination rates for potential parental pairs that were compared with observed values. This estimation procedure largely resembled the one used for the selection of progenies,

with the exception that self-fertilisation was excluded instead of assuming a minor fraction of selfing.

Data analyses were carried out using Microsoft Excel, Genstat 6 (release 6.1.0.200) and custom-designed computer programs developed with Turbo Pascal.

## Results

### *Field experiment*

All 49 plants of the study population flowered during the field experiment, starting with plant 9 on May 24 and ending with plant 16 on July 12, resulting in field data over a 50-day period. The duration of flowering ranged from 20 days for plant 42 to 36 days for plant 16 and 44, while simultaneous flowering of all 49 plants was observed for a period of 10 days from June 14 until June 23. This 10-day period comprised the flowering of 54.3% of the total number of recorded inflorescences. Overlap in flowering time for any combination of two plants ranged from 17 to 30 days. Peak flowering was observed on June 14 when a total number of 3871 inflorescences with anthers releasing pollen were recorded. The total number of inflorescences with open flowers ranged from 427 for plant 27 to 2017 for plant 13. Mean wind speed per day within the plot ranged from 0.30 to 1.52 m/s, while the prevailing wind direction during the flowering season was from south-west/west.

### *Microsatellite genotyping of parental plants*

The parental plants were screened for 38 microsatellites, of which 13 were found non-informative due to amplification problems, interpretation difficulties or monomorphism. The observed number of alleles of the remaining 25 microsatellites ranged from 2 to 13, while the estimated resolving power for paternity exclusion ranged from 0.5 to 25.5 potential pollen donors expected to be excluded per marker (Table 1). For the 12 selected progenies used for paternity analysis, a mean number of 45.7 potential pollen donors were expected to be excluded per offspring using the combined set of 25 microsatellites. This was close to the maximum value of 48 that equals unambiguous paternity identification.

Consistent absence of amplified products after repeated PCR and large deficiencies of heterozygotes compared to Hardy-Weinberg expectations suggested the occurrence of null-alleles at 11 microsatellite loci (Table 1). Genotypic data also suggested plants 4, 6, 21, 25, 34 and 46 to be tetraploid instead of diploid (Figure 1). For these plants often three or four alleles were observed for the majority of microsatellites. The six presumed tetraploids often carried alleles that were absent from the other parental plants. Except for plant 4, morphological data were in line with presumed tetraploidy, as plants 6, 21, 25, 34 and 46 exhibited typical tetraploid characteristics such as more vigorous growth, wider leaves and a more light green leaf colour in comparison with the other parental plants. The flowering periods of the 6 plants also differed from the other parental plants, starting relatively late between June 8 and June 14 and ending relatively late between July 7 and July 10. Apart from plant 4, the one-thousand grain weight of the presumed tetraploids ranged from 3.49 to 4.01 g, which was markedly higher than the population mean of 2.54 g. Finally, to ascertain the tetraploid nature of plants 4, 6, 21, 25, 34 and 46, 5 offspring per plant were subjected to flow cytometry (Galbraith et al., 1983, Barker et al., 2001), which indicated tetraploidy for all six progenies.

### *Paternity analysis*

Morphological comparison of the seedlings raised from the 12 selected progenies for paternity analysis indicated clear tetraploid characteristics for progenies 21 and 46. With respect to ploidy level, all 12 progenies appeared homogeneous, suggesting absence of cross-fertilisation between plants of different ploidy level. This also suggested that the study population in fact consisted of two reproductively isolated subpopulations.

Examination of mother-offspring combinations confirmed the occurrence of null-alleles at the 11 microsatellite loci indicated in Table 1. This was concluded from the fact that progenies of mother plants having only a single allele could include offspring for which no alleles were observed and offspring with a single allele that did not match the mother plant. Genotypic data obtained from the other 14 microsatellites were in line with absence of null-alleles.

Based on the microsatellite data, paternity was identified in 451 cases out of the 551 offspring examined (81.9%). Exclusion of all 49 known pollen donors was observed for 9 offspring that subsequently were designated contaminants. Alleles unknown to the parental population were observed at multiple loci in all of these cases. The five offspring matching the mother

plant were considered pollen contaminants, whereas the four offspring showing mismatches with the mother plant were regarded seed contaminants. Of the remaining 91 offspring, ambiguous paternity was observed between 2 (62×), 3 (15×), 4 (9×), 5 (3×) and 7 (2×) known pollen donors. Thus, excluding the 9 identified contaminants, the microsatellite analyses excluded per offspring a mean number of 47.7 known plants as actual pollen donor, which was close to the predicted value of 45.7. Remaining ambiguities were resolved with AFLP analysis, except in four cases (Table 2).

As expected, no cross-fertilisation was observed between diploid and tetraploid plants, indicating that the study population indeed consisted of two reproductively isolated subpopulations. Among the 459 investigated offspring from diploid plants, four cases of self-fertilisation (0.87%) were observed. Surprisingly, genotypic data of the offspring of the tetraploid plant 21 indicated 15 self-pollinations (32.6%).

