Comparative Analysis of Genetic Similarity between Perennial Ryegrass Genotypes Investigated With AFLPs, ISSRs, RAPDs and SSRs

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Abstract: Perennial ryegrass (*Lolium perenne* L.) is the most important grass species used in temperate grassland agriculture. Our objective was to obtain an overview of the genetic relationships between 20 individual genotypes of perennial ryegrass of diverse origins, using amplified fragment length polymorphism (AFLP), inter-simple sequence repeat (ISSR), random amplified polymorphic DNA (RAPD) and two sets of simple sequence repeat (SSR) markers. All 20 individuals were uniquely fingerprinted by all four marker systems and comparisons were made on the basis of 85 markers each. Mean genetic similarities were estimated at 0.31, 0.43, 0.23 and 0.15 for AFLPs, ISSRs, RAPDs and SSRs, respectively. Cophenetic values resulted in good (AFLP and SSR-B = 0.88) to moderately good fits (ISSR = 0.76, RAPD = 0.70, and SSR-A = 0.79). Comparing the four marker systems to each other, AFLP and SSR-A were correlated best (r = 0.57). All other comparisons revealed rather low correlation coefficients in the Mantel Z test. With twice as many markers cophenetic values increased to a very good fit for AFLPs (0.90) and SSRs (0.92).

Keywords: marker comparison; AFLP; ISSR; RAPD; SSR; *Lolium perenne* L.

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 as turf grass for amenity purposes. Europe is generally considered as a centre of diversity for this species. It has been introduced to almost all the rest of the world. Compared to other field crops, grass breeding has a rather short history starting in the 1920s. Perennial ryegrass is a diploid outbreeding species with a strong self-incompatibility system (Cornish *et al.* 1979), which assures recombination of alleles in each cycle of mating, causing a high degree of genetic variation within populations.

To date several different marker systems have been applied to perennial ryegrass populations: RAPDs (Huff

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1997; Bolaric *et al.* 2005), AFLPs (Roldan-Ruiz *et al.* 2000), ISSRs (Ghariani *et al.* 2003) and SSRs (Kubik *et al.* 2001). No comparison of these marker types has been done in perennial ryegrass so far.

The first comparative analysis of different marker types (RFLPs, RAPDs, AFLPs, SSRs) was published by Powell *et al.* (1996), investigating the soybean germplasm. Similar investigations were carried out in other species, mainly inbreeders like barley (Russell *et al.* 1997), wheat (Bohn *et al.* 1999), rice (Virk *et al.* 2000), or inbred lines of maize (Pejic *et al.* 1998; Lübberstedt *et al.* 2000). A comparison of different markers in an outcrossing species was published recently (Budak *et al.* 2004).

The objectives of this investigation were to analyse the molecular similarity between individual genotypes and to compare the discriminatory power of four marker systems with an equal number of markers.

MATERIALS AND METHODS

Plant materials

A total of 20 genotypes of perennial ryegrass (*Lolium perenne* L.) originating from various sources (Table 1) were included in this study.

DNA isolation

Genomic DNA was isolated from leaf material using a modified CTAB protocol (Saghai-Maroof *et al.* 1984 modified by CIMMYT Applied Molecular Genetics Laboratory).

Table 1. Description of the *Lolium* Test Set (LTS)

Code	Name	Type	Specificity	Reference	Provided by
LTS01	G00612	forage	parent mapping population in NIMGRASS		1
LTS02	G00559				1
LTS03	Oko Falster	forage	parent mapping population in a national project	Jensen *et al.* (2005)	2
LTS04	Veyo				2
LTS05	DLF5	turf			3
LTS06	DLF6				3
LTS07	G00851	forage	pParent mapping population		1
LTS08	G00852				1
LTS09	WSC17-03	forage	sSelected genotype from WSC mapping population	Thorogood *et al.* (2004)	4
LTS10	ILGI 80		ILGI mapping population	Thorogood *et al.* (2002)	4
LTS11	Lp 34-551	turf	colchicine induced mutant	Pasakinskiene (2005)	5
LTS12	INRA1	forage	parent mapping population	Barre *et al.* (2001)	6
LTS13	INRA2				6
LTS14	INRA3	turf			6
LTS15	INRA4	ecotype	mediterranean origin: Greece		6
LTS16	INRA5		nordic origin: Sweden		6
LTS17	WSC 22/9	forage	selected genotype from WSC mapping population	Turner *et al.* (2006)	4
LTS18	WSC 23/9				4
LTS19	ILGI P150/112 74	forage	selected genotype from ILGI mapping population		4
LTS20	ILGI P150/112 166				4

