Molecular genetic diversity within and among German ecotypes in comparison to European perennial ryegrass cultivars

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Abstract

Perennial ryegrass (Lolium perenne L.) is the most important grass species for temperate grassland agriculture. The level and distribution of genetic variation in gene bank ecotype collections is still largely unknown but of great interest for the planning of breeding programmes. The objectives of this study were to (i) assess the molecular variance and population structure of German ecotypes at the regional and population level, (ii) assign ecotypes to germplasm pools and (iii) compare the relationship between German ecotypes and previously-investigated European cultivars of perennial ryegrass. A total of 22 ecotypes originating from three geographic areas in Germany, each with a sample size of 20 individual plants, were investigated with 156 polymorphic RAPD markers. Genetic distance among ecotypes ranged from 0.27 to 0.48. An analysis of molecular variance (AMOVA) revealed a much larger variation within populations (71%) than among them (29%). Ecotypes from North Germany were significantly different from those of South and Middle Germany. Thus, two distinct germplasm pools could be identified. The 22 ecotypes and 22 previously investigated cultivars shared 98% of the molecular variance.

Key words: Lolium perenne—AMOVA—diversity—ecotypes—genetic distance—geographic distance—RAPD marker

Perennial ryegrass (Lolium perenne L.) is the most important grass species in temperate climates of the world. It is extensively cultivated for forage and amenity purposes. Like other crop species, perennial ryegrass originates from the Near East and has invaded most parts of Europe (Balfourier et al. 2000). Perennial ryegrass is a diploid species (2n = 2x = 14) with a two-locus self-incompatibility system, which ensures a high degree of genetic variation in populations. Grass breeders collected their base materials from permanent grassland and until now ecotypes are still intensively used in breeding programmes for cultivar development. Information on the level and distribution of genetic diversity in crop species is important for the selection of parental materials and optimum strategies of preservation of germplasm in gene banks (Gunter et al. 1996). Instead of maintaining thousands of accessions in gene banks, it was suggested by Brown (1989) to extract ca. 10% of the accessions into ‘core collections’ which would cover most of the variation. Based on a collection gathered all over France, Charmet and Balfourier (1995) demonstrated that a 5% core collection of perennial ryegrass (25 ecotypes) contained 92% of the total variation as estimated by phenotypic and geographical clustering. For plant breeding purposes, it was suggested to pool accessions according to ‘ideotypes’ (Guy et al. 1989, Paul 1989). In practice, time of flowering is the most important character for the grouping of germplasm to establish gene pools. Following this approach, breeders combined both closely related and unrelated materials. This might be one of the reasons for the rather low genetic diversity among perennial ryegrass cultivars (Roldan-Ruiz et al. 2001).

The usefulness of the stored accessions depends on sufficient characterisation. While the value of morphological or other traits as evaluation measures may vary according to the intended use of the material, molecular characterization of genetic diversity provides base information which can be used to select a promising range of accessions for different breeding programmes (Roldan-Ruiz et al. 2001). This is the first study to probe molecular genetic diversity in perennial ryegrass ecotypes collected in Germany. Hitherto, description and classification of perennial ryegrass cultivars and ecotypes was mainly based on morphological traits (Balfourier and Charmet 1991, Loos 1994, Fernando et al. 1997) or isozymes (Charmet et al. 1993, Loos 1994, Fernando et al. 1997). Molecular DNA markers such as Random Amplified Polymorphic DNA (RAPD; Huff 1997, Bolaric et al. 2005) and Amplified Fragment Length Polymorphism (AFLP; Roldan-Ruiz et al. 2001) have been used for diversity and identification studies in perennial ryegrass cultivars. Far more genetic variation has been found within than between populations in allogamous species. Thus the diagnostic use of markers for the discrimination of populations is generally not possible, in contrast to the discrimination of single individuals within and among populations. Only a few studies have investigated the molecular genetic diversity among perennial ryegrass ecotypes (Creswell et al. 2001, Skøt et al. 2002, Ghesquiere et al. 2003). It would seem that no comparison between cultivars and ecotypes of perennial ryegrass has been published so far.

The objectives of this study were to (i) assess the molecular variation and population structure of ecotypes from Germany at the regional and population level, (ii) assign ecotypes to germplasm pools and (iii) compare the genetic relationships between ecotypes and cultivars of perennial ryegrass.

