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A comparative study of molecular and morphological methods of describing relationships between perennial ryegrass (*Lolium perenne* L.) varieties

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Abstract A sample set of registered perennial ryegrass varieties was used to compare how morphological characterisation and AFLP®[1] and STS molecular markers described variety relationships. All the varieties were confirmed as morphologically distinct, and both the STS and AFLP markers exposed sufficient genetic diversity to differentiate these registered ryegrass varieties. Distances obtained by each of the approaches were compared, with special attention given to the coincidences and divergences between the methods. When correlations between morphological, AFLP and STS distances were calculated and the corresponding scatter-plots constructed, the variety relationships appeared to be rather inconsistent across the methods, especially between morphology and the molecular markers. However, some consistencies were found for closely related material. An implication could be that these molecular-marker techniques, while not yet suited to certain operations in the traditional registration of new varieties, could be suitable methods for investigating disputable distinctness situations or possible EDV[2] relationships, subject to establishing standardised protocols and statistical techniques. Some suggestions for such a protocol, including a statistical test for distinctness, are given.

Keywords Perennial ryegrass · AFLP (amplified fragment length polymorphism) · STS (sequence tag sites) · Morphology · Similarity

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Introduction

Revealing identity differences between varieties of plants is a vital determinant in their commercialisation. It is required, for example, by breeders to assess the diversity of breeding stocks, by testing-authorities to determine their uniqueness prior to registration, and by certifying-authorities to control the subsequent multiplication and marketing of seed lots.

The protection of intellectual property rights, through the national registration of varieties and plant breeders rights (PBR) schemes, is probably one of the most-stringent uses of difference assessment. In many countries, particularly in the EU, the controlling legislation complies with the Convention of the International Union for the Protection of New Varieties of Plants (UPOV 1991). This involves testing for distinctness, uniformity and stability (DUS) by growing candidate and previously registered varieties together. Crop-specific morphological characteristics are measured to test for the prescribed statistical levels of difference. In many crops, including ryegrasses, this is becoming an increasingly arduous task, mainly due to the fact that the size of reference collections is continuously increasing (Camlin and McMichael 1990). As the number of registered varieties requiring protection increases, so

[1] AFLP® is a registered trademark of Keygene N.V.
[2] EDV= essentially derived variety. An EDV is a variety being clearly distinct from, but conforming in the expression of the essential characteristics of, an 'initial variety' (IV) from which it is found to have been predominantly derived

does the number of controls needing to be morphologically described annually, in order to ensure that the candidate varieties are genuinely distinct.

Identifying and quantifying the genetic similarity of varieties of perennial ryegrass (*Lolium perenne* L.) presents a complex problem. The main difficulty arises from the variability among individuals within these genetically heterogeneous populations. This can be further exacerbated by the possibility of shared or closely related breeding ancestry among commercially registered varieties, due to the common practice of selective breeding from within existing varieties. There has been extensive research into ryegrass variety identification, and published work has demonstrated the separation of varieties with varying degrees of success by using morphological data (Camlin and McMichael 1990; Loos 1993; Gilliland et al. 2000), protein variation (Ostergaard et al. 1985; Moller and Spoor 1993; Charmet and Balfourier 1994) and molecular markers (Warpeha et al. 1998a; Roldán-Ruiz et al. 2000a). The sensitivity levels of various molecular techniques have been found to differ greatly. Some methods have been successful only at the species level (Stammers et al. 1995; Warpeha et al. 1998b), others have proven useful at the level of natural populations (Xu et al. 1992), while still other methods have been successfully used to identify registered varieties (Huff 1997) and even very closely related accessions (Roldán-Ruiz et al. 2000b). Only two of these studies (Gilliland et al. 2000; Roldán-Ruiz et al. 2000b) analysed the correspondence between morphology derived and DNA-marker derived inter-variety similarities in a systematic way. Nevertheless, the general applicability of the conclusions reached in these two studies, namely a close correspondence between morphology and DNA-marker-derived similarities in perennial ryegrass varieties, must be qualified by the fact that only closely related varieties were compared.

Genetic differences between varieties find their ultimate basis in differences between DNA sequences. Currently, an absolute measure of genetic difference is not technically feasible by any technique, as arguably this would require a comparison of entire genome sequences and probably an understanding of their incremental impact on the phenotype. Consequently, any methodology used to study variety differences will be a sampling strategy, so that estimations of similarity or dissimilarity often reflect the methodology used as well as the plant material being examined. Potential sampling strategies can involve comparisons based on morphological and / or biochemical characters as currently used for DUS testing, and comparisons based on molecular markers. The latter promises to provide a less-noisy reflection of the underlying DNA sequences than the more-traditional morphological characters. An important issue concerns the properties of the various sampling techniques. In comparisons between synthetic varieties of an outcrossing species, the variance of a genetic-distance estimator will be principally affected by both the genetic differences between plants within the varieties (level of heterogeneity) and the precision with which the genome of individual plants is sampled (ignoring technical errors due to imperfections in the measurement processes). Furthermore, when using morphological traits, the developmental and genotype by environment noise that is superimposed on the genetic basis needs to be accounted for, plus the various measurement limitations and inaccuracies.

The motivation for this work was to investigate the usefulness of molecular markers as a supplement, a complement and / or an alternative for distinctness testing based on morphological characters. The morphology based standard distinctness tests for ryegrass are based on a crop-specific set of characters that comply with the UPOV guidelines (UPOV 1991). These characters are assessed over a 3 year testing period. A candidate variety is defined as distinct when it is found to differ significantly in at least one character from each of the existing registered varieties. The statistical procedure to assess distinctness consists of a *t*-test in which the 'variety × year' interaction mean square serves as the basis for the standard error of the difference between the candidate and the reference variety. In the case of markers, every marker locus represents a discrete variable, where the alleles constitute the possible outcomes. In principle, individual marker loci might be treated as individual morphological characters. However, the power to distinguish between ryegrass varieties by *t*-tests on the frequency of bands (or alleles) for specific markers is rather low. Tests for distinctness using DNA-markers should thus combine the information present in a series of markers in order to attain an equivalent power for the distinctness tests. A convenient way to combine information over markers, when comparing pairs of varieties, is to calculate (genetic) distances that reflect the proportion of bands (or alleles) different between the two varieties. When the distance between a candidate and the most-similar reference variety exceeds a certain threshold, the candidate may be said to be distinct. A problem with the use of distances is that their statistical properties are little known. There are few closed expressions for the calculation of the standard errors of distances.

In this paper the relationships between a number of ryegrass varieties were explored by using morphological information, AFLPs and STSs. Distances obtained by each of these approaches were compared, with special attention given to the coincidences and divergences between the methods. The suggestion of a statistical test for distinctness based on AFLP and STS data is also offered. Finally, the feasibility of distinctness-testing based on marker data exclusively, or in combination with traditional morphological evaluations, is discussed.