Provided by: 1 – PRI, Wageningen, NL; 2 – DIAS, Slagelse, DK; 3 – DLF, Store Heddinge, DK; 4 – IGER, Aberystwyth, UK; 5 – LIA, Kedainiai, LT; 6 – INRA, Lusignan, FR

DNA amplification

AFLPs. PCR amplifications were performed on a PTC 100 thermocycler (MJ Research). The AFLP kit from GIBCO-BRL LifeTechnologies with labelled primers from LI-COR Biosciences was used following the manufacturer's instructions. The electrophoresis was performed on a LI-COR 4200 IR² (Sciencetech). Five primer pairs were used: E32M50, E35M47, E35M60, E38M60 and E41M50.

ISSR. The PCR mix contained 50 ng DNA, 0.2μM primer, 0.2mM of each dNTP, 0.5 u DyNAzyme™ II polymerase, 2mM $MgCl_2$ and 1× PCR buffer (all Finnzymes Oy) in a 25-μl volume. PCR reactions were carried out with a GeneAmp® 2700 thermocycler (Applied Biosystems) and the following cycle profile: initial denaturation – 2 min at 95°C, followed by 40 cycles of 30 s at 95°C, 1 min at 50°C and 30 s at 72°C, final extension – 6 min at 72°C. PCR products were separated on a 1.5% agarose gel with ethidium bromide added. Samples were run for 2 h at 4V/cm in TAE buffer. Twelve anchored primers were used to generate ISSRs, out of which 8 primers had dinucleotide repeat motifs: $(TC)_8A$, $(TC)_8C$, $(TC)_8G$, $(AC)_8T$, $(AC)_8G$, $(AC)_8YA$, $(CT)_8RG$, $(TG)_8GA$; 2 primers – trinucleotide ones: $(TCC)_5GT$, $(ATG)_5GA$; and 2 primers – tetranucleotide motifs: $(GACA)_4GT$ and $(AGAC)_4GC$.

RAPD. The analysis was carried out with six Operon primers (A-09, A-11, B-01, B-12, C-04, and C-13; Roth GmbH) as described by Bolaric *et al.* (2005). Amplified DNA products were separated on a 1.4% agarose gel in 0.5× TBE buffer, stained with ethidium bromide, visualised on a UV transilluminator, photographed and digitised.

SSR. Published *Lolium* SSR primers (M4-136, M4-213, M12-52, M15-185, M16-B (Kubik *et al.* 1999); LP165, LP194, M144, M10138, PR10, PR14, PR24 (Kubik *et al.* 2001)) were used in the comparative study (set A). A second set of primers (set B) included not yet publicly available (ViaLactia Bioscience, NZ) ones. Published SSRs were analysed following the procedures provided with them and visualised with Silver Sequence DNA staining. Gels were digitised and scored by eye. The ViaLactia SSRs were amplified individually with fluorescent primers (thermal cycling was performed beginning with 10 min at 95°C, followed by 10 cycles of 60 s at 94°C, 30 s at annealing temperature, 60 s at 72°C with the annealing temperature decreasing by 1°C per cycle, followed by 25 cycles of 30 s at 94°C, 30 s at final annealing temperature, 30 s at 72°C and 10 min at 72°C; the annealing temperatures were dependent on the primer pair used) and multiplexed on an ABI 3100 sequencer. Marker bands were identified with ABI Prism Genotyper 3.7 software.