Materials and Methods

Plant materials: A total of 22 perennial ryegrass (Lolium perenne L.) ecotypes were analysed in this study. They originated from a collection covering three different geographic regions of Germany (Oetmann 1994) and were specified according to their origin from the North (N),

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Morphological traits: Oetmann (1994) assessed the morphological traits used in this study to estimate morphological distances. The phenotypic data were derived from the plants raised from the tiller collection. Measured traits were: date of ear emergence (days), length of flag leaf (cm), width of flag leaf (cm), ear length (cm), number of spikelets, length of the uppermost internode (cm). Scored traits were: winter hardness, growth type, number of tillers, regeneration ability after first harvest, leaf width and number of ears after first harvest.

DNA isolation: Genomic DNA was isolated from a total of 440 individuals (Bolaric et al. 2005). The presence of high molecular weight genomic DNA was verified using 0.8% agarose gel. The DNA resuspension was diluted to a concentration of 2 ng DNA/μl and used for RAPD-PCR amplification.

DNA amplification: DNA amplification was carried out with six Operon primers (Roth GmbH) (Table 2) as previously used for the European perennial ryegrass cultivars (Bolaric et al. 2005). Amplified DNA products were separated on a 1.4% agarose gel in 0.5X TBE buffer. The DNA resuspension was diluted to a concentration of 2 ng DNA/μl and used for RAPD-PCR amplification.

Results

Degree of polymorphism

A total of 156 polymorphic marker bands were detected among the 440 individual plants of the 22 ecotypes. For the European cultivars, 165 polymorphic bands were found (Bolaric et al. 2005). In the joint analysis of ecotypes and European cultivars, 169 polymorphic bands were used (Table 2). The additional bands in the joint analysis were derived from the European cultivars. From these, 20 marker bands were common across all 22 ecotypes and 17 across ecotypes and cultivars. The number of scorable markers in individual ecotypes of the three regional groups ranged from 51 to 80 (N), 50 to 67 (M) and 52 to 65 (S), respectively.

Modified Rogers’ distance: Modified Rogers’ distance (MRD) was calculated as described previously (Bolaric et al. 2005):

\[ d_{MRD} = \sqrt{\frac{\sum (Y_{ai} - Y_{aj})^2}{A}} \]

where \( Y_{ai} \) and \( Y_{aj} \) denote the frequency of a band ‘a’ in populations \( i \) and \( j \), and summation is over the bands (\( a = 1, 2, \ldots, A \), with \( A = 156 \)). In this case, MRD is proportional to the Euclidean distance on band frequencies. MRD was computed with SAS (SAS 1990).

Mantel test: The correspondence between pairs of matrices based on geographic, phenotypic or molecular distances were tested using the Mantel Z-statistic (Rohlf 1998).

Cluster and principle coordinate analyses: The distance matrices were used as input data for cluster analysis based on the unweighted pair group method of arithmetic averages (UPGMA) and to perform a principle coordinate analysis (PCoA) with the NTSYS-pc software program (Rohlf 1998).

AMOVA: An analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was carried out using the ARLEQUIN 1.1 software (Schneider et al. 1997). The level of significance for variance component estimates was determined by non-parametric permutation procedures using 1000 permutations.

Table 1: Description of 22 perennial ryegrass ecotypes originating from the North (N), Middle (M) and South (S) of Germany based on 156 RAPD markers, sum of scorable RAPD marker bands and within-ecotype diversity expressed as mean-square deviations (MSD)

