Genetic diversity in European perennial ryegrass cultivars investigated with RAPD markers

S. Bolaric1, S. Barth2, A. E. Melching3 and U. K. Posselt1,4

1 State Plant Breeding Institute, University of Hohenheim, D-70593 Stuttgart, Germany; 2 Teagasc, Crops Research Centre, Oak Park, Carlow, Ireland; 3 Institute of Plant Breeding, Population Genetics and Seed Science, University of Hohenheim, D-70593 Stuttgart, Germany; 4 Corresponding author, E-mail: posselt@uni-hohenheim.de

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Abstract
Perennial ryegrass (Lolium perenne L.) is the most important grass species for temperate grassland agriculture. The genetic relationship and distance among cultivars is largely unknown but of great interest for breeding programmes. The objectives of this study were to (i) investigate the molecular variation and structure of population cultivars, (ii) describe the relationship among cultivars in terms of the modified Rogers’ distance, and (iii) determine the minimum sample size required for characterization of cultivars of L. perenne using random amplified polymorphic DNA (RAPD) markers. A total of 22 ryegrass cultivars, mainly of European origin, were investigated with RAPD markers. The minimum sample size required for the characterization of cultivars was about 20 individuals per population. An analysis of molecular variance (AMOVA) revealed a much larger genetic variation within cultivars (66%) than between them (34%).

Key words: Lolium perenne — RAPD marker — diversity — cultivars — Rogers’ distance — minimum sample size

Perennial ryegrass is the most important pasture grass species for temperate grassland agriculture and the most important species within the genus Lolium. It is cultivated as forage grass for grazing and cutting and for amenity purposes as turf grass. Europe is generally considered as a secondary centre of origin for this species. It has been introduced to almost all the rest of the world. Compared with other field crops, grass breeding has a rather short history starting in the 1920s. Grass breeders collected their base material from permanent grassland and hitherto, ecotype collections still play an important role in broadening the genetic basis. A relevant question in this context is whether there is still sufficient genetic diversity within presently used cultivars for future breeding progress.

Perennial ryegrass is a diploid outbreeding species with a strong self-incompatibility system (Cornish et al. 1979), which assures reshuffling of alleles in each cycle of mating causing a high degree of genetic variation within populations. Homogeneity for morphological traits is an important criterion in testing for distinctness, uniformity and stability. According to the rules of the International Union for the Protection of New Varieties of Plants (UPOV), the distinction of ryegrass cultivars in plant variety protection is based on morphological characteristics and requires extensive observations during the whole growing season. As a result of selection by plant breeders, the genetic distance among new cultivars is expected to become smaller, which further hampers their identification on the basis of morphological traits (Roldan-Ruiz et al. 2001).

Isozymes have been applied to describe differences among ryegrass cultivars (Nielsen et al. 1985). Morphological traits and isozyme markers can be used to distinguish particular ryegrass populations, however, their limited number restricts their use for the distinction of closely related cultivars (Loos 1994, Roldan-Ruiz et al. 2000). In contrast, molecular markers are abundant and several studies have been reported for perennial ryegrass cultivars (Sweeney and Danneberger 1994, 1997, Huff 1997, Warpeha et al. 1998, Koelliker et al. 1999, Roldan-Ruiz et al. 2000, Guthridge et al. 2001, Kubik et al. 2001). In perennial ryegrass, random amplified polymorphic DNA markers (RAPD) have been successfully applied to distinguish among and within accessions of cultivars (Sweeney and Danneberger 1994, Huff 1997, Koelliker et al. 1999). A major advantage is their cost-effectiveness in comparison with isozymes, restriction length polymorphism (RFLP) and other PCR-based marker systems.

To distinguish populations of allogamous outbreeding species with a large amount of genetic variation, a sufficiently large number of individuals must be analysed for precise estimation of genetic distances. The minimum number of individual plants statistically required for an RAPD marker analysis is a function of the marker system (number of scorable bands, level of polymorphism) and the diversity of the cultivars sampled. For morphological discrimination, 60 individual plants per population are requested, and a sample size of 60–100 is required for isozyme analysis (UPOV 1991). However, the minimum sample size for investigation of molecular genetic diversity among and within populations of perennial ryegrass based on RAPD markers is still unknown.

The objectives of this investigation were to survey the molecular variation among and within cultivars, describe the relationship among cultivars based on genetic distances and determine the minimum sample size required for RAPD marker investigations in perennial ryegrass.