Statistical analysis

The banding patterns of all four marker systems were scored in a dominant manner as present (1) or absent (0) for each fragment. Only polymorphic markers were considered in the data analysis even though monomorphic bands were scored. Genetic similarity (*GS*) between genotypes was measured according to Jaccard (GS_J):

$$GS_{ij} = N_{ij}/(N_i + N_j - N_{ij})$$

where:

N_i – number of detected bands in genotype *i* and not in genotype *j*

N_j – number of detected bands in genotype *j* and not in genotype *i*

N_{ij} – number of bands common to genotypes *i* and *j*

Dendrograms of 20 LTS genotypes were constructed from the GS_J similarity matrix by the UPGMA clustering method and cophenetic values were calculated. The correspondence between pairs of matrices was tested with the Mantel Z statistic based on 1000 runs. All computations were performed with NTSYSpc (Rohlf 2000).

RESULTS AND DISCUSSION

AFLPs

The five primer pairs generated 222 bands, out of which 166 were polymorphic (75%). Genetic similarities between 20 genotypes ranged from 0.52 to 0.89 with a mean of $GS_J = 0.59$. On the basis of 85 markers, GS_J ranged from 0.09 to 0.82 with a mean of 0.31 (Table 2). Powell *et al.* (1996) and Lübberstedt *et al.* (2000) also found high *GS* estimates for AFLPs as compared to other markers. For an AFLP dendrogram (Figure 1a), the cophenetic correlation was estimated at $r = 0.88$, corresponding to a good fit. Using all markers available, the cophenetic correlation yielded $r = 0.90$, corresponding to a very good fit. Both in wheat (Bohn *et al.* 1999) and in inbred lines of maize