Materials and methods

Plant Material

The plant material consisted of 16 registered diploid perennial ryegrass (*L. perenne* L.) varieties (Table 1). This was a diverse group

Table 1 Description of the plant material analysed. 'Class' refers to the maturity class used in DUS trials in the UK; 'First test year' refers to the year of the first DUS test in the UK; 'Country of origin' refers to the home country of the submitting breeder, and the number of plants used in the AFLP experiment is given in the last column

Variety		Type	Class	First test year	Breeder	Country of origin	Genetic base	Nos. in AFLP data set
A	Barlet	Forage	Inter	1980	Barenbrug	Holland	4 clones from diverse material	41
B	Barpolo	Forage	Early	1989	Barenbrug	Holland	4 clones from old pastures	50
C	Barylou	Forage	Late	1982	Barenbrug	Holland	Mass selection of Dutch ecotypes	44
D	Blazer	Turf	Late	1981	Pickseed West Inc.	USA	33 clones from 3 separate lines	19
E	Concile	Forage	Late	1994	Force Limagrain	France	19 clones from Dutch material	20
F	Herbie	Forage	Late	1987	Advanta (Van der Have)	Holland	4 families from NW Europe	54
G	Magella	Forage	Inter	1980	Cebbeco Zaden	Holland	Selection from wild population	20
H	Merbo	Forage	Inter	1986	D. V. P.	Belgium	8 clones from Belgium ecotypes	43
J	Merganda	Forage	Inter	1987	D. V. P.	Belgium	4 clones from Belgium ecotypes	51
K	Mikado	Forage	Inter	1993	D. L. F. Trifolium	Denmark	Danish ecotypes × Chantal clones	51
L	Mongita	Forage	Inter	1990	Advanta (Mommersteeg)	Holland	6 clones from mowing selection	45
M	Morimba	Forage	Inter	1989	Advanta (Mommersteeg)	Holland	6 clones from mowing selection	48
N	Pacage	Forage	Late	1985	Carneau Freres	France	7 clones	20
O	Paddok	Forage	Inter	1991	D. V. P.	Belgium	6 clones selected for performance	49
P	Repell	Turf	Late	1986	Sodisem (R.A.G.T.)	France	27 clones from USA material	20
Q	Yatsyn	Forage	Early	1988	N. Zealand Agriseeds	N. Zealand	4 clones from Auckland ecotypes	18

Table 2 Descriptive names of the morphological characters examined. A detailed description of how these characteristics were measured is provided by MAFF (1999). ID No. is the standard character number used in UK Plant Breeders Rights testing scheme, in compliance with UPOV guidelines

ID no.	Code	Character name and description
4	ANGLEYOS	Growth habit in year of sowing (degrees)
5	SPRINGHGHT	Spring height (cm)
8	DATEOFEE	Date of ear emergence (days)
9	SPANG	Spring angle (degreees)
10	HGHTEE	Plant height at ear emergence (cm)
11	WIDTHEE	Plant width at ear emergence (cm)
14	LGTHFL	Flag leaf: length (cm)
15	WIDTHFL	Flag leaf: width (mm)
17	LLSEE+30	Stem length at ear emergence + 30 Days (cm)
24	EARLGTH	Ear length (cm)
31	SPKLTNO	Number of spikelets / spike (number)
34	GLUMELGH	Length of glume (mm)
35	LGHBSP-A	Length of spikelet less awn (mm)
60	NATSPHT	Natural spring height (cm)
70	SPWIDTH	Spring width (cm)

of varieties comprising both forage (14) and turf (2) types, with representatives from all three maturity classes (two Early, eight Intermediate and six Late) and originating from breeding programmes in continental Europe (Belgium, Denmark, France and Holland), continental America (USA) and the Antipodes (New Zealand). The breeding construction of the varieties varied greatly, with varieties being based on between 4 and 33 clones or on mass selection, and they also represented examples of breeding progress over the 15 year period of 1980–1994.

Morphological characterisation

An experiment was performed that mimicked official registration trials to describe the varieties morphologically and to quantify their overall distance based on morphological characters. The methods used complied with the UK protocol for national listing and the PBR registration of ryegrasses by reproducing the 3 year test system used to evaluate the distinctness, uniformity and stability (DUS) of candidate varieties (MAFF 1999). In each test year (1996, 1997 and 1998), the same seed-lot was used to raise 60 new spaced plants of each variety, which were planted in a randomised block design of six replicates of ten spaced plants. Fifteen morphological characters were assessed on each spaced plant. This configuration produced 2,700 observations for each variety and generated a total data set of over 43,200 recordings for the 16 varieties over the 3 years. The morphological characters (Table 2) comprised the approved set used for the UK National Listing and the PBR registration of ryegrasses (MAFF 1999) and are based on the UPOV guideline (UPOV 1991). UPOV-approved statistical methods for determining the distinctness uniformity and stability of candidate varieties, involving a'COYD' (Combined Over-Years Distinctness) analysis, were based on the methods of Weatherup (1994) and incorporated a modified joint regression analysis (MJRA). This MJRA model took account of systematic annual increases or decreases in character expression across all varieties by fitting extra terms, one for each year, in the analysis of variance. Each term represented the linear regression of the observations for the year against the variety means over all years, as described by Digby (1979).

Molecular analyses

All 16 varieties were fingerprinted with AFLP markers and the number of plants used to represent each is presented in Table 1. In addition, ten of these varieties (Barpolo, Blazer, Concile, Herbie, Merbo, Merganda, Morimba, Pacage, Repell and Yatsyn) were also fingerprinted with eight STS markers. For each of these ten varieties, the STS procedure was conducted using DNA extracted from 20 of the plants used in the AFLP tests. All the plants used in these molecular analyses were generated from seed and grown in the greenhouse. After approximately 2 months, 100 mg of young leaf material was harvested for DNA extraction. The plants used in the molecular experiments were a different random set of plants from those used in the morphological experiment.

DNA extractions

For plants of the varieties Barlet, Barpolo Barylou, Hamlet, Herbie, Merbo, Merganda, Mikado, Mongita, Morimba and Paddok, the plant material was freeze-dried and ground using a mechanical mill before DNA extraction. DNA was isolated following a standard CTAB extraction protocol (Weising et al. 1991). For plants of

the varieties Blazer, Repell, Pacage, Yatsyn, Magella and Concile, the plant material was ground in liquid nitrogen and DNA was extracted using the protocol described in Cheung et al. (1993). DNA concentration and quality was checked by comparing with a standard series of λ-DNAs on 1.5% agarose-gels after electrophoresis.

AFLP analysis

The AFLP reactions were conducted on DNA samples from individual plants. The methodology was according to Vos et al. (1995), using a commercial kit (GIBCO-BRL) and following the protocol of Roldán-Ruiz et al. (2000a). The enzymes *Eco*RI and *Mse*I were used for DNA digestion. Each individual plant was fingerprinted with two primer combinations. The primer extensions used were *Eco*-ACG / *Mse*-CAA and *Eco*-AGG / *Mse*-CTT. Fragment separation and detection took place on an ABI Prism 377 DNA sequencer on 36-cm denaturing gels using 4.25% polyacrylamide (4.25% acrylamide / bisacrylamide 19 / 1, 6 M Urea in 1 × TBE). A GS-500 Rox labelled size standard (Perkin Elmer) was loaded in each lane.

The fluorescent AFLP patterns were assessed using Genotyper (Perkin Elmer 1996). A total of 117 polymorphic markers, generated by the two AFLP primer combinations, were scored over the entire set of 642 individuals. For the primer combination *Eco*-ACG / *Mse*-CAA, 54 polymorphic bands were scored ranging from 72 to 418 bp, whereas for the primer combination *Eco*-AGG / *Mse*-CTT, 63 polymorphic bands were scored between 85 and 406 bp. Each marker was coded as 1 or 0, whether present or absent in an individual plant, to form a binary data matrix. Each individual was thus represented by a vector of 1 s and 0 s. As ryegrass varieties are genetically heterogeneous, typically not all plants in a given variety had the same combination of present and absent markers. In fact, each of the individual plants analysed displayed a unique banding pattern. The differences among varieties were attributable to frequency differences in variable markers, rather than markers being exclusive to specific varieties.

STS analysis

The STS reactions were conducted on DNA samples from individual plants. Reaction volumes of 25 µl were made up of *Taq* buffer (1 × is 50 mM KCL, 10 mM Tris HCl), 1.75 mM $MgCl_2$, 100 µM of each dNTP, 0.5 µM of the primers, 20 ηg of genomic DNA and 0.12 U of *Taq* polymerase.