Materials and Methods
Plant materials: A total of 22 cultivars of perennial ryegrass (Lolium perenne L.) originating mainly from European breeding companies were included in this study (Table 1). Plants from seed lots were raised in a greenhouse, and after 8 weeks leaf material was harvested and lyophilized. From each of three cultivars (‘Loretta’, ‘Gremie’ and ‘Vigor’), leaf material from 60 individual plants was harvested to determine the minimum sample size prior to cultivar screening. The
three cultivars were chosen without prior knowledge of molecular diversity and distance. Following the initial primer screening for all other cultivars, 20 individual plants per cultivar were harvested.

**DNA isolation:** Genomic DNA was isolated from a total of 560 individuals. DNA isolation was performed according to Schweizer et al. (1995) with the following modifications: approximately 40 mg of lyophilized leaf material was milled in a microcentrifuge tube using a bead mill (Retsch MM 200, Retsch, Haan, Germany) to obtain fine lyophilized leaf material was milled in a microcentrifuge tube using a microcentrifuge tube and treated with RNAse (ROTH) at 37°C temperature. The aqueous supernatant was transferred to a new microcentrifuge tube and resuspended in 100 μl 1x TE buffer (10 mM TRIS-HCl, pH 8.0; 1 mM ammonium acetate/75% ethanol, and 10 mM sodium acetate/75% ethanol), dried at room temperature under sterile conditions, and finally stained with ethidium bromide, visualized on a UV transilluminator, and separated on a 1.4% agarose gel in 0.5x TBE buffer, DNA was diluted to the concentration of 2 ng DNA/μl and used for RAPD PCR amplification.

**Choice of primers:** To select suitable RAPD primers, 40 10-mer primers (ROTH GmbH, Karlsruhe, Germany) were initially screened, using five individual plants from each of the three cultivars ‘Loretta,’ ‘Gremie’ and ‘Vigor.’ Eleven primers polymorphic for all 15 individuals were subsequently tested for stability and reproducibility. Stability was tested using different amounts of DNA (5, 10 and 20 ng) as template. Reproducibility was tested using two sets of DNA being either freshly extracted or stored for 7 months. On the basis of these results, six primers were selected for routine screening (Table 2).

**DNA amplification:** Amplification reactions (Williams et al. 1990) were performed in volumes of 25 μl containing 10x PCR reaction buffer (Pharmacia, Amersham Biosciences Europe, Freiburg, Germany; 10 mM Tris-HCl, pH 8.0; 50 mM KCl, 1.5 mM MgCl2), 100 μM each of dATP, dCTP, dGTP and dTTP (Pharmacia, Amersham Biosciences Europe, Freiburg, Germany), 0.2 μM primer, 10 ng of genomic DNA and 1 unit of Taq DNA polymerase (Pharmacia). DNA amplification was performed in a PTC-100 thermocycler (MJ Research Inc., Waltham, MA, USA) for 45 cycles in the following steps: 1 min at 92°C (denaturation); 1 min at 36°C (annealing) and 2 min at 72°C (extension). RAPD analysis was carried out with the six most stable and reproducible Operon primers (ROTH GmbH) (Table 2). Amplified DNA products were separated on a 1.4% agarose gel in 0.5x TBE buffer, stained with ethidium bromide, visualized on a UV transilluminator, photographed and digitalized. Fragment size of each RAPD marker was determined by comparison with the 100 bp ladder (Pharmacia) as a standard. RAPD marker bands within the range of 300–1800 bp were scored for presence (1) or absence (0).

**Modified Rogers’ distance:** For RAPD data, the modified Rogers’ distance (MRD) was calculated according to Roldan-Ruiz et al. (2001):

\[
d_{\text{MRD}} = \sqrt{\frac{1}{4} \sum (Y_{ij} - Y_{ij}^*)^2}
\]

Table 1: Description of 22 perennial ryegrass cultivars, minimum and maximum squared Euclidean distance (E) based on RAPD marker bands, variance of E, number and sum of scorable RAPD marker bands