*Relationship between paternity and field data*

Mating within the study population was clearly not random, but skewed to plants at closer distance as high frequencies of paternity involved neighbouring plants in nearly all cases. For example, high frequencies of cross-pollination were observed between the neighbouring plants 11 and 19 and between 33 and 39 (Table 2). Out of the total number of identified pollen donors, 61.9% were located within 1 m distance from the mother plant. Considering that inter-plant distance is an important factor affecting pollination rates, the observed absence of cross-fertilisation between the neighbouring plants 9 and 11 and between 19 and 33 was quite surprising. These results could not be ascribed to non-overlapping flowering periods or strong differences in the number of inflorescences during the overlap in flowering periods, which may indicate the involvement of reproductive barriers between plants, such as an incompatibility system.

No pollen contributions to any of the analysed mother plants were observed for plants 4, 7, 16 and 34. Pollen production of these plants ranged from low to moderate with 473, 529, 1035 and 850 recorded inflorescences, respectively. However, in contrast to the effect of inter-plant distance, the influence of pollen production and temporal overlap on pollination rates in general was less clear. To investigate how accurate the variation in pollination rates could be predicted from the spatial arrangement and flower characteristics of plants, several functions were fitted to the observed pollination rates. In calculating expected pollination rates, diploid and tetraploid plants were treated as two separate, reproductively isolated populations. Expected pollination rates were calculated as the absolute number of pollinations based on the progeny sample size and were estimated for each combination of analysed mother plant and potential pollen donor. Data from different progenies were then combined and classified into observed distance categories. Because of the discrete distribution of observed inter-plant distances, pollination rates were presented cumulatively (Figure 3). When only spatial arrangement was considered, observed pollination rates were very well described by an inverse quadratic function of inter-plant distance (Figure 3a). Using this function the sum of squared differences between observed and expected values of all 430 potential mating pairs was 1575. This value indicated a 51.0% better fit than the random mating model, a 44.7% better fit than the model based on the data of Giddings et al. (1997a), a 28.4% better fit than the inverse distance function and a 9.5% better fit than the inverse third power distance function. When the flowering characteristics were included in the calculation of expected values, the fit of the inverse quadratic function slightly improved (Figure 3b), as the sum of squared differences reduced to 1563 (0.77%). Thus, spatial arrangement alone already explained the main part of the observed variation in pollination rates, while the recorded flowering characteristics contributed only to a negligible extent.

## Discussion

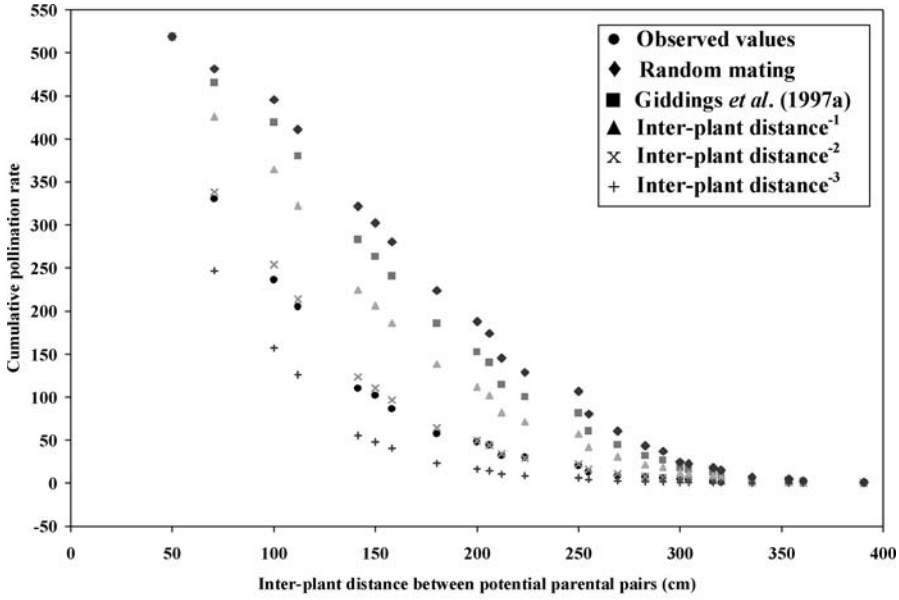
*Microsatellite genotyping*

Because of their codominant inheritance and high allelic diversity, microsatellites are considered ideal markers in population genetic studies. However, the occurrence of null-alleles at microsatellite loci may lead to erroneous genotyping, which is particularly troublesome in parentage studies (Pemberton et al., 1995). Population studies have reported null-allele frequencies as high as 15% and occurrence in up to 25% of microsatellite loci analysed (Jarne & Lagoda, 1996). In the present study 11 out of the 25 analysed microsatellites appeared to harbour null-alleles. In paternity identification the occurrence of null-alleles was taken into account by concentrating on offspring alleles not present in the mother plant, a straightforward approach that is independent of the presence of