<table>
<thead>
<tr>
<th>Name</th>
<th>Collection area</th>
<th>Genebank Accession</th>
<th>Oetmann population</th>
<th>Sum of scorable bands</th>
<th>MSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>Wittmund</td>
<td>GR 5097</td>
<td>OE 38</td>
<td>80</td>
<td>19.60</td>
</tr>
<tr>
<td>N2</td>
<td>Westerstede</td>
<td>GR 5111</td>
<td>OE 88</td>
<td>70</td>
<td>22.23</td>
</tr>
<tr>
<td>N3</td>
<td>Leer</td>
<td>GR 5114</td>
<td>OE 99</td>
<td>76</td>
<td>24.66</td>
</tr>
<tr>
<td>N4</td>
<td>Cuxhaven</td>
<td>GR 5110</td>
<td>OE 85</td>
<td>51</td>
<td>21.27</td>
</tr>
<tr>
<td>N5</td>
<td>Emden</td>
<td>GR 5099</td>
<td>OE 48</td>
<td>70</td>
<td>25.67</td>
</tr>
<tr>
<td>N6</td>
<td>Emden</td>
<td>GR 5100</td>
<td>OE 52</td>
<td>52</td>
<td>14.93</td>
</tr>
<tr>
<td>N7</td>
<td>Norden</td>
<td>GR 5113</td>
<td>OE 98</td>
<td>71</td>
<td>28.49</td>
</tr>
<tr>
<td>N8</td>
<td>Leer</td>
<td>GR 5091</td>
<td>OE 1</td>
<td>74</td>
<td>27.21</td>
</tr>
<tr>
<td>N9</td>
<td>Malchow/Poel</td>
<td>GR 5092</td>
<td>OE 17</td>
<td>65</td>
<td>22.16</td>
</tr>
<tr>
<td>N10</td>
<td>Stade</td>
<td>GR 5098</td>
<td>OE 41</td>
<td>68</td>
<td>24.61</td>
</tr>
<tr>
<td>N11</td>
<td>Bremervörde</td>
<td>GR 5101</td>
<td>OE 65</td>
<td>51</td>
<td>16.80</td>
</tr>
<tr>
<td>N12</td>
<td>Cuxhaven</td>
<td>GR 5102</td>
<td>OE 66</td>
<td>59</td>
<td>23.69</td>
</tr>
<tr>
<td>M1</td>
<td>Hersfeld</td>
<td>GR 5093</td>
<td>OE 20</td>
<td>54</td>
<td>20.45</td>
</tr>
<tr>
<td>M2</td>
<td>Eschwege</td>
<td>GR 5109</td>
<td>OE 83</td>
<td>50</td>
<td>19.70</td>
</tr>
<tr>
<td>M3</td>
<td>Eschwege</td>
<td>GR 5104</td>
<td>OE 70</td>
<td>67</td>
<td>21.56</td>
</tr>
<tr>
<td>M4</td>
<td>Eschwege</td>
<td>GR 5103</td>
<td>OE 68</td>
<td>58</td>
<td>21.79</td>
</tr>
<tr>
<td>M5</td>
<td>Kempten</td>
<td>GR 5105</td>
<td>OE 74</td>
<td>62</td>
<td>24.71</td>
</tr>
<tr>
<td>M6</td>
<td>Kempten</td>
<td>GR 5094</td>
<td>OE 27</td>
<td>59</td>
<td>16.24</td>
</tr>
<tr>
<td>S3</td>
<td>Weilheim</td>
<td>GR 5106</td>
<td>OE 76</td>
<td>65</td>
<td>21.24</td>
</tr>
<tr>
<td>S4</td>
<td>Kempten</td>
<td>GR 5107</td>
<td>OE 77</td>
<td>62</td>
<td>25.52</td>
</tr>
<tr>
<td>S5</td>
<td>Kempten</td>
<td>GR 5095</td>
<td>OE 28</td>
<td>60</td>
<td>22.76</td>
</tr>
<tr>
<td>S6</td>
<td>Kempten</td>
<td>GR 5108</td>
<td>OE 79</td>
<td>52</td>
<td>18.62</td>
</tr>
</tbody>
</table>
In cultivars, 49–78 scorable markers were found. The mean number of polymorphic markers per primer ranged from 9 to 13 (cultivars: 11–13). Primer A-11 generated on average only nine bands in ecotypes, but 12 bands in cultivars. The Operon primers B-01 and C-04 were most informative in both sets of materials. PIC values ranged from 0.33 to 0.40, being lower than in cultivars (0.39–0.44).

### Genetic distance and molecular variance

Mean MRD among all 22 ecotypes was 0.38. The largest MRD (0.48) occurred between combinations N6 and S2, while the smallest (0.27) was obtained between M4 and S6. The group means for MRD were 0.38 (N), 0.34 (M) and 0.35 (S). In the dendrogram based on MRD (figure not shown), no clear grouping of the ecotypes according to their regions of origin was found.

The molecular variance among the group of cultivars and ecotypes accounted for only 2% of the variation (Table 2). The variance among populations of both groups (ecotypes and cultivars) accounted for 31% of the total molecular variance, while 67% of the variance was within populations. The molecular variance within cultivars (67%) and within ecotypes (71%) was almost identical. However, in absolute values, cultivars displayed a higher molecular variance than ecotypes (18.33 vs. 15.98). In a separate AMOVA (data not shown) the three regional groups of ecotypes were compared. Those from Middle and Southern Germany were not significantly different from each other, and thus can be classified as one group or gene pool. Comparing this pool with ecotypes from North Germany, a significant difference of 4% of the variance could be detected. Mean-square deviations (MSD) extracted from the AMOVA partitioning of the variance within ecotypes provided a measure of genetic variation within each population (Table 1). MSDs ranged from 14.93 (N6) to 28.48 (N7).