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbrev.</th>
<th>Type</th>
<th>Country of release</th>
<th>Year of release</th>
<th>E</th>
<th>Operon A-09</th>
<th>Operon A-11</th>
<th>Operon B01</th>
<th>Operon B12</th>
<th>Operon C04</th>
<th>Operon C13</th>
<th>Sum of scorable bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aber Elan</td>
<td>Abe</td>
<td>Forage(T)</td>
<td>UK</td>
<td>1993</td>
<td>12</td>
<td>35</td>
<td>20.24</td>
<td>12</td>
<td>8</td>
<td>14</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>Arabella</td>
<td>Ara</td>
<td>Turf(T)</td>
<td>D</td>
<td>1994</td>
<td>16</td>
<td>39</td>
<td>22.28</td>
<td>11</td>
<td>13</td>
<td>17</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Arion</td>
<td>Ari</td>
<td></td>
<td>CH</td>
<td>1993</td>
<td>18</td>
<td>36</td>
<td>16.52</td>
<td>10</td>
<td>11</td>
<td>11</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Calibra</td>
<td>Cal</td>
<td></td>
<td>F (4x)</td>
<td>1995</td>
<td>10</td>
<td>28</td>
<td>12.85</td>
<td>9</td>
<td>5</td>
<td>11</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Fennema</td>
<td>Fdn</td>
<td></td>
<td>D</td>
<td>1987</td>
<td>11</td>
<td>35</td>
<td>18.49</td>
<td>8</td>
<td>8</td>
<td>13</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Gremie</td>
<td>Gre</td>
<td></td>
<td>D</td>
<td>1974</td>
<td>16</td>
<td>37</td>
<td>15.57</td>
<td>11</td>
<td>14</td>
<td>11</td>
<td>13</td>
<td>10</td>
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<tr>
<td>Lihera</td>
<td>Lih</td>
<td></td>
<td>F</td>
<td>1981</td>
<td>11</td>
<td>32</td>
<td>15.86</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Limes</td>
<td>Lim</td>
<td></td>
<td>F</td>
<td>1984</td>
<td>13</td>
<td>41</td>
<td>22.22</td>
<td>13</td>
<td>8</td>
<td>13</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>Lipondo</td>
<td>Lip</td>
<td></td>
<td>F</td>
<td>1988</td>
<td>9</td>
<td>26</td>
<td>11.07</td>
<td>6</td>
<td>7</td>
<td>12</td>
<td>12</td>
<td>4</td>
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<tr>
<td>Livonne</td>
<td>Liv</td>
<td></td>
<td>T</td>
<td>1989</td>
<td>8</td>
<td>31</td>
<td>15.18</td>
<td>9</td>
<td>13</td>
<td>10</td>
<td>9</td>
<td>11</td>
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<tr>
<td>Loretta</td>
<td>Lor</td>
<td></td>
<td>T</td>
<td>1975</td>
<td>11</td>
<td>35</td>
<td>18.82</td>
<td>7</td>
<td>17</td>
<td>9</td>
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<td>9</td>
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<tr>
<td>Liprinta</td>
<td>Lpr</td>
<td></td>
<td>F</td>
<td>1989</td>
<td>17</td>
<td>42</td>
<td>18.00</td>
<td>12</td>
<td>10</td>
<td>10</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>Lipresso</td>
<td>Lps</td>
<td></td>
<td>D</td>
<td>1993</td>
<td>13</td>
<td>28</td>
<td>10.28</td>
<td>8</td>
<td>8</td>
<td>9</td>
<td>4</td>
<td>8</td>
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<tr>
<td>Loretanovana</td>
<td>Lrt</td>
<td></td>
<td>T</td>
<td>1996</td>
<td>11</td>
<td>29</td>
<td>15.66</td>
<td>9</td>
<td>6</td>
<td>10</td>
<td>8</td>
<td>11</td>
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<tr>
<td>Marika</td>
<td>Mar</td>
<td></td>
<td>F</td>
<td>1992</td>
<td>9</td>
<td>28</td>
<td>13.24</td>
<td>7</td>
<td>7</td>
<td>9</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Matura</td>
<td>Mat</td>
<td></td>
<td>F</td>
<td>1981</td>
<td>16</td>
<td>42</td>
<td>23.83</td>
<td>13</td>
<td>11</td>
<td>13</td>
<td>12</td>
<td>13</td>
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<tr>
<td>Moly</td>
<td>Moly</td>
<td></td>
<td>F</td>
<td>1991</td>
<td>18</td>
<td>37</td>
<td>16.07</td>
<td>11</td>
<td>8</td>
<td>12</td>
<td>13</td>
<td>11</td>
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<tr>
<td>Sirius</td>
<td>Sir</td>
<td></td>
<td>F (4x)</td>
<td>1995</td>
<td>9</td>
<td>32</td>
<td>14.98</td>
<td>10</td>
<td>11</td>
<td>15</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td>Synthetic</td>
<td>Syn</td>
<td></td>
<td>D</td>
<td></td>
<td></td>
<td>no release</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Table 2: Primer name, primer sequence, mean number of polymorphic markers and polymorphic information content (PIC) detected in 22 perennial ryegrass cultivars