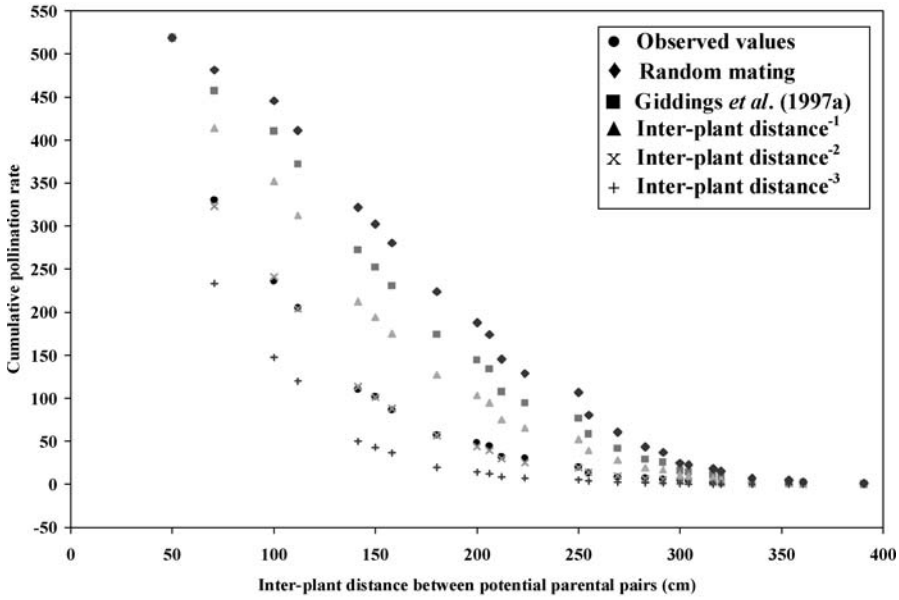
*Table 2*. Number of seeds sired by each of the study plot plants for each investigated progeny array based on a paternity exclusion analysis using molecular markers. Tetraploid plants are indicated in boldface. Study plot plants for which no contribution was observed to any of the analyzed progenies are not presented. "No match" indicates that all 49 study plot plants were excluded as pollen donor.

| Study plot plants | Investigated progeny arrays | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 9 | 11 | 19 | **21** | 22 | 30 | 33 | 35 | 39 | 41 | 43 | **46** |
| 1 | | | | | | 1 | | | | | | |
| 2 | | | | | | | | | | | 1 | |
| 3 | | | | | | | | | | | 1 | |
| 5 | | 2 | | | | 5 | | | | | | |
| **6** | | | | 3 | | | | | | | | 40 |
| 8 | | | 3 | | 5 | 1 | 2 | 1 | 1 | | 1 | |
| 9 | | | 4 | | | | | 10 | 1 | 10 | | |
| 10 | | | 1 | | 4 | | | | 1 | | | |
| 11 | | | 10 | | | 1 | 4 | | | 5 | 2 | |
| 12 | 2 | | 3 | | | | | 1 | 2 | 1 | | |
| 13 | 1 | 2 | 2 | | 1 | 1 | 8 | | | 3 | | |
| 14 | | | | | | | | | 1 | 1 | | |
| 15 | 1 | | | | | 1 | | 7 | | | 1 | |
| 17 | 3 | | | | | 2 | 1 | | 3 | | | |
| 18 | | | | | | 1 | | | | | | |
| 19 | 9 | 10 | 1 | | 1 | | | | | 3 | 6 | |
| 20 | | | 2 | | | | | 3 | | 2 | 1 | |
| **21** | | | | 15 | | | | | | | | 1 |
| 22 | | | 3 | | | | 1 | | | | | |
| 23 | | | | | 1 | | | | | 1 | | |
| 24 | 1 | | 3 | | 6 | | 1 | 1 | | 1 | 4 | |
| **25** | | | | 13 | | | | | | | | 5 |
| 26 | | | | | | | | | | 1 | | |
| 27 | | | 1 | | 2 | | 3 | 1 | 3 | 1 | 6 | |
| 28 | | 2 | 1 | | 1 | | | 1 | 1 | | 4 | |
| 29 | 1 | | | | 4 | | | 1 | | 1 | 1 | |
| 30 | 1 | 2 | 1 | | | 3 | 5 | | 3 | 1 | | |
| 31 | | | | | 1 | | | 2 | | | 1 | |
| 32 | | | | | | | | | | 1 | | |
| 33 | 5 | 9 | | | | 4 | | | 7 | 2 | | |
| 35 | 5 | 2 | | | | 1 | | | | | | |
| 36 | 2 | 2 | | | | | | 4 | | 5 | | |
| 37 | | 1 | | | | 6 | 5 | | 10 | | 1 | |
| 38 | 1 | | 1 | | 15 | 1 | 1 | | | 1 | 7 | |
| 39 | 1 | 1 | | | | 5 | 11 | | | 1 | 1 | |
| 40 | | | 1 | | 2 | 1 | | | 1 | | | |
| 41 | 6 | 7 | | | | 2 | 2 | 4 | | | | |
| 42 | | | | | 1 | | | | | | | |
| 43 | 3 | 2 | | | 1 | 1 | | | 1 | | | |
| 44 | 1 | 1 | | | | 4 | | | 6 | | | |
| 45 | | | 8 | | 1 | | | 5 | 1 | 1 | 4 | |
| **46** | | | | 14 | | | | | | | | |
| 47 | | | | | | | | 3 | | | 1 | |
| 48 | | 1 | | | | | | 1 | | 2 | 1 | |
| 49 | 1 | 1 | | | | 2 | 1 | | 3 | 1 | | |
| No match | | [a]1 | [b]1 | [b]1 | | [ab]2 | [a]1 | | | [a]1 | [ab]2 | |
| Ambiguity | [c]1 | | | | | [d]1 | | [e]1 | [f]1 | | | |
| Total | 45 | 46 | 46 | 46 | 46 | 46 | 46 | 46 | 46 | 46 | 46 | 46 |