Primer sequences were chosen to amplify introns of the following genes (the centigrade annealing temperature used is given between brackets): Alcohol dehydrogenase (53°); Triosephosphate Isomerase (55°); ADP-glucose glycosyl transferase (57°); Pollen allergen (57°); ''late embryogenesis abundant'' gene (60°); ADP glucose phosphorylase (50°); Catalase (55°); Serin carboxypeptidase (55°). The thermal treatment was 10 min at 94°C followed by 40 cycles of 30 s at the annealing temperature, 1 min at 72°C and 30 s at 94°C, with a final cycle of 30 s at the annealing temperature and 6 min at 72°C.

Length polymorphisms were detected by separating the amplification products on 5% polyacrylamide gels or 1.8% agarose gels. The gels were stained using silver nitrate (acrylamide gels) or ethidium bromide (agarose gels). The number of alleles per STS locus varied between two and seven noted as letters (a, b, c,...) or figures (1, 2, ...).

Data analysis

To summarise the relations between pairs of varieties Euclidean distances were calculated from the morphological data, from the AFLP data, and from the STS data.

The morphological variables were standardised as

$$y_m = \frac{(x_m - \bar{x}_{m)}}{sd_{x_m}},$$

with x_m the m-th morphological character before standardisation (m = 1...M, with M = 15), y_m the standardised character, $\bar{x}_m$ the mean of x_m, and sd_{xm} the standard deviation of x_m. An individual value for the variety i (i = 1...16) on the variable x_m, i.e. x_{im}, represented the 3-year average from 1996 until 1998. The (average) Euclidean morphological distance between variety i and j, d_{ij}^{Morph}, was defined by $d_{ij}^{Morph} = \frac{\left[\sum_{m=1}^{M}(y_{im} - y_{jm})^2\right]^{1/2}}{4M}$. The normalizing constant 4 M (= 60) brings the morphological distances on a scale from 0 to 1, where 4 roughly represents the range of a standardised variable (a standardised variable will have about 95% of its values between –2 and 2, so that the approximate maximum difference between two varieties on a standardised variable becomes $\sqrt{(-2-2)^2 = 4}$).

For the AFLP data, Rogers distances were calculated as $d_{ij}^{AFLP} = \frac{\left[\sum_{a=1}^{A}(y_{ia} - y_{ja})^2\right]^{1/2}}{A}$, where y_{ia} and y_{ja} stand for the frequency of band 'a' in the varieties i and j, respectively, and summation is over the bands (a = 1...A, with A = 117). In this case the Rogers distance is equivalent to the Euclidean distance on band frequencies.

For the STS data an adapted form of Rogers distance was used, to account for the fact that STS loci vary in the number of alleles per locus, i.e., $d_{ij}^{STS} = \frac{\sum_{s=1}^{S}\left[\frac{1}{2}\sum_{g=1}^{n_s-1}(y_{isg} - y_{isg})^2\right]^{1/2}}{S}$ where y_{isg} is the frequency of the allele g of the marker locus s for the variety i. Averaging takes place over loci (s = 1...S, with S = 8). Within loci summation goes over alleles, g = 1...(n_s -1), i.e. the last allele is not included as its frequency follows from the other alleles (n_s varied between 2 and 6). The normalisation constant 1 / 2 secures that the distance for an individual locus stays between 0 and 1.

Morphological, AFLP and STS distances were compared in various ways; by scatter plots, comparison of rankings, Pearson correlations and biplots. The significance of Pearson correlations was assessed by, a permutation test for distance matrices (see Manly 1997). The correlations were calculated on the basis of the ten varieties for which all three types of measurements (morphology, AFLP and STS) were available.

Biplots are graphical devices that simultaneously represent objects (= varieties) and variables (= morphological characters, AFLP band frequencies and STS allele frequencies). They serve to identify the main features in the variety by variable data matrices: distances between varieties, correlations between variables and values of varieties on variables. As distances were Euclidean in nature, a standard principal-components biplot (Digby and Kempton 1987) was accepted as the most-appropriate choice. In such a biplot, distances between varieties are two-dimensional approximations to the higher-dimensional observed distances. Variables are represented by directed lines (from the origin) and projections of varieties on these lines give approximations to the original values of the varieties on the variables. Projections on the positive side of the origin represent above-average values for a variable. Conversely, projections on the negative side represent below-average values. The angle between variable lines / directions is related to the correlation between them: acute angles indicate positive correlations, obtuse angles indicate negative correlations, orthogonal angles indicate absence of a correlation. Biplots were constructed by the special purpose package SC-Biplot (http: / / www.smitconsult.nl / biplot1.htm). One of the special features of this package is the imposition of original observation scales on the variable representations in the biplot, according to a technique that is described in chapter 2 of Gower and Hand (1996).

Biplots were based on the ten varieties for which morphological, AFLP and STS data were available. There were morphological and AFLP data available on six additional varieties. These varieties were added passively to the biplots based on the ten

common varieties. This means that the six additional varieties were fitted to a configuration determined by the ten common ones (another special feature of the SC-Biplot). Passive varieties can be recognised in the biplots by a black square in the centre of the larger and lighter-shaded square representing the variety itself. Biplots for the morphological and AFLP data in which all 16 varieties were used to perform the biplot analysis deviated only slightly from the ones presented in the results section.

The biplot for the STS data was somewhat special in that it was constructed on the allele frequencies of the loci, with omission of the last allele of every locus. The omitted alleles were added passively afterwards, i.e. they were fitted in the plot constructed on the basis of the other alleles. Every locus was given the same weight but, due to differences in the number of alleles per locus, alleles from different loci could differ in weights.

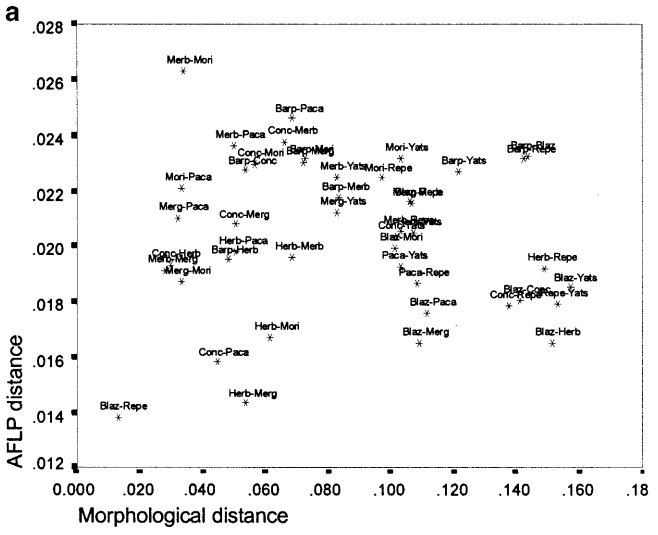
Results

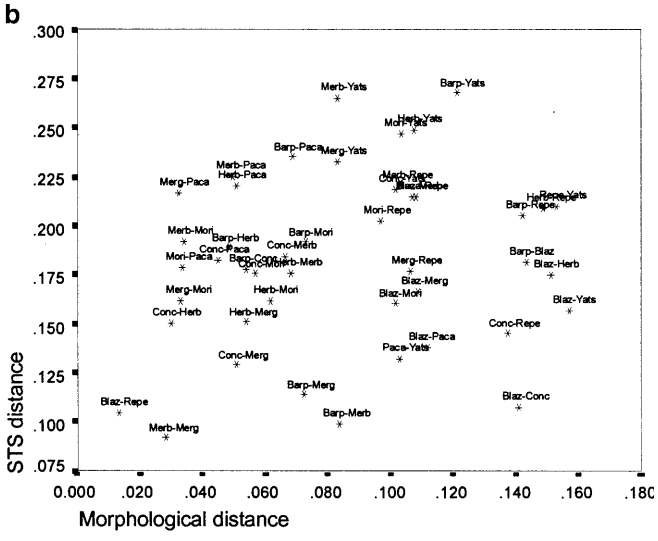
Comparison of distance estimates by scatter plots, rankings, and correlations