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Mean number of polymorphic markers</th>
<th>PIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>OperonA-09</td>
<td>5-GGG TAA CGC C-3</td>
<td>12</td>
<td>0.44</td>
</tr>
<tr>
<td>OperonA-11</td>
<td>5-CAATCGGCT T-3</td>
<td>12</td>
<td>0.39</td>
</tr>
<tr>
<td>OperonB-01</td>
<td>5-GTGTCCGCT T-3</td>
<td>13</td>
<td>0.42</td>
</tr>
<tr>
<td>OperonB-12</td>
<td>5-CCTGATCGC A-3</td>
<td>12</td>
<td>0.41</td>
</tr>
<tr>
<td>OperonC-04</td>
<td>5-CCCTCACTA T-3</td>
<td>13</td>
<td>0.41</td>
</tr>
<tr>
<td>OperonC-13</td>
<td>5-AAGCTGTCG T-3</td>
<td>11</td>
<td>0.39</td>
</tr>
</tbody>
</table>
where $Y_{ai}$ and $Y_{aj}$ denote the frequency of a band ‘a’ in the cultivars $i$ and $j$, respectively, and summation is over the bands ($a = 1, 2, \ldots A$, with $A = 165$). In this case, MRD is proportional to the Euclidean distance based on band frequencies. MRD was computed using SAS (SAS Institute 1990). The MRD matrix was used as input data for cluster analysis based on the unweighted pair-group method of arithmetic averages (UPGMA), and to compute a principle coordinate analysis (PCoA) with the NTSYS-pe software program (Rohlf 1998). Separate PCoAs were conducted with (1) MRD values among 180 individuals of the three cultivars ‘Loretta’, ‘Gremie’ and ‘Vigor’, and (2) MRD values among the 22 cultivars.

**AMOVA:** The AMOVA was carried out with the ARLEQUIN 1.1 software (Schneider et al. 1997). The level of significance for variance component estimates was calculated by non-parametric permutation procedures (1000 permutations).

**Sample size:** Sample sizes of 5, 10, 15, 20, 25, 30 and 35 individuals were randomly chosen from an original data set consisting of 60 individuals from each of the three cultivars ‘Loretta’, ‘Gremie’ and ‘Vigor’. Euclidean distance ($E$) calculated from RAPD marker bands was determined for each pair of individuals within each cultivar. The effect of sample size $N$ on the sampling variance (var($Y_{ai}Y_{aj}$)) of the mean ($Y_{ai}$) of $E$ values was investigated with a bootstrap analysis according to Weir (1996):

$$\text{Var}_{Y}(Y_{ij}) = \frac{(n-1)/n^2 \sum (X_d - X)^2}{n}$$

where $X_d$ is the sample mean, $X$ the total mean of all samples and $n$ the number of samples used for bootstrapping. For variance estimation about $n = 100$ samples are generally regarded as sufficient (Weir 1996).

**PIC value:** The polymorphism information content (PIC) for each RAPD marker was calculated with the formula described by Roldan-Ruiz et al. (2000): PIC = $2(f(1-f))$, where PIC is the polymorphic information content of marker $i$, $f_i$ the frequency of the marker bands which were present, and $1-f_i$ the frequency of marker bands which were absent. PIC values for dominant marker bands such as RAPD markers have a maximum of 0.5 for $f_i = 0.5$ (De Riek et al. 2001).