[a]Pollen contamination; [b]Seed contamination; [c]Pollen donor 16 or 28; [d]Pollen donor 44 or 47; [e]Pollen donor 9 or 47; [f]Pollen donor 11 or 14.




(a) Inter-plant distance




(b) Inter-plant distance combined with flowering characteristics

*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 various distance functions. Estimated values were based solely on inter-plant distance (a) and on a combination of inter-plant distance and flowering characteristics (b).

null-alleles. Drawback of this method is that resolving power is sacrificed compared to genotypic data analysis. Only when all parental and offspring data suggested absence of null-alleles at a microsatellite locus, plants showing a single allele were considered homozygotes and genotypic data were used in paternity analysis. However, the occurrence of rare null-alleles remaining undetected cannot be completely ruled out.

For the 12 selected progenies used for paternity analysis, an average of 45.7 potential pollen donors

were expected to be excluded per offspring based on the simulation model and microsatellite data of the parental population. This value was over-estimated because of disregarding the occurrence of null-alleles and considering plants with a single allele as homozygotes. Therefore, microsatellites harbouring null-alleles displayed the largest expected resolving power (Table 1). Underestimation of the resolving power was caused by the fact that paternity was considered ambiguous when different pollen donors *a priori* could give rise to identical genotypes while pollinating the same plant. However, whether pollen donors can be excluded as actual father will depend on the alleles that are actually transmitted by the parents to the offspring. All possible mating combinations within the study population were considered in the estimation of resolving power. However, non-random mating may affect the actual resolving power positively or negatively depending on the genotypes involved. Despite violation of the assumptions underlying the simulations, the estimated resolving power was close to the observed value of 47.7. The simulations proved useful in estimating the overall resolving power of the investigated microsatellites and in identifying informative combinations of markers for the selected progenies. Variation at microsatellites PR3 and PR24 appeared not independent. Correlation of variation was also observed between PR14 and PRG and between PR8, PRE and PR25. Published data of the microsatellites indicated that the 5' flanking region primers of PR8 and PR25 were in fact identical in nucleotide sequence (Kubik et al., 2001).

All offspring of the selected progenies were initially investigated for 12 microsatellites. However, in many cases paternity identification could already be accomplished with only a few markers, depending on the alleles received by the offspring and their frequencies among the parental plants. Excess of resolving power was used in validating obtained results and in identifying errors. For example, in cases where initially no paternity could be identified, the generation of pseudoreplicate datasets each containing all but one of the original marker loci (jackknifing) proved useful to trace scoring errors.

Three, and in one case even four alleles per locus were observed for 11 offspring at one to four microsatellite loci per plant, although both the mother plant and the identified pollen donor displayed a maximum of two alleles per locus. Repeated analysis showed identical results in all cases, which ruled out the possibility of PCR artefacts underlying these observations. Contamination of samples during laboratory experimentation was also considered unlikely because in that case exclusion of all 49 potential pollen donors might have been expected for offspring in paternity analysis. Moreover, data analysis without the involved microsatellite(s) did not alter paternity identification. Therefore, these findings do not seem to have a methodological cause but may point towards the involvement of chromosomal rearrangements, such as translocation events and the occurrence of duplication-deficiencies.

All 49 plants of the study population were excluded as actual father for 9 out of the 551 offspring examined (1.6%), which were designated either pollen contaminants or seed contaminants. In all these cases alleles were observed at multiple microsatellite loci that were unknown to the study population. In the absence of other perennial ryegrass germplasm in and around the plot, gene flow by pollen may have occurred from perennial ryegrass accessions that were rejuvenated simultaneously with the study population. Although pollen is largely deposited in the vicinity of the donor plant, small fractions of long distance pollen dispersal are common in perennial ryegrass (Giddings et al., 1997a,b; Cunliffe et al., 2004). Assuming the absence of plants other than those of the study population within the plot, seed contamination must have occurred during seed handling after harvesting of the mother plants. For one additional offspring, paternity was initially excluded for all 49 potential pollen donors based on the single fact that an unknown allele of 215 bp was observed for microsatellite M4-136 in the progeny of a diploid mother plant displaying alleles of 194 and 213 bp, respectively. Repeated PCR ruled out the possibility of a PCR artefact. Since the size of the other offspring allele was 188 bp, the data suggested seed contamination. However, at other microsatellites no unknown alleles were observed and offspring genotypes matched those of the mother plant. Furthermore, data analysis without M4-136 identified a single, known parental plant. Given the fact that microsatellite M4-136 consists of dinucleotide repeat units (Kubik et al. 1999), a mutation was assumed from maternal allele 213 to an allele of 215 bp. Mutation at microsatellite loci has been estimated to occur at the high rate of $10^{-3}$ to $10^{-2}$ per locus per gamete per generation (Weber & Wong, 1993). Therefore, the observation of mutation is not unlikely considering the scale of the present study. AFLP analysis was carried out for additional validation, which showed compatibility with the identified parental combination and did not support contamination.