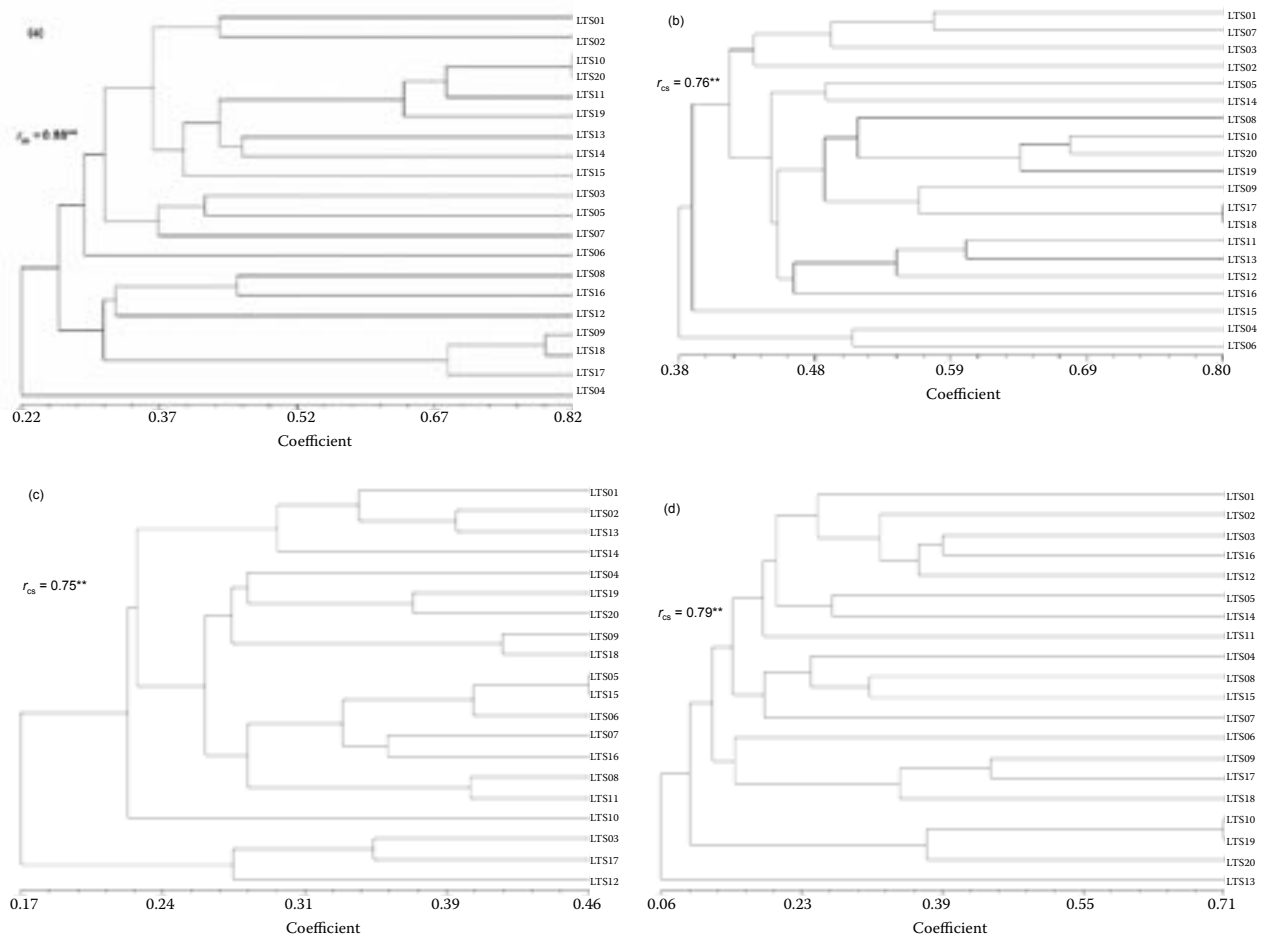

Figure 1. Dendrograms and cophenetic values (r_{cs}) of 20 individual genotypes of perennial ryegrass revealed by UPGMA cluster analysis based on genetic similarities (GS_J) calculated from (a) AFLP, (b) ISSR, (c) RAPD, and (d) SSR (set A) marker data from 85 markers each

(Pejic *et al.* 1998), the cophenetic correlation of r = 0.83 was estimated for AFLPs, based on 117 and 232 polymorphic markers, respectively.

The highest similarity (GS_J = 0.82) was found between genotypes LTS 10 and 20. Together with LTS 19 they formed a distinct subcluster (Figure 1a). All three genotypes originate from the ILGI-mapping population. A second distinct subcluster contained LTS 9, 17 and 18, belonging to the WSC-mapping population.

ISSRs

12 primers generated a total of 88 fragments, out of which 85 were polymorphic (97%). GS_J ranged from 0.25 to 0.80 with a mean of 0.43. The highest similarity was found for LTS 17 and 18 (r = 0.80). For an ISSR dendrogram (Figure 1b) the cophenetic correlation was estimated at 0.76. This is much lower than the value (0.90) given by Budak *et al.* (2004) investigating buffalo grasses. However, these authors used 207 markers generated from 30 ISSR primers. As with AFLPs, ISSRs identified related genotypes (members of the ILGI and WSC populations), which grouped into particular subclusters in the dendrogram (Figure 1b).

When all the AFLP and ISSR data were combined in one similarity matrix to construct a common dendrogram, the calculated cophenetic value of r = 0.87 was higher than for ISSRs alone, but still lower than for AFLPs (0.90). A comparable result was presented by Talhinhas *et al.* (2003) in lupins. The cophenetic correlation of the combined analysis was smaller than for the better marker system.

RAPDs

Six primers generated 93 polymorphic bands, out of which 85 were used in this study. Genetic

similarity ranged from 0.07 to 0.46 with a mean of 0.23. For an RAPD dendrogram (Figure 1c) the cophenetic correlation was estimated at r = 0.75. Discrimination according to *GS* was not high enough to group related genotypes into subclusters. Budak *et al.* (2004) found a much broader range of *GS* estimates and also a higher cophenetic value (0.86). This may be due to the use of twice as many markers as in the current study.