**Results**

**Minimum sample size**

A total of 121 polymorphic markers for 180 individuals of three cultivars (‘Loretta’, ‘Gremie’ and ‘Vigor’) were generated with six primers. The discrimination power was sufficiently high to assign each individual to its respective cultivar and to distinguish the three cultivars from each other in the PCoA (Fig. 1). Mean values of squared Euclidean distance ($E$) for each sample size were rather similar in magnitude (data not shown). Bootstrap values of the sampling variance of mean $E$ decreased from about 0.045 (five individuals/sample) to 0.005 (20 individuals/sample) (Fig. 2). Increasing the sample size from 20 to 35 resulted only in a marginal reduction of the sampling variance. Optimal minimum sample size for RAPD marker investigations in *L. perenne* to distinguish cultivars unequivocally from each other was therefore determined as 20 individuals per cultivar. A parallel investigation with three ecotypes gave similar results.

**Degree of polymorphism**

A total number of 165 polymorphic marker bands was detected among the 440 individual plants of the 22 cultivars (Table 1). The mean number of polymorphic markers per primer ranged from 11 to 13, with Operon B-41 and Operon C-04 being most informative (Table 1). The number of scorable markers in individual cultivars varied from 49 (‘Marika’ and ‘Lipresso’) to 78 (‘Weigra’) (Table 1).

**Genetic distances and molecular variation**

Mean MRD among all 22 cultivars was 0.43. The smallest MRD (0.31) occurred between combinations ‘Arabella’ × ‘Weigra’, while the largest (0.58) was obtained for ‘Lipresso’ × ‘Limes’. In the UPGMA cluster analysis (data not shown), no clear grouping according to the utilization (forage or turf) or country of origin of cultivars was observed. Likewise, the two tetraploid cultivars (‘Sirius’ and ‘Calibra’) did not cluster together.

‘Lipresso’ had the lowest variance (10.28) with $E$ values ranging from 13 to 28 among individuals, while ‘Matura’ had the highest variance (23.83) with $E$ values ranging from 16 to 42 (Table 1). The intravarietal variance of ‘Arion’ with 16.52 (Table 1) was similar to the data (17.1) reported by Koelliker et al. (1999) also based on RAPDs.

The main source of molecular variation was within cultivars (66%) and only 34% was found between cultivars (Table 3).
Table 3: Analysis of molecular variance for 22 perennial ryegrass cultivars, each with 20 individuals using 165 RAPD marker bands

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>SSD(^1)</th>
<th>Variance component</th>
<th>% Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among cultivars</td>
<td>21</td>
<td>2846</td>
<td>6.17**</td>
<td>34</td>
</tr>
<tr>
<td>Within cultivars</td>
<td>418</td>
<td>5099</td>
<td>12.20</td>
<td>66</td>
</tr>
</tbody>
</table>

\(^1\) SSD, sum of squared differences. ** Significant at P = 0.01.

Fig. 3: Association among 22 perennial ryegrass cultivars revealed by PCoA analysis based on modified Rogers’ distance (MRD) calculated from RAPD data (T = turf type, 4x = tetraploid) of 20 individuals per cultivar

Principle coordinate analysis
The first (PC1) and second principle coordinates (PC2) explained 12.2 and 11.3% of the total variation among cultivars (Fig. 3). Cultivars were spread across all quadrants. A group of five cultivars was found close to the centre of the graph (‘Arion’, ‘Gremie’, ‘Weigra’, ‘Arabella’ and ‘Matura’). ‘Marika’ and the two tetraploids ‘Calibra’ and ‘Sirius’ located in quadrant II formed a separate cluster. The turf types ‘Livonne’, ‘Loretta’ and ‘Lorettanova’ were separated by PC2 and were located in quadrants I and IV. The most distinct cultivars ‘Limes’ and ‘Fennema’ were also only separated by PC2. ‘Moy’ and ‘Aber Elan’, both originating from the UK, grouped together.

Discussion
Experiments with *Lolium* cultivars have demonstrated the potential of RAPD markers as a rapid, reproducible and useful method for distinguishing among different cultivars and clustering of genotypes in the *Lolium* complex (De Loose et al. 1993) and other turf grass species (Huff et al. 1993, Wu and Lin 1994, Caetano-Anolles et al. 1995). Furthermore, their usefulness has been demonstrated in a wide range of species (Wolfe and Liston 1998). If RAPD markers are applied with appropriate controls and adjustment of the experimental conditions as carried out in this study, RAPDs can still be considered as a useful, cost-effective and highly informative marker system (Wolfe and Liston 1998). RAPDs are neutral DNA markers similar to amplified fragment length polymorphism (AFLP) markers and are not necessarily linked to functional genes like cleaved amplified polymorphic and RFLP markers. Other DNA markers such as simple sequence repeat markers (SSR, microsatellites) are also straightforward and economical in their application; however, they require a high advance input for their development because the markers have to be generated from genomic DNA sequences. While during recent years extensive genomic and EST sequence data from most species of economic importance including perennial ryegrass have been generated, this information is largely proprietary to companies and research institutions, and is not publicly available. Thus, for a first overall estimate of the genetic diversity of a species, RAPDs are a valuable tool, as no prior knowledge of the genomic sequence is necessary. Additionally, in an RAPD experiment with a number of different primers the complete genome is scanned for marker polymorphisms, whereas in RFLP or SSR experiments only a smaller and potentially biased part of the genome is sampled, depending on the choice and number of these markers.