Six tetraploids were identified among the parental plants, although the studied accession was supposed to consist of diploid plants. To test whether these could have been present in the original seed lot used for the present study, a random sample of 30 plants raised from the remaining seeds were tested by flow cytometry, which indicated diploidy in all cases. Assuming that tetraploids occur in a frequency of 6/49 within the original seed population, the probability that a random sample of 30 seeds contains only diploids is less than 2%. Therefore, it was considered unlikely that the tetraploid plants originated from accession BA 11894. Additional data suggested contamination during the preparatory phase of the regeneration. In fact, the study population formed part of an experiment to investigate variation in seed production at 4 European sites where 2 replicate plots per accession including BA11894 were examined at each location. For that purpose, plants derived from accessions were first cloned and then distributed in order to assure that the same sets of genotypes were studied at all sites (ICONFORS 2001). It appeared that the six tetraploid genotypes found in the present study were heading very late, not only in the studied plot, but also in each of the other 7 plots. Given that heading date usually displays a high heritability (e.g. Elgersma, 1990), this indicated that the 8 plots still consisted of the same set of genotypes. This means that the source of the contamination was not related to the study plot, but must have taken place between seed sowing and the cloning of plants. In paternity analysis of the progenies of the tetraploid mother plants 21 and 46, it was assumed that two copies of the same allele could be transmitted to the gametes. This mode of segregation is relatively common for autotetraploids such as perennial ryegrass, and is known as double reduction (Darlington, 1929; Mather, 1936; Ramsey & Schemske, 2002). Without the possibility of double reduction, paternity analysis would have indicated an increased frequency of contamination.

Self-fertilisation in perennial ryegrass is assumed to occur at a very low rate due to the existence of a self-incompatibility system (Thorogood et al., 2002). Four cases of self-fertilisation were observed among the 459 diploid offspring examined, which corresponds to a rate less than 1%. These 4 offspring were also analysed for all 3 AFLP primer pairs, together with the mother plants and the potential pollen donors remaining after microsatellite analysis. For each of these 4 offspring, all AFLP bands observed in the offspring appeared to be present also in the mother plant. Although self-fertilisation cannot be proved using a dominant marker, probability estimation of the observed results supported self-fertilisation. First, bands were identified in the remaining potential pollen donors that were absent in mother plant. Second, self-fertilisation was disregarded and it was assumed that the actual father was among the known remaining pollen donors. This means that all the identified paternal bands must have been heterozygous, and that in each case the null-allele must have been transmitted to the offspring. Third, considering the number of AFLP bands involved, the probability of not observing a single paternal band in the offspring, given that outcrossing had occurred, ranged from $2.4 \times 10^{-4}$ to $1.5 \times 10^{-8}$ for the pair-wise combinations of offspring and potential pollen donors. Surprisingly, paternity identification for progeny 21 indicated 15 self-fertilised offspring. For seven of these offspring additional AFLP data for a single primer combination were available, which corresponded to self-fertilisation. Polyploidy in angiosperms has frequently been found associated with increased self-compatibility because chromosome doubling may produce new allelic interactions in pollen that result in breakdown of self-incompatibility (De Nettancourt, 2000). The rather isolated position of plant 21 within the plot, relative to other tetraploid plants, may have contributed to the observed selfing rate. By means of unreduced gametes, diploid genotypes of perennial ryegrass may give rise to diploid pollen that are able to fertilise tetraploid plants (Jansen & Den Nijs, 1993). However, in the present study, the diploid and tetraploid plants appeared to constitute two reproductively isolated populations.

*Paternity in relation to field data*

Spatial distribution of the parental plants and recorded flowering data were used to estimate pollination rates between plants and to select progenies for paternity analysis. In the selection of progenies, the estimated distribution of pollination rates was affected by violation of at least two assumptions. First, instead of 49 plants assumed to be capable of pollinating each other, the study population appeared to consist of two reproductively isolated subpopulations. Second, pollination rates appeared to be more related to an inverse quadratic function of inter-plant distance than to a function derived from the experimental data of Gidddings et al., (1997a).