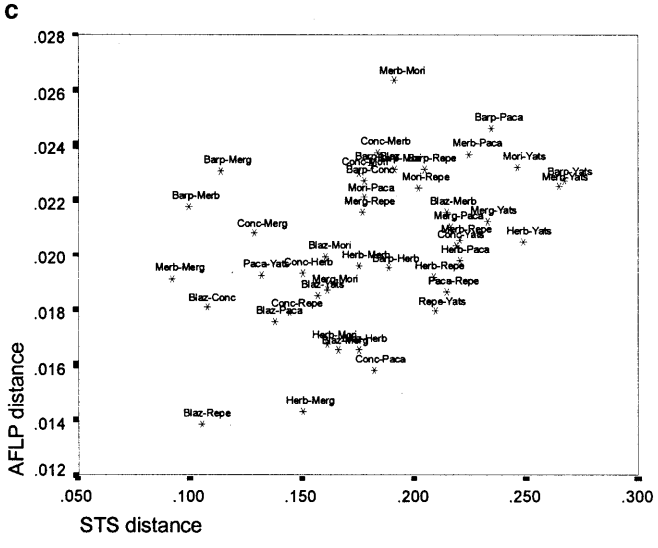
Distances between pairs of varieties and their rankings are given in Table 3. Although the distances were defined in such a way as to scale them between 0 and 1, the actual AFLP, STS and morphological distances should not be compared to their absolute values. As is obvious from Table 3, the absolute distances were quite-different between the three measurement systems (AFLP, STS and morphology). However, not only were the absolute distances clearly different, but the rankings also appeared to be quite different. Of course, some examples of consistency can be found. Some pairs of varieties were consis-

Table 3 Pair-wise distances between varieties computed using AFLP, STS and morphology information. Next to each distance value its rank order is given

Varieties		AFLP		STS		Morphology	
		Distance	**Rank**	**Distance**	**Rank**	**Distance**	**Rank**
Blazer	Repell	0.01383	1	0.1048	3	0.0130	1
Herbie	Merganda	0.01432	2	0.1506	11	0.0539	13
Concile	Pacage	0.01581	3	0.1825	24	0.0449	8
Blazer	Merganda	0.01652	4	0.1662	16	0.1090	35
Blazer	Herbie	0.01653	5	0.1751	17	0.1513	43
Herbie	Morimba	0.01673	6	0.1613	14	0.0614	16
Blazer	Pacage	0.01758	7	0.1375	8	0.1116	36
Concile	Repell	0.01788	8	0.1448	9	0.1377	38
Repell	Yatsyn	0.01794	9	0.2099	32	0.1532	44
Blazer	Concile	0.01808	10	0.1073	4	0.1413	39
Blazer	Yatsyn	0.01850	11	0.1567	12	0.1572	45
Pacage	Repell	0.01865	12	0.2146	34	0.1085	34
Merganda	Morimba	0.01871	13	0.1615	15	0.0331	5
Merbo	Merganda	0.01911	14	0.0917	1	0.0279	2
Herbie	Repell	0.01922	15	0.2087	31	0.1491	42
Pacage	Yatsyn	0.01928	16	0.1319	7	0.1030	28
Concile	Herbie	0.01934	17	0.1501	10	0.0296	3
Barpolo	Herbie	0.01951	18	0.1885	26	0.0482	9
Herbie	Merbo	0.01960	19	0.1756	19	0.0684	18
Herbie	Pacage	0.01982	20	0.2204	38	0.0508	12
Blazer	Morimba	0.01992	21	0.1600	13	0.1016	26
Concile	Yatsyn	0.02034	22	0.2186	36	0.1018	27
Herbie	Yatsyn	0.02047	23	0.2492	43	0.1074	33
Merbo	Repell	0.02053	24	0.2202	37	0.1033	29
Concile	Merganda	0.02078	25	0.1287	6	0.0507	11
Merganda	Pacage	0.02101	26	0.2168	35	0.0320	4
Merganda	Yatsyn	0.02121	27	0.2327	40	0.0829	22
Blazer	Merbo	0.02155	28	0.2145	33	0.1069	32
Merganda	Repell	0.02158	29	0.1767	20	0.1064	31
Barpolo	Merbo	0.02175	30	0.0992	2	0.0834	24
Morimba	Pacage	0.02207	31	0.1783	22	0.0332	6
Morimba	Repell	0.02246	32	0.2025	29	0.0971	25
Merbo	Yatsyn	0.02248	33	0.265	44	0.0830	23
Barpolo	Yatsyn	0.02268	34	0.2675	45	0.1216	37
Barpolo	Concile	0.02273	35	0.1779	21	0.0539	14
Concile	Morimba	0.02297	36	0.1752	18	0.0568	15
Barpolo	Merganda	0.02304	37	0.1137	5	0.0721	20
Barpolo	Morimba	0.02314	38.5	0.1913	27	0.0728	21
Barpolo	Repell	0.02314	38.5	0.2051	30	0.1426	40
Morimba	Yatsyn	0.02318	40	0.2468	42	0.1033	30
Barpolo	Blazer	0.02324	41	0.1813	23	0.1435	41
Merbo	Pacage	0.02365	42	0.225	39	0.0498	10
Concile	Merbo	0.02376	43	0.1841	25	0.0663	17
Barpolo	Pacage	0.02461	44	0.2351	41	0.0688	19
Merbo	Morimba	0.02635	45	0.1917	28	0.0340	7







tently close over the three methods: most notably Blazer and Repell and, to a lesser extent, Herbie and Merganda, Herbie and Morimba, Merganda and Morimba, and Merbo and Merganda. Other pairs were consistently far apart: Morimba and Yatsyn, Barpolo and Repell, and Barpolo and Yatsyn. Consistently intermediate were the pairs Morimba and Repell, Merganda and Repell, and Blazer and Merbo. Most variety pairs, however, behaved rather irregularly from one system of measurement to another. This irregularity, evident in Table 3, is more-clearly exhibited by scatter plots (Fig. 1) and correlation values. The Pearson correlation between AFLP and morphological distances was $r = -0.06$ by the permutation test $p < 0.375$ (Fig. 1a); between the STS and morphological distances the values were $r = 0.18$, $p < 0.12$ respectively (Fig. 1b) and between AFLP and STS distances were $r = 0.42$, $p < 0.003$ (Fig. 1c) (each based on 1,000 permutations). These tests indicated a complete absence of any relationship between morphological and either AFLP or STS distances, but they provided positive evidence that some correspondence existed between the two molecular methods. This observation suggested that these two molecular methods were sampling similar genetic information.

Several of the variety relationships matched with the expectations due to their breeding histories (Table 1). For example, Blazer and Repell were the only ‘turf’-type varieties and were also both derived from American germplasm; Merbo and Merganda were selected from Belgian ecotypes by the same breeder; and Yatsyn was a very unique type, derived from a Southern-hemisphere genepool.

As the comparisons between the different distance-measures showed no precise agreement (between morphology, AFLP and STS) a more-detailed examination of variety relationships was performed using graphical depictions in the form of biplots for each system.