### Principle coordinate analysis (PCoA)

The first (PC1) and second principle coordinate (PC2) explained 16.1 and 11.3% of the total molecular variation of the ecotypes (Fig. 1). Ecotypes from the Middle and the South of Germany formed a cluster mainly assigned to quadrant IV. The northern group had a markedly wider spread across PC1 than the Middle and South German groups. The most distinct ecotypes N6 and N11 were separated from all other North German accessions by both PC1 and PC2, and were located in quadrant I. Ecotypes closely positioned in the two-dimensional PCoA were clearly separated by the third principle coordinate which accounted for 7.5% of the total molecular variation.

In the PCoA based on morphological distances among the ecotypes, the first (PC1) and second principle coordinate (PC2) explained 85.5 and 8.7% of the total variation (graph not shown). PC1 reflected the grouping of the collection according to flowering time as observed in the field from the earliest (S1) to the latest (N6) ecotype. The cluster analysis did not separate the Northern populations from those from the Middle and the South of Germany.

In the joint PCoA of the molecular data of 22 European cultivars and 22 German ecotypes, PC1 and PC2 explained 8.6 and 7.6% of the total molecular variation (Fig. 2). Most ecotypes formed a cluster in quadrants II and III, whereas most cultivars clustered in quadrants I and IV. However, there was also substantial overlap among the two clusters in the centre of the graph.
Mantel test
For the ecotypes, the Mantel Z-statistic was significant (P < 0.01) for the comparison between morphological vs. molecular distance (r = 0.10), and also for the comparison between morphological vs. geographic distance (r = 0.36). Associations between MRD and geographic distance (r = 0.08) were not significant.

Discussion
Genetic resources in perennial ryegrass
Permanent pastures in Europe are less than 1000 years old (Scholz 1975) and with intensified land use, land-races have been used for re-seeding. Systematic grass breeding in Germany started around 1920. Ecotypes from native grassland species were collected and, after phenotypic selection and recombination, open-pollinated varieties were created. These improved populations were used for over seeding or re-sowing old pastures. Cultivars were widely spread by the seed trade, and also used in regions far beyond their origin. During the past 40 years a large area (about 300 000 ha p.a.) of old permanent grassland in Germany was re-sown with new cultivars (Posselt 2000). In some areas, heavily grazed pastures are re-established every 3-5 years. Consequently, a large number of ecotypes have been ousted and replaced by more uniform cultivars. At the same time, breeding programmes based on heterogeneous populations of perennial ryegrass largely depend on sufficient genetic variation. To meet this challenge, the remaining natural genetic diversity has to be monitored, and measures have to be taken for its conservation in order to sustain future breeding objectives and changing requirements.

Genetic diversity: ecotypes
In this study, genetic diversity was investigated in 22 ecotypes using 20 individual plants per ecotype and six RAPD primers, which gave 156 polymorphic markers. Each pair of individuals was distinguished by at least seven markers. Similarly, in an earlier perennial ryegrass RAPD study with 33 polymorphic markers (Huff 1997), all individual plant pairs were distinguished by at least four markers. In this new study, plants from the same population have clustered together without any overlap between populations (dendrogram not shown). Thus, cultivar-breeding programmes based on heterogeneous populations of perennial ryegrass were expected to have high molecular diversity. However, no overlap between populations was found. Some groups of polymorphic bands formed patterns, which were characteristics of populations, individuals within these populations were distinguished by other independently varying bands. Cresswell et al. (2001) reported a similar mode of marker variation in an investigation of three perennial ryegrass accessions with AFLPs. All individuals clustered according to their accession and no population-specific markers were found.

The genetic distance between accessions from different regions, e.g. M4 from Middle Germany vs. S6 from the South of Germany (MRD 0.27), was smaller than the smallest genetic distance between accessions from the same region, e.g. S6 vs. S4 (MRD 0.30). Similar results were reported by Cresswell et al. (2001).

Based on the geographical origin of the ecotypes, a structured hierarchical AMOVA was applied to the data set. The largest fraction of variation was found within ecotypes (71%) and a smaller variation among ecotypes (29%), which is in accordance with an ecotype study in fescue (Kölliker et al. 1999), and is expected for an allogamous species. In an AFLP marker study of 80 perennial ryegrass ecotypes originating from 17 European countries, Ghesquiere et al. (2003) detected 93.8% of the variation within ecotypes. This high percentage of within accession variation could be due to the fact that only 10 individual plants per population were investigated. Differences among accessions within countries accounted for 3.6% and between countries for only 2.5% of the total variation.