As could be expected based on the generally observed leptokurtotic distribution of pollen dispersal in perennial ryegrass (Giddings et al., 1997a,b; Cunliffe

et al., 2004), inter-plant distance had a large effect on pollination rates. Observed pollination rates were very well described by an inverse quadratic function of inter-plant distance between potential mating pairs. This function corresponds to a two-dimensional dispersal of a pollen cloud that dilutes rapidly with increasing distance. Although incorporation of flowering data in the calculation of expected pollination rates improved the goodness of fit with observed values by 0.77%, inter-plant distance alone was found to be an accurate predictor of pollination rates. Obviously, the relative importance of inter-plant distance on pollination rates depends on the variation in flowering intensity and flowering periods between plants. Data from other regenerated perennial ryegrass populations (ICONFORS 2001) showed that about 50% of these populations displayed variance values in heading date that were lower or similar to that of the study plot. This may indicate that the relative importance of inter-plant distance found for the study plot may also apply to other populations.

In order to compare expected and observed values, pollination rates of different progenies were combined into observed distance classes and presented cumulatively. Combining results from different progenies has the advantage that a strong relationship is not obscured by low statistical power of individual progenies. The cumulative presentation of pollination rates was followed because observed inter-plant distances did not display a continuous distribution. Despite the fact that inter-plant distance was found to be an accurate predictor of pollination rates, analysis of individual progenies suggested that incompatibility for specific parental combinations was at least one additional factor influencing pollination rates. For practical reasons, pollen production was roughly estimated by counting the number of inflorescences with open flowers. However, differences in actual pollen production between plants may have been influenced by variation in number of flowers per inflorescence, in the fraction of opened flowers per inflorescence and in number of pollen grains per flower. To estimate pollen production the study plot was visited on a daily basis, which may have caused some interference with natural pollination patterns. Results from earlier studies in perennial ryegrass suggested the influence of climatic parameters on pollen dispersal, such as wind direction, wind speed and turbulence (Giddings et al., 1997a,b). In the present study, graphical representation of observed paternity (results not shown) did not reveal a clear relationship with the recorded wind parameters. Because observed pollinates rates were already very well described by inter-plant distance, no attempt was made to further improve the fit by including wind parameters in the estimation of pollination rates.

*Relevance to genebanks*

Ideally, the genetic composition of a rejuvenated genebank accession is an exact copy of the previous generation. Achievement of this goal requires infinite population size, absence of mutation, selection and gene flow from other germplasm, equal family sizes and completely random mating. Because these conditions are not very likely to apply to regeneration populations, the question is not whether genetic integrity is lost during regeneration, but rather how seed multiplication protocols can be optimised in order to reduce loss of genetic integrity as much as possible. In a comprehensive research project directed to the optimisation of regeneration protocols for European forage species, several important factors that may influence loss of genetic integrity were studied, including variation in seed production and its genetic basis, gene flow among populations and variation in pollination rates between plants (ICONFORS 2001). The present study was carried out within the framework of this project.

Differential pollination rates between plants may lead to unequal contributions of plants to the next generation, resulting in reduced effective population size ($N_e$) and increment of the inbreeding coefficient (Falconer 1981). In the present study, spatial distribution appeared the key factor influencing pollination rates between plants, which were best described by an inverse quadratic function of inter-plant distance. Based on this function and assuming a population of 49 plants spatially arranged as in the present study, the relative paternal contribution to the total seed population ranged from 1.2% for plants at the corners of the plot to 2.6% for the central plant. At least two different modifications of the regeneration protocol may be considered that, based on the function of inter-plant distance, are expected to reduce variation in paternal contributions. First, the spatial arrangement of the parental plants could be modified. For example, a single row of 49 plants is expected to reduce the variance in relative paternal contributions by 72% compared to the plot design of the present study, while a single circle of 49 plants would result in a reduction of 100%. However, it remains to be determined whether the observed distance function will apply to such modified plot designs

as well. Moreover, single linear or circular plot designs may have potential negative side-effects. For instance, the number of different pollen donors contributing to individual progenies is expected to decrease, making the pollination of plants more dependent on specific pollen donors. In addition, overall pollination rates may be reduced considerably in case of unfavourable prevailing wind directions. Second, unequal paternal contributions could be corrected by differential seed harvesting of individual plants. This could be particularly relevant to genebanks that already practise single-plant harvesting and composing of balanced bulks to correct for differential seed production by plants. In that case, correction for differential pollination rates is easily achieved by taking differential seed numbers from plants based on the observed distance function, instead of sampling equal seed numbers from each plant. Compared to plants with a more central position, higher seed numbers are sampled from border plants to compensate for their lower pollen contributions (Table 3). Although organellar DNA is substantially smaller than the nuclear DNA, it should be noted that differential seed harvesting from plants introduces unequal contributions by plants in organellar DNA. Moreover, harvesting higher seed numbers from border plants may be accompanied by an increased contamination rate in case border plants have a higher probability of receiving foreign pollen. This will be tested by data from experiments on gene flow between regeneration plots (ICONFORS 2001). To implement modifications of the regeneration protocol, it would be worthwhile to test the general validity of the results of the present study. It would be interesting to investigate whether similar results can be obtained for other populations and locations and to determine across which range the observed relationship between inter-plant distance and pollination rates will hold. For example, maximum increase in the density of plants may make pollination rates less dependent on inter-plant distance, resulting in a more uniform dispersal of pollen. This could apply to seed multiplications in isolation chambers where plants typically occupy less than 1 square meter (Maurice Hinton-Jones, pers. comm.).