SSRs

A total of 209 polymorphic markers were generated by all 23 primer pairs of set A. 85 markers used for the comparative data analysis originated from 12 primer pairs. Genetic similarity (GS_J) ranged from 0.01 to 0.83 with a mean of 0.16. For an SSR dendrogram (Figure 1d) the cophenetic value was estimated at r = 0.79 (and at r = 0.85 for all available set A markers). With set B 8 and 15 primer pairs generated 85 and 160 polymorphic markers, respectively (Table 2). Cophenetic values of these two data sets displayed a good and very good fit with r_{cs} = 0.88 and 0.92, respectively.

Published cophenetic correlations are of similar magnitude: wheat r = 0.87 with 33 markers (Bohn *et al.* 1999), maize r = 0.80 with 183 markers (Pejic *et al.* 1998), and buffalo grass r = 0.88 with 168 markers (Budak *et al.* 2004). Clustering was very similar to the AFLP dendrogram. The members of the ILGI (LTS 10, 19 and 20) and WSC (LTS 9, 17, 18) populations were grouped into their respective clusters.

Comparison of marker systems

The Mantel matrix correspondence test yielded the correlation coefficients presented in Table 3. A moderate correspondence was found between AFLPs and SSRs (0.57 and 0.32) for sets A and B, respectively. ISSRs had only a low correspondence to SSRs (0.18). RAPDs had a very low correspondence to all other marker systems.

Budak *et al.* (2004) calculated Spearman's rank correlation coefficients between diversity indices and found a good correlation (0.66) between ISSRs and SSRs. The reported ISSR-RAPD correlation was lower at 0.41, but still much better than in

Table 2. Marker system, assay units, polymorphism and genetic similarity (GS_J) of 20 *Lolium perenne* genotypes

Marker system	No. of primers	No. of markers	No. of bands per primer/marker		Mean GS_J
AFLP	2[a]	85	273[a]	6.4	0.313
ISSR	12	85	57	8.0	0.432
RAPD	6	85	92	6.4	0.239
SSR[1]	12[b]	85	22[b]	2.9	0.155
SSR[2]	8	85	37	3.4	0.143
AFLP	5[a]	166	238[a]	7.1	0.599
SSR[1]	23[b]	209	30[b]	3.2	0.184
SSR[2]	15	160	32	2.9	0.120

[1]set A; [2]set B; [a]primer combination; [b]primer pair

Table 3. Correlations (Mantel-Z-Test) between genetic similarity (GS_J) matrices based on different marker systems with 85 markers each