Morphology comparisons

The mean values for the 15 morphological characters are presented in Table 4. There was a wide range of expression across the varieties for many of these characters, including a 36-day range in heading date and a 25.6-cm range in height at ear emergence. Given this diversity, all 16 varieties were found to differ from each other in one or more individual characters at or above the ‘combined over years’ significance level necessary to discriminate varieties in the UK DUS trials. This confirmed that all the varieties were morphologically distinct according to UPOV guidelines, as was expected given that they were all registered varieties.

Fig. 1a–c Scatter plots comparing Morphological, AFLP and STS distances. **a** Morphology vs AFLP; **b** Morphology vs STS; **c** STS vs AFLP. Variety codes: *Barp* = Barpolo, *Blaz* = Blazer, *Conc* = Concile, *Herb* = Herbie, *Merb* = Merbo, *Merg* = Merganda, *Mori* = Morimba, *Paca* = Pacage, *Repe* = Repell, *Yats* = Yatsyn

Table 4 Three year mean values for 15 measured morphological characters (1996–1998) of 16 perennial ryegrass varieties. Values in boxes and underlined are the upper and lower extremes for each character. The full descriptive names of the characters are given in Table 2, the method of measurement is described by MAFF (1999) and the regression probability (Regr. prob.) was used as an indicator for the MJRA analysis as described by Digby (1979)

Varieties															
ID Number Characters	4 angleyos	5 springhght	8 dateofee	9 spang	10 hghtee	11 widthee	14 lgthfl	15 widthfl	17 llsee+30	24 earlgth	31 spkltno	34 glumelgh	35 lghbsp-a	60 natspht	70 spwidth
Barlet	48.69	35.81	94.81	48.62	45.42	61.30	19.78	6.18	79.35	25.56	25.01	11.52	22.53	26.16	43.75
Barpolo	46.08	33.51	108.70	39.02	30.44	83.39	20.40	6.60	84.34	27.33	26.58	13.27	18.01	19.85	49.07
Barylou	34.05	39.58	63.73	32.05	19.85	75.67	17.74	6.20	67.24	19.99	21.70	11.78	17.76	18.79	64.55
Blazer	41.95	26.98	91.31	43.55	30.94	54.26	14.00	5.27	65.25	19.50	23.62	9.22	14.96	17.91	39.47
Concile	44.75	35.10	101.67	41.83	41.60	72.48	21.40	6.11	85.02	26.99	25.58	13.75	20.52	22.94	48.28
Herbie	41.50	36.39	101.28	40.75	41.49	78.59	22.21	6.41	86.17	27.34	26.21	13.21	19.80	22.46	52.48
Magella	45.60	34.79	94.96	48.43	42.62	61.37	18.81	6.03	78.09	23.36	24.23	11.95	20.12	25.22	43.34
Merbo	40.89	37.64	83.71	40.92	37.21	68.69	19.19	6.10	77.88	23.81	22.73	11.75	18.41	23.08	53.55
Merganda	41.91	37.24	91.45	41.39	41.55	68.95	18.96	6.11	79.72	24.29	24.62	11.71	18.45	24.39	50.96
Mikado	41.83	37.26	92.56	42.46	42.17	74.75	20.34	6.40	83.45	26.22	25.54	11.69	19.81	23.06	51.52
Mongita	42.43	40.78	89.38	41.50	43.32	71.58	18.52	6.25	81.98	23.52	26.11	10.23	18.83	25.76	52.26
Morimba	41.00	33.40	95.54	41.42	37.35	71.82	19.62	5.96	81.81	24.49	23.08	10.99	19.08	21.66	47.89
Pacage	42.50	34.76	105.46	43.07	44.99	70.15	20.37	5.90	81.66	25.43	24.52	11.45	18.23	22.42	48.90
Paddok	39.00	35.73	98.78	39.12	37.53	75.27	20.88	6.20	81.82	26.32	27.17	12.26	18.56	20.57	52.04
Repell	42.56	26.92	90.64	43.67	31.33	54.33	14.59	5.25	66.02	19.68	22.82	9.34	15.51	18.71	37.85
Yatsyn	49.42	45.30	72.56	49.54	39.39	64.23	18.93	6.82	80.42	22.71	23.12	12.85	18.59	32.32	55.47
Variety.MS	47.90	28.75	395.08	74.48	134.75	114.08	4.19	0.17	60.68	11.15	9.56	2.57	4.23	40.07	73.53
Variety year.MS	3.77	3.08	1.72	2.64	4.68	7.08	0.83	0.05	8.10	0.75	0.51	0.19	0.31	1.36	3.93
Regr. prob.	1.84	4.81	0.00	41.39	35.68	23.52	0.11	20.51	35.48	75.90	4.04	87.96	98.90	0.01	47.94

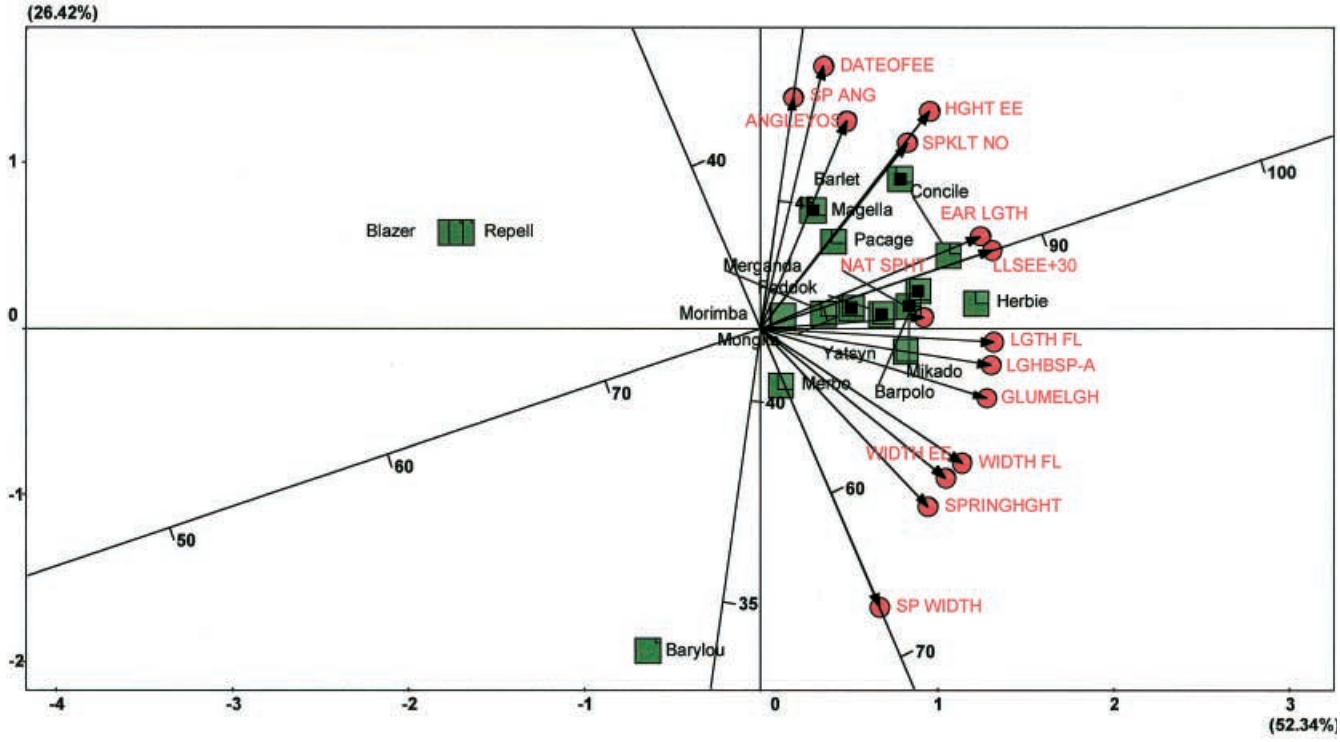


Fig. 2 Principal components analysis based on morphological data. The spread of the varieties and the morphological characters on the plane defined by the first two components is shown. The varieties are represented by *green squares* and the morphological characters by *red circles*. The actual scale values are shown for the characters 'Spring width' (SP *WIDTH*), 'Stem length at ear emergence + 30 Days' (*LLSEE+30*) and 'Growth habit in year of sowing' (*ANGLEYOS*). Passive varieties can be recognised in the biplot by a *black square* in the centre of the *larger and lighter shaded square* representing them. For further details on the interpretation of the symbols, see text