Contamination during seed multiplication is not a major threat to the genetic integrity of accessions as long as contaminants have a low probability of being used as parent during the next seed multiplication. Otherwise, contaminants can easily spread through the accession by pollen dispersal, which is even more troublesome in case contaminants have a selective advantage over the original germplasm. In the present study, contamination was observed in 9 cases among the 551 investigated offspring (1.63%). This contamination level corresponded to a 57% probability of selecting at least one contaminant as parental plant for the next seed multiplication, indicating the need for improved measures to avoid gene flow from other germplasm. However, specific recommendations are difficult to give because the origin of the contaminations could not be identified with absolute certainty. Pollen contaminations were probably due to gene flow from other accessions regenerated along with the study population. Studies on gene flow among populations that were carried out within the project ICONFORS will be used to evaluate the potential for pollen contamination of accessions that are regenerated simultaneously. Seed contaminants indicated the need for safety precautions to avoid mix-up of seeds of different germplasm during post-harvest handling of the seeds.

*Table 3.* Number of seeds per plant to assemble balanced bulks (a) and the number of paternal gametes contributed by plants to the bulk (b). Data were derived from an optimisation procedure* using a 7×7 matrix plot design and assuming that pollination rates are an inverse quadratic function of inter-plant distance

a. Number of seeds per plant for the assembly of balanced bulks

| | | | | | | |
|---|---|---|---|---|---|---|
| 156 | 123 | 127 | 128 | 127 | 123 | 156 |
| 123 | 99 | 103 | 103 | 103 | 99 | 123 |
| 127 | 103 | 105 | 105 | 105 | 103 | 127 |
| 128 | 103 | 105 | 105 | 105 | 103 | 128 |
| 127 | 103 | 105 | 105 | 105 | 103 | 127 |
| 123 | 99 | 103 | 103 | 103 | 99 | 123 |
| 156 | 123 | 127 | 128 | 127 | 123 | 156 |

b. Number of paternal gametes contributed per plant

| | | | | | | |
|---|---|---|---|---|---|---|
| 77.2 | 110.4 | 106.0 | 105.6 | 106.0 | 110.4 | 77.2 |
| 110.4 | 133.8 | 130.3 | 129.9 | 130.3 | 133.8 | 110.4 |
| 106.0 | 130.3 | 128.4 | 128.1 | 128.4 | 130.3 | 106.0 |
| 105.6 | 129.9 | 128.1 | 128.0 | 128.1 | 129.9 | 105.6 |
| 106.0 | 130.3 | 128.4 | 128.1 | 128.4 | 130.3 | 106.0 |
| 110.4 | 133.8 | 130.3 | 129.9 | 130.3 | 133.8 | 110.4 |
| 77.2 | 110.4 | 106.0 | 105.6 | 106.0 | 110.4 | 77.2 |

*Optimisation included calculation of the variance in the sum of maternal and paternal gametes contributed by plants to the next generation and minimisation of this variance by varying the number of seeds per plant. Different solutions may be found depending on the start values of the number of seeds per plant and the algorithm used to minimise the variance. However, results obtained from different optimisations showed similar ratios of seed numbers between plants. The seed numbers presented in this Table resulted in a variance value of 0.05, which was a reduction of 99.98% compared to a bulk of similar size but consisting of equal numbers of seeds per plant.

The detection of 6 tetraploid plants among the parental plants used for seed multiplication was particularly worrisome since they were not likely to belong to the original germplasm and indicated contamination during the preparatory phase of the seed multiplication. Thus, contamination may occur during various stages of the regeneration of accessions, and can be regarded more threatening to the genetic integrity of perennial ryegrass germplasm than variation in pollination rates between plants.

## Acknowledgments

The experiments described in this paper were carried out within the framework of a research project aiming at the optimisation of regeneration protocols for perennial European forage species (ICONFORS 2001). We are grateful to Ruaraidh Sackville Hamilton who initiated ICONFORS, which was funded by the European Union (QLRT-1999–30621). We are also grateful to Julie King and Ian King for making unpublished microsatellites from the Institute of Grassland & Environmental Research (Aberystwyth, UK) available for the project. We would also like to thank Maurice Hinton-Jones who was responsible for the flow cytometry tests, Dick Klein Geltink who examined ploidy levels of offspring plants, Theo van Hintum and Marco van Veller who assisted in the analysis of the data, and Oene Dolstra who contributed to the interpretation of the results. The comments of Ruaraidh Sackville Hamilton and Oene Dolstra on an earlier version of this manuscript are highly appreciated.

## References

Barker, R.E., J.A. Kilgore, R.L. Cook, A.E. Garay & S.E. Warnke, 2001. Use of flow cytometry to determine ploidy level of ryegrass. Seed Sci Technol 29: 493–502.