In the PCoA graph based on RAPD data, the first two dimensions accounted for 27% of the total variation. This was sufficient to group the ecotypes roughly according to geographical origin (Fig. 1). The ecotypes from the North were clearly separated from those from the Middle and the South of Germany, which built a mixed group. This clustering of the molecular data in the study was not confirmed by a similar clustering of the morphological data from the original collection (graph not shown). In an AFLP marker study analysing 54 populations of perennial ryegrass, Skot et al. (2002) found some clustering according to geographic origin in their PCoA graph representing 23% of the total variation, but overall the association between molecular and geographical data was weak. In an investigation with several Lolium species collected in Portugal using AFLP markers (Cresswell et al. 2001) no association between molecular and geographic distance was found. In the present study too, a very poor association (r = 0.08) between MRD and geographic distance was observed. The morphological data of Oetmann (1994) were only weakly associated with the molecular distances in the current study (r = 0.10). This is in agreement with a perennial ryegrass cultivar study using AFLP and STS molecular markers (Roldan-Ruiz et al. 2001). These authors argued, that AFLPs are neutral markers, not being linked to genes underlying the phenotypic traits. The same is valid for RAPD markers.

Genetic diversity: ecotypes vs. cultivars
The overlap in molecular diversity between cultivars and ecotypes (Table 2) was unexpectedly high (98%). In a joint UPGMA cluster analysis of ecotypes and cultivars, only the cultivars ‘Loretta’, ‘Fennema’ and ‘Limes’ were clearly separated (dendrogram not shown). Thus, cultivar-breeding programmes largely exhausted the molecular variation present in the three German regions investigated. On the other hand, one could argue that plant breeders’ activities maintained the level of molecular diversity present in ecotypes. During the selection process, breeders have to consider DUS (distinctness, uniformity, stability) requirements. In particular, the potential parents for a cultivar are selected for uniformity. One can speculate that this assortative mating resulted in directional selection which reduced the within population variance of cultivars as compared to ecotypes (67% vs. 71%, respectively) (Table 2). For individual populations, the within ecotype variation (Table 1) expressed as mean square deviations (MSD) ranged from 14.93 (N6) to 28.48 (N7), while the MSDs for cultivars (Bolaric et al. 2005) ranged from 10.28 (‘Lipresso’) to 23.83 (‘Matura’). The cultivar ‘Lipresso’ was developed from a single ecotype population collected in the same area as the South German accessions of this study (Feuerstein, Pers. communication) and it is interesting to note, that they cluster closely together (Fig. 2). Since the ecotypes...
had been multiplied at the gene bank in populations of 100 plants, the large differences between within and among population variance reflect the evolution of these ecotypes. In contrast to current results, in a recent AFLP study with meadow fescue (Festuca pratensis Huds.) less variation within Norwegian ecotypes was found (69.2%) as compared to cultivars (79.6%) (Fjellheim et al. 2003). The authors explained this result by the spatial isolation of individual ecotypes and by the creation of new cultivars from intermating different ecotypes.

Gene pool structure and utilization

According to the results of this study, German ecotypes can be divided into two major groups based on RAPD markers. They correspond to two possible gene pools: North German accessions vs. Middle and South German accessions. However, it still needs to be shown whether or not these groups are gene pools in the sense that combinations would give a positive heterotic response in breeding programmes. For quantitative characters such as yield, heterotic response is expected to increase with the parental genetic distance (Melchinger 1999). Following this assumption, maximal heterosis could be expected by crossing ecotypes from North Germany with genetically distant ecotypes from Middle or Southern Germany. The molecular characterization of populations and elite materials could be a useful pre-screening method in a breeding programme to fully exploit existing diversity and to maximise heterosis in newly developed cultivars. Kolliker et al. (2004) carried out a molecular assessment of elite clones as potential parents of new cultivars within maturity groups using AFLPs. Based on the molecular genetic distances, synthetics with low and high diversity among their parents were constructed. So far, no difference in phenotypic heterogeneity was evident among the offspring.

Furthermore, genetic resources from other gene pools might be useful in introducing quality traits, e.g., high sugar levels, into cultivars. Consequently, to gain a broader knowledge of perennial ryegrass genetic resources, ecotypes from other regions of Germany and Europe should be investigated with the same marker system. According to the results of this study, RAPDs would provide a valuable tool for the molecular characterisation of gene bank accessions.

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