Examination of the highest and lowest values for each character revealed that some varieties were frequently expressing values at the outer limits of the character ranges (Table 4). Yatsyn was most extreme (greatest or least) in 5 of the 15 characters, Barylou was also an extreme variety in six characters. Blazer and Repell were together at the upper or lower extremities of the range in seven characters and were individually the most-extreme in two other characters. Furthermore, expression at the extreme of the range was produced in three characters by Barpolo and Herbie, and in two by Barlet. The remaining varieties were either consistently within the range of expression for each character, or had the extreme expression in no more than one character.

To study the inter-relationships between all the varieties, as indicated by their morphology, a principal components analysis was performed (Fig. 2). The varieties were represented by squares. The smaller the cut-out in the upper right, the better the representation of the variety in the biplot. Most of the varieties clustered together near the origin in the upper right quadrant, with only three varieties clearly outside this group: Blazer and Repell, which were very similar to each other, and Barylou, which was very different from all others. Yatsyn, which displayed extreme values for five characters (Table 4), was poorly represented in the biplot and, therefore, did not look like an extreme variety. Blazer, Repell, Barylou, Herbie, and Concile were very well represented and appeared to be dominant in determining the biplot configuration in which Yatsyn did not fit well. Morimba was also poorly represented on this biplot.

To help interpret the basis of these relationships, the morphological character variables were included as circles in Fig. 2, with the mean for each character at the centre or origin of the plot. For clarity reasons the original scale values were presented for only three variables, with only the direction arrows provided for the others. The arrows indicate the directions for increasing character expression, and the angles between the lines reflect the correlations between characters (90^{o} being a null relationship). However, as the biplot represents 15 character axes simultaneously in only two-dimensions, some distortion is inevitable and extreme values are better approximated than the intermediate ones.

For the three variables with scale values it is possible to read off the approximated character values for varieties. Therefore, the 'Stem length at ear emergence +30 days' should be close to 85 cm for Herbie and approximately 65 cm for Blazer and Repell. The actual measured values from Table 3 were 86.17, 65.25 and 66.02, respectively. Furthermore, Concile, Barlet and Magella were among the varieties with the largest 'Spring angle', Blazer and Repell were at the lower extreme while Barylou was at the higher extreme of the measured range. An understanding of the physiological interactions between the characters can also be deduced. For example, 'Number of spikelets / Spike' and 'Plant height

at ear emergence' displayed a correlation close to one. 'Date of ear emergence', 'Spring angle' and 'Angle in year of sowing' were highly positively correlated but were all negatively correlated to 'Spring width'. In contrast, there were no detectable relationships between the former three characters and head-geometry characters like 'Flag leaf length' and 'Length of basal spiklet'. These observations are understandable, as earlier-maturing plants tend to be more-erect growing, whereas later varieties tend to be wider and more prostrate and there are no biological links between growth habit and seed head structure.

Overall the interpretation from the biplot was largely consistent with the observations on extreme character expression by the varieties, with the exception of Yatsyn and Morimba, which were poorly represented in the biplot. Furthermore, by using a fitting-constants analysis to re-calculate the variety means from a 10 year incomplete data set (data not shown), these morphologically perceived variety relationships were found to be robust. The correlation between the Euclidean distances based on the 3 and the 10 year data-sets was 0.99.

AFLP analysis of relatedness

For the sake of clarity, the biplot representation of the AFLP data was separated into two figures, one for varieties (Fig. 3a) and the second for markers (Fig. 3b). These can be superimposed and are interpreted in a similar way as the morphological biplot, except that the size of the marker symbols (circles in Fig. 3b) indicate the frequency of each marker across all the varieties. Overall, the variety relationships were different from those indicated by the morphological study. In Fig. 3a, the most-similar variety couple appeared to be Herbie and Merganda, whereas Morimba and Merbo were the most-dissimilar pair and, most notably, Repell appeared to be more-closely related to Yatsyn than to Blazer. However, both Blazer and Repell were represented very badly in this plot, indicating that they probably exhibited a frequency pattern that was highly incompatible with that of the varieties dominating the configuration (Morimba, Barpolo, Merbo and Pacage).

The bands generated from the two primer combinations were distributed somewhat differently on the plot of Fig. 3b with the 'Bar' bands (*Eco*-AGG / *Mse*-CTT generated) lying more to the right, whereas the 'Gen' bands (*Eco*-ACG / *Mse*-CAA generated) were distributed more-equally dispersed over the whole plot. An above-average frequency of a marker in a variety is indicated if both marker and variety have coinciding directions; a below-average frequency is indicated by opposing directions. For example, Concile and Pacage in general appeared to have above-average frequencies of 'Bar' bands (especially Bar47), whereas Merbo and Barpolo displayed below-average frequencies of Bar bands (especially Bar31), with the exception of Bar02, which appeared to have higher than average frequencies in Merbo and Barpolo. It is important to distinguish between absolute marker frequencies and relative marker frequencies. The latter refer to the average frequency over all varieties. In the biplot the absolute frequency of a marker is expressed by the size of the circle representing the marker, whereas the relative frequency of a marker in a particular variety is given by the distance to the origin of the variety projection on the marker direction. Thus, Morimba had a high relative frequency of the on-average medium-frequent Gen31 band, while Morimba had also a high relative frequency of the on-average low-frequency Mor26 band (*Eco*-AGG / *Mse*-CTT generated).

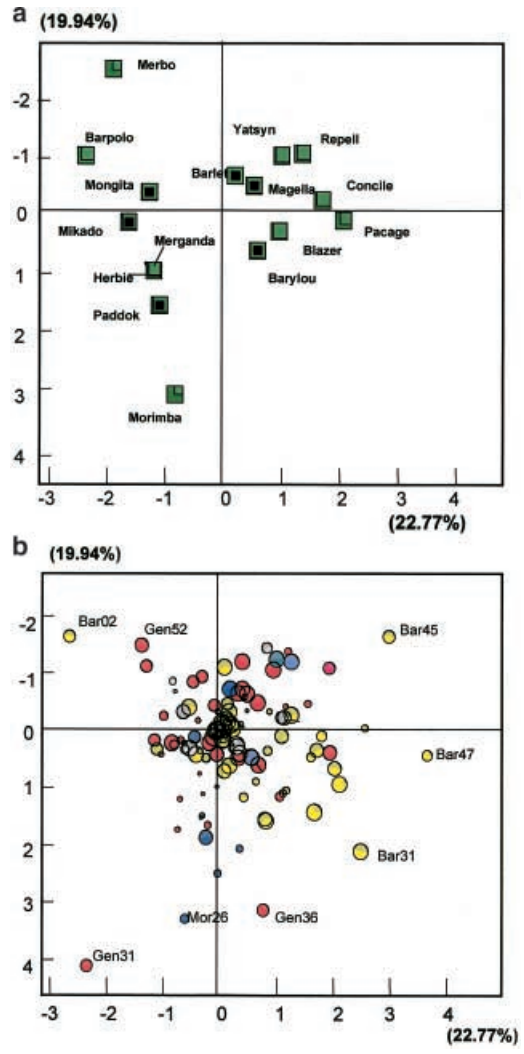


Fig. 3a, b Principal components analysis based on AFLP data. **a** Spread of the varieties according to the first two components. Passive varieties can be recognised in the biplot by a *black square* in the centre of the *larger and lighter shaded square* representing them. **b** Spread of the AFLP markers according to the first two components. Red circles represent AFLP markers generated using the primer combination *Eco*-ACG / *Mse*-CAA. *Yellow, blue and grey circles* represent AFLP markers generated using the primer combination *Eco*-AGG / *Mse*-CTT. For further details on the interpretation of the symbols see text