Brownstein, M.J., J.D. Carpten & J.R. Smith, 1996. Modulation of non-templated nucleotide addition by *Taq* DNA polymerase: Primer modifications that facilitate genotyping. Biotechniques 20: 1004–1010.

Cunliffe, K.V., A.C. Vecchies, E.S. Jones et al., 2004. Assessment of gene flow using tetraploid genotypes of perennial ryegrass (*Lolium perenne* L.). Austr J Agr Res 55: 389–396.

Darlington, C.D., 1929. Chromosome behaviour and structural hybridity in the *Tradescantiae*. J Genet 21: 207–286.

De Nettancourt, D., 2000. Incompatibility and Incongruity in Wild and Cultivated Plants. Springer-Verlag, Berlin.

Elgersma, A., 1990. Heritability estimates of spaced-plant traits in three perennial ryegrass (*Lolium perenne* L.) cultivars. Euphytica 51: 163–172.

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, pp. 299–320. CABI Press.

Fulton, T.M., J. Chunwongse & S.D. Tanksley, 1995. Microprep protocol for extraction of DNA from tomato and other herbaceous plants. Plant Mol Biol Rep 13: 207–209.

Galbraith, D.W., K.R. Harkins, J.M. Maddox et al., 1983. A rapid flow cytometric analysis of the plant cell in intact plant tissues. Science 220: 1049–1051.

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*. Theor Appl Genet 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. Theor Appl Genet 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.

ICONFORS, 2001. Improving germplasm conservation methods for perennial European forage species. http://www.igergru.bbsrc.ac.uk/iconfors/projectannouncement.htm.

Jansen, R.C. & A.P.M. den Nijs, 1993. A statistical method for estimating the proportion of unreduced pollen grains in perennial ryegrass (*Lolium perenne* L.) via the size of the pollen grains. Euphytica 70: 205–215.

Jarne, P. & P.J.L. Lagoda, 1996. Microsatellites, from molecules to populations and back. Trends Ecol Evol 11: 424–429.

Johnson, R.C., V.L. Bradley & M.A. Evans, 2002. Effective population size during grass germplasm seed regeneration. Crop Sci 42: 286–290.

Johnson, R.C., V.L. Bradley & M.A. Evans, 2004. Inflorescence sampling improves effective population size of grasses. Crop Sci 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.). Theor Appl Genet 102: 405–415.

Kubik, C., W.A. Meyer & B.S. Gaut, 1999. Assessing the abundance and polymorphism of simple sequence repeats in perennial ryegrass. Crop Sci 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 Sci 41: 1565–1572.

Lexer, C., B. Heinze, H. Steinkellner, S. Kampfer, B. Ziegenhagen & J. Glossl, 1999. Microsatellite analysis of maternal half-sib families of *Quercus robur*, pedunculate oak: Detection of seed contamination and inference of the seed parents from the offspring. Theor Appl Genet 99: 185–191.

Marum, P., I.D. Thomas & M. Veteläinen, 1998. Summary of germplasm holdings. In: 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), pp. 184–190. International Plant Genetic Resources Institute, Rome, Italy.

Mather, K., 1936. Segregation and linkage in autotetraploids. J Genet 32: 287–314.

Pemberton, J.M., J. Slate, D.R. Bancroft & J.A. Barrett, 1995. Non-amplifying alleles at microsatellite loci: A caution for parentage and population studies. Mol Ecol 4: 249–252.

Queller, D.C., J.E. Strassmann & C.R. Hughes, 1993. Microsatellites and kinship. Trends Ecol Evol 8: 285–288.

Ramsey,J. & D.W. Schemske, 2002. Neopolyploidy in flowering plants. Ann Rev Ecol Syst 33: 589–639.

Sackville Hamilton, N.R., 1998. The rationalization of regeneration methods: how far can we go? The example of forage grasses. In: T. Gass, L. Frese, F. Begemann & E. Lipman (Eds.), Implementation of the Global Plan of Action in Europe – Conservation and Sustainable Utilization of Plant Genetic Resources for Food and Agriculture. IPGRI, Rome, Italy.

Thorogood, D., W.J. Kaiser, J.G. Jones & I. Armstead, 2002. Self-incompatibility in ryegrass 12. Genotyping and mapping the *S* and *Z* loci of *Lolium perenne* L. Heredity 88: 385–390.

Van Treuren, R., N. Bas, P.J. Goossens, H. Jansen & L.J.M. van Soest, 2005. Genetic diversity in perennial ryegrass and white clover among old Dutch grasslands as compared to cultivars and nature reserves. Mol Ecol 14: 39–52.

Vos, P., R. Hogers, M. Bleeker et al., 1995. AFLP: a new technique for DNA fingerprinting. Nucl Acids Res 23: 4407–4414.

Weber, J.L. & C. Wong, 1993. Mutation of human short tandem repeats. Hum Mol Genet 2: 1123–1128.

Zeven, A.C. & J.M.J. de Wet, 1982. Dictionary of Cultivated Plants and their Regions of Diversity. Pudoc, Wageningen, the Netherlands.