In allogamous crop species, a high intrapopulation variation is expected, which requires a suitable minimum sample size per population (Marshall and Brown 1975). According to the results of this study the minimum population size for an RAPD marker genetic diversity study was 20 individuals based on the variance of estimates by bootstrapping and determination of the standard error. For the three cultivars involved in the minimum sample size test, no genotype duplicates were found. In the PCoA, the individual plants clustered into three groups corresponding to the cultivars investigated (Fig. 1). With closely related cultivars, it might be necessary to increase the sample size, although the three cultivars underlying Figs 1 and 2 were located in close vicinity in Fig. 3, suggesting that their genetic distance was only moderate.

In clone or line cultivars, one to several individuals will be representative of a specific cultivar because they are genetically homogeneous. However, more than 15 plants per population in outcrossing species are suggested (Morell et al. 1995). Therefore, in outcrossing cultivars, only occasionally are there differences for fixed bands and frequency differences accounting for most of the genetic divergence. In the allogamous tetraploid crop alfalfa (*Medicago sativa* L.), 25 RAPD markers and 40 individuals were sufficient to differentiate closely related populations (Gherardi et al. 1998) using the variance of the genetic distance as an estimator in four hypothetical systems like RFLP (alfalfa: Labombada et al. 2000) and SSRs (perennial ryegrass: Kabik et al. 2001), different optimal sample sizes were found. This indicates the dependency of the minimum sample size on the marker system. In the latter marker types, the appropriate number of markers also depends on the choice of the specific markers, because the targeted regions should represent the whole genome. As indicated above, this is the case with RAPDs if a larger number of different primers is applied which take potential codon bias into account. Thus, for each species and specific marker system, a sample size test is required prior to a diversity study.

To analyse ryegrass genomes with RAPD markers, a selection of primers that provide sufficient and reliable information is required. The six primers in this study amplified 165 polymorphic markers in 22 cultivars, which is comparable to a study with perennial and Italian ryegrass genotypes using seven RAPD primers (Depicker et al. 1994). The degree of polymorphism in the present study was 88%, which is slightly
higher than the findings of Roldan-Ruiz et al. (2000) for AFLPs (83%). For individual cultivars the number of polymorphic markers varied from 49 to 78. The PIC values for six primers were on average 0.41 indicating their high discriminative power. This value is much higher than the value given by Roldan-Ruiz et al. (2000) for AFLPs (PIC = 0.28). In the current study the smallest difference between a pair of individuals was 11 markers compared with a turf grass cultivar study with 33 RAPD markers (Huff 1997), where a minimum difference of four markers was found. The overall within-cultivar variation accounts for 66%, which is much lower than the results reported by Kubik et al. (2001) with 85.35% and Guthridge et al. (2001) with 79.6% based on SSRs and AFLPs respectively. One reason could be the larger number of populations investigated in the study reported here. A second reason could be the genetic structure of the material itself and the marker types used (Ferdinandez et al. 2001).

In the PCoA (Fig 3) the turf types ‘Loretta’ and ‘Loretta-nova’ were not clearly distinguishable from forage type cultivars, which is in accordance with the findings of Roldan-Ruiz et al. (2000). These authors deemed an association of AFLP markers with specific traits unlikely because AFLPs are neutral DNA markers not necessarily linked to particularly AFLP markers with specific traits unlikely because AFLPs are neutral DNA markers not necessarily linked to particularly distinguishing features of the cultivar. On the other hand, a larger number of populations were used in the present study. Therefore, for a more detailed classification of these cultivars, the use of RAPD markers is necessary. A method to measure genetic distance between allogamous populations of alfalfa (Medicago sativa) using RAPD markers was presented by Huff et al. (1997).


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