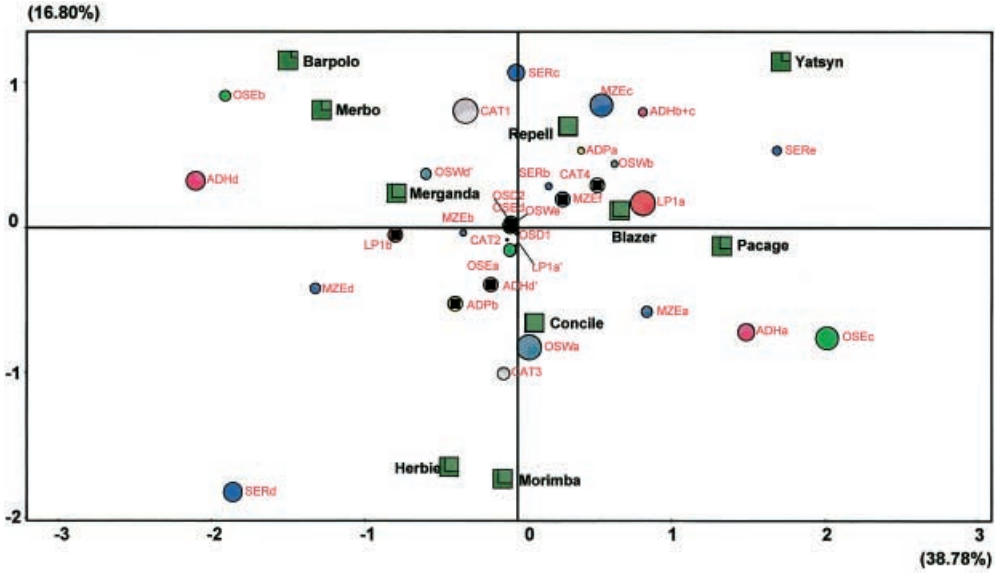


Fig. 4 Principal components analysis based on STS data. The spread of the varieties and the STS alleles on the plane defined by the first two components is shown. The varieties are represented by *green squares* and the STS markers by *circles of different colours*. Passive alleles (see text) can be recognised in the biplot by a *black square* in the centre. STS marker codes: *LP1* = Pollen allergen, *ADP* = ADP-glucose phosphorylase, *OSW* = ADP-glucose glycosyl transferase, *SER* = Serin carboxypeptidase, *OSE* = ''late-embryogenesis abundant gene'', *ADH* = alcohol dehydrogenase, *CAT* = Catalase, *MZE* = Triosephospate isomerase

STS analysis of relatedness

The biplot for the STS markers (Fig. 4) is interpreted in the same way as the AFLP biplot. The distribution of the ten varieties on this biplot was notably different from that on the morphology biplot, but had some interesting similarities with the AFLP biplot. For example, in both biplots, Merbo, Barpolo, Merganda, Herbie and Morimba were distributed to the left of the origin, while the remainder Yatsyn, Repell, Concile, Pacage and Blazer were all located on the right. Similarly, the separation along the second component located Merbo, Barpolo, Yatsyn and Repell above, and Herbie, Pacage and Morimba below, the origin. Only the remaining two varieties, Concile and Merganda, were located notably differently in the STS and AFLP biplots. However, unlike in the AFLP biplot, Repell and Yatsyn were not closely associated in the STS biplot, nor was Repell closely associated with Blazer as in the morphology biplot, but Yatsyn was an outlying or extreme type. With regard to the distribution of the alleles, the biplot indicated that Barpolo and Merbo had a comparatively high frequency for *OSEb* (overall a low-frequency allele) and *ADHd* (overall a high-frequency allele), and a low frequency for *ADHa* and for *OSEc* (overall high-frequency alleles). Barpolo and Merbo appeared to be totally uncorrelated with Yatsyn. Yatsyn had a comparatively high-frequency of the low-frequency allele *SERe* and a low-frequency of the high-frequency allele *SERd*. Morimba and Herbie showed similar frequencies for *CAT3* and *OSWa* (above average), and *CAT1*, *SERc* and *MZEc* (below average).

Discussion

The varieties used in this study were a small but representative sample of existing commercial diploid ryegrass varieties, and so typified the kind of diversity encountered by the testing authorities conducting registration tests. They were all found to be morphologically distinct, as would be expected. The biplots provided a visual impression of how the varieties interrelated within each of the taxonomic methods (morphology, AFLP and STS), but inevitably these displays were two-dimensional compromises of multi-dimensional relationships. The scatter diagrams provided a more-accurate representation of the variety relationships but only for pairwise comparisons. Therefore, interpretation is facilitated by a combination of both types of analyses.

When the overall morphological relationships were examined, the dominating pattern was that of the two 'turf' varieties on the one hand and the 'forage' varieties (with the exception of Barpolo) on the other hand. This contrast reflected the breeders targetting a diverse phenotypic goal for 'forage' types (high shoot productivity for a plethora of growing systems) and a more-common phenotypic goal for 'turf' varieties (a fine leaf, high tillering and low prostrate-growth ideotype). Therefore, 'turf' type varieties might be expected to display even greater morphological similarity than 'forage' types. This was the case with Blazer and Repell but, as these were the only two 'turf' type varieties included in the study, this observation is not sufficient to be conclusive. Despite these complicating factors, morphological characterisation is an effective discriminating tool for ryegrass varieties, with a high success reported (Camlin and McMichael 1990). However, this approach is inefficient on account of the time and cost involved (Camlin and Gilliland 1994).

Recently, an increasing number of studies have demonstrated the capacity of molecular markers to be highly discriminating between varieties in a range of species, including tomato (Noli et al. 1997), oilseed rape (Lee et al. 1996; Lombard et al. 2000a), maize (Pejic et al. 1998) and evergreen azaleas (De Riek et al. 1999). In the

present study, the molecular markers also exposed useful genetic diversity, and the visual displays appeared to disperse the varieties somewhat more-evenly over the plot than the morphological method, particularly for STS. However, there was little agreement on variety relationships between the morphology and the molecular methods. Other workers have reported a similar disparity between phenotypic and molecular distances, for example in maize (Ben-Har et al. 1995; Burstin and Charcosset 1997; Smith et al. 1997; Senior et al. 1998) and tomato (Noli et al.1997). Such observations should not be regarded as indicating a weakness or limitation of these systems. Varieties that display high phenotypic similarity need not be genetically similar as different genepools can be manipulated to create similar phenotypes. Had close-matching of molecular and morphological relationships been found, it might have indicated a very restricted commercial genepool. It would also have meant that the practice of interbreeding between successful ryegrass varieties might be eroding the genetic diversity in this crop. However, this was not the case, and so the relationships between varieties are likely to be multifaceted and complex to interpret, as comparisons may correctly display similarities in neither, both or either morphological and molecular phenotypes. Therefore, consistency should only be expected if varieties had shared genetic resources and parallel breeding objectives or, conversely, were very different in both gene-pool source and selection targets.