	ISSR	RAPD	SSR-A	SSR-B
AFLP	0.46**	0.09	0.32**	0.57**
ISSR		–0.10	0.18	0.37**
RAPD			0.01	0.07
SSR-A				0.39**

Significance levels: **P = 0.001

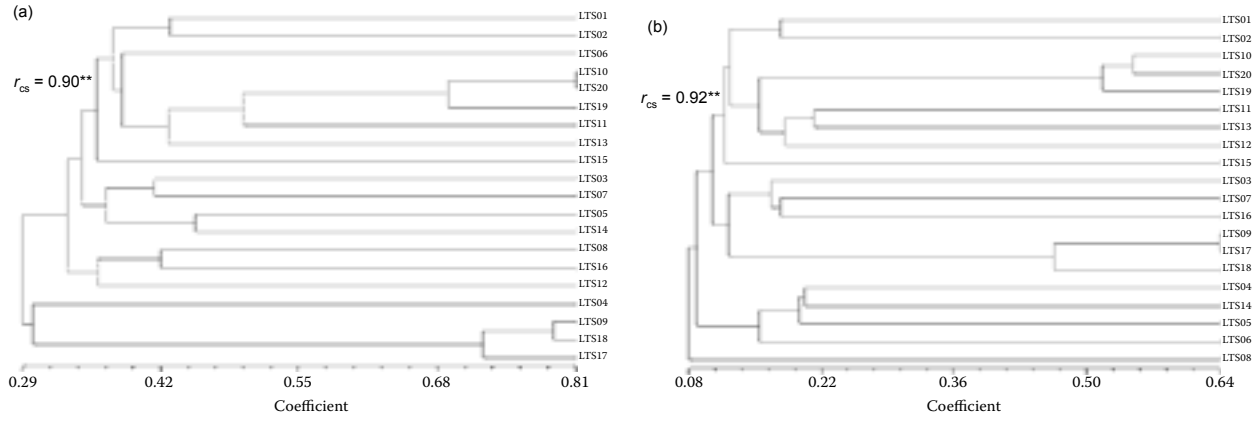

Figure 2. Dendrograms and cophenetic values (r_{cs}) of 20 individual genotypes of perennial ryegrass revealed by UPGMA cluster analysis based on genetic similarities (GS_j) calculated from (a) 166 AFLP, and (b) 160 SSR-B marker data

the current study. The AFLP-SSR relationship from the Mantel Z test in other crops was mostly intermediate: barley 0.51 (Russell *et al.* 1997), wheat 0.34 (Bohn *et al.* 1999), maize inbred lines 0.67 (Pejic *et al.* 1998), but higher in soybeans at 0.86 (Powell *et al.* 1996). Applying all AFLP and SSR-B markers available in this study, a good correspondence (0.60) could be observed.

For ISSRs, comparable correlation coefficients to both AFLPs and RAPDs were found in rice (Virk *et al.* 2000). The correlation between RAPDs and other marker systems varies from very low (AFLPs and SSRs in barley; Russell *et al.* 1997) through medium size coefficients (maize; Pejic *et al.* 1998) to more significant values (> 0.68) with AFLPs and SSRs in soybeans (Powell *et al.* 1996). In the study of Pejic *et al.* (1998) it became evident that the *GS* estimates from different marker systems were mainly correlated only between related inbred lines. Besides a too low number of markers, the non-relatedness of most of the LTS genotypes might be responsible for the low correspondence between different marker similarities.

RAPDs were not able to separate genotypes into clear groups. For the class of these markers neither the number of primers nor the number of genotypes was sufficiently high to obtain robust relationships between genotypes. Using the same six primers in an investigation of perennial ryegrass cultivars, Bolaric *et al.* (2005) found reliable *GS* estimates on the basis of 165 markers.

For AFLPs, the five primer combinations used (i.e. all markers) generated a good number and distribution of fragments, comparable to the investigation of Pejic *et al.* (1998) in maize. The total number of SSR-A markers was needed to give good *GS* estimates. The reduction to only 85 markers did not give satisfactory results, which might also be due to an incomplete coverage of the genome. However, no information on the linkage map position of these SSRs is available for the time being. Even with the more reliable SSR-B primers, 15 loci with 160 markers are required to construct a robust dendrogram. Warburton *et al.* (2002) showed in maize that a reduction from 85 to 53 SSR loci did not alter the dendrogram construction. For wheat, You *et al.* (2004) calculated that 550 alleles (73 loci) were sufficient to construct a robust dendrogram with high certainty. The comparison between the two sets of SSR primers demonstrates that the quality of the primers is of even greater importance than their number *per se*.

The genetical and mathematical properties of ten similarity and dissimilarity coefficients were compared by Reif *et al.* (2005). For allelic non-informative molecular data, coefficients like *GS* (Jaccard) must be applied. The same holds true if SSR data is scored as present or absent, like in this study. However, this should only be done for comparison reasons. To fully exploit the molecular information content, SSR data should normally be treated as allelic informative.

On the basis of the presented data and restricted to 85 markers, it can be concluded that only AFLPs and high quality SSRs have the discriminatory power to reflect genetic relationships between unknown accessions with good certainty. However, with twice as many markers a further improvement in the reliability of the dendrogram and the

cophenetic values (Figure 2) could be observed. This is in accordance with Pejic *et al.* (1998), who concluded that 150 markers were sufficient for reliable estimates of genetic similarity.

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