Several workers have reviewed the use of molecular markers for registration testing in various crops, including Camlin and Gilliland (1994) and Law et al. (1999). The observations from the present study have implications for the practical application of molecular markers for this function in ryegrass. There are three separate functions that need to be considered: determination of variety distinctness; performance as a pre-screening tool; and the capacity to indicate putative Essential Derivation (ED) relationships. Since molecular differences between varieties cannot also imply equivalent morphological differences, the same distinction, and thus registration decisions, cannot be expected with molecular markers as with the current morphology based DUS methods. Clearly these molecular markers would have the capacity to distinguish ryegrass varieties but, if adopted for the registration of ryegrasses, then the definition of 'variety' would need to be adapted accordingly. This would be outside the current views being expressed in UPOV regarding ryegrasses (Camlin 1999). Furthermore, the results of this study show that the molecular techniques used are unlikely to be suitable for pre-screening ryegrass varieties. The purpose of pre-screening would be to subdivide candidate varieties into groups, so reducing the number of controls and pairwise comparisons that have to be examined in the morphology tests. However, this process assumes that the pre-screening characters guarantee that varieties placed in different groups are distinct in the morphological characters used for registration. Clearly, this would not be the case as the present study showed that marker differences and morphological differences were not correlated. Therefore, using molecular markers as grouping-characters would, by default, require acceptance of their use as a distinguishing character, at least for the most-divergent varieties. An alternative way to deal with the poor correlation between genetic and morphological distances could be to select only molecular markers linked to phenotypic traits in DUS testing. The prediction of phenotypic distances through this reduced set of well-characterised markers using a linear model is the basis of the work of Nuel et al. (2000). Applied to maize data, it resulted in 29% of field-trial savings for less than 5% of errors in the pre-screening of varieties. The advantage of this approach is that it fits better into the current views being expressed within UPOV, by combining the phenotypic and the genotypic assessment of varieties. However, the application of this approach in ryegrass is not feasible at present, due to the lack of enough molecular markers known to be linked to specific phenotypic traits in this crop. The developments which are expected to take place within the next few years in this field will probably make this approach more feasible.

Where molecular markers appear to have the most immediate potential is in indicating variety associations in which EDV relationships may be implicated. Investigating a possible EDV relationship requires a creditable methodology for indicating genetic conformity between an established and a newer variety. It is unlikely that any of the varieties studied were at a similarity level typical of an EDV relationship; but the most similar variety pair (Blazer – Repell) was the most robust and consistently reproduced relationship across all three methods. To a lesser extent also the close pair Herbie – Merganda was scored more or less consistently over the three different systems. This complies with the observations of Gilliland et al. (2000) and Roldán-Ruiz et al. (2000b) who found that when varieties with shared genepools were examined using AFLP markers, extremely high similarity measures were produced and were also linked to morphological similarities. Therefore, the molecular methods described in this study might be used to indicate genetically conforming variety pairs, with potentially shared genepools.

Before either the AFLP or STS methods could, for example, be used to estimate genetic relationships among ryegrass varieties, the robustness and reproducibility of these systems across a wider range of varieties would need to be demonstrated and an accepted statistical protocol established for quantifying the degrees of difference. With respect to this it was notable that while the STS and AFLP methods displayed some similarities, there were also notable differences. Weak correlations between RAPD and AFLP distances, and also with pedigree, have been reported before (Graner et al. 1994; Melchinger et al. 1994; Manninen and Nisala 1997). The observation that the most-closely linked variety pair was consistently described as very close by both methods is important, as this is the kind of relationship that is likely

to involve EDV or distinctness claims and so requires accurate description. A similar result was found by Roldán-Ruiz et al. (2000b), who also found that, in ryegrass, the repeatability and sensitivity of AFLP distances were less dependant on the number of plants examined than the number of markers and the primer combinations involved. Based on these findings and the current study, it is expected that a suitable protocol could be developed to achieve high and repeatable variety discrimination using AFLPs. In this context it is interesting to mention that a bootstrap-assessed mean coefficient of variation [mean of the (bootstrap standard error / bootstrap estimate for distance)] for AFLP amounted to 0.105, whereas it amounted to 0.305 for STS. Therefore, the AFLP system could serve as a benchmark for the STS system. To complete the test system, standardised statistical analyses would need to be established. It would be of great interest to know the map position of the molecular markers used to compute genetic similarities, in order to be sure that the genome is homogeneously covered.

Moreover, the knowledge of the position of the markers allows one to compute the BLUE (Best Linear Unbiased Estimation) of genetic distance. Unfortunately, for most of the markers used in this study the map position is unknown. Computed on rapeseed cultivars using AFLP markers, the sampling variance of the BLUE was found to be 23% lower than the Rogers variance (Lombard et al. 2000b). This gain in precision might be of great importance in the context of EDV.

Based on the evidence of the data generated in this study, a simple but useful statistical procedure for distinctness based on markers could be proposed as follows. Where two varieties i and j were to be compared, a number of plants from each would be fingerprinted with the same set of AFLP markers. The information on variety membership for individual plants could be represented by two 'indicator' variables. The first indicator would contain 1 every time a plant of variety i was recorded, and 0 otherwise. The second indicator would contain 1 every time a plant of variety j was recorded and 0 otherwise. The information on variety membership would be summarised in a plant × variety membership incidence matrix (plants × 2). The information on the markers would be summarised in a plants × markers incidence matrix. Both the variety membership matrix and the marker incidence matrix would then be transformed into a similarity matrix (or distance matrix = 1- similarity or $\sqrt{1-\text{similarity}}$). In the similarity matrix based on variety membership, two plants from the same variety would have a similarity of 1, and 0 otherwise. In the similarity matrix based on the marker information, the similarity between two plants could be calculated using measures like Jaccard's similarity coefficient or the simple matching coefficient. The correlation between these two matrices could be calculated (leaving out the diagonal) and a Mantel test used to assess the *p*-value. Effectively, variety membership would have to be permuted a number of times, after which a new variety membership similarity matrix is calculated and the correlation calculated again. After, for example, 999 permutations the position of the original correlation is determined in the set of sorted permutation correlations. When the original correlation is among the 5% most-extreme permutation results, it is assumed that the original correlation was significant, and that the varieties are distinct. This test for distinctness is based on the multi-response permutation test developed by Mantel and Varland, and described by Manly (1997). For Blazer – Repell the original correlation between membership similarity and AFLP similarity was 0.175, and the minimum value of 999 permutations was –0.074, while the maximum was 0.098. The original value of 0.175 is thus extreme and so it can be concluded that Blazer and Repell are distinct on the basis of AFLPs.

The same test could equally well be used for STS, morphology, or combinations of morphology and markers, by using a similarity or distance matrix based on observed characters that can be compared with a similarity matrix based on variety membership. An alternative procedure based on a permutation test of a bias-corrected form of the Roger distance was presented by Ghérardi et al. (1998). The null distribution of the Rogers distance was constructed by permuting the membership vector, and the position of the original distance is looked up in the set of sorted permutation distances. More work in this direction was presented by Nuel et al. (2000).

Conclusion

The overall findings from this study were that, using STS or AFLP markers, sufficient genetic diversity could be detected to differentiate registered ryegrass varieties. Although both marker methods did not provide exactly the same description of relationships between the tested varieties, there was some consistency. Little correspondence was found between either of the marker methods and morphological characterisation. The best agreement between all three methods was present for variety pairs that were very distant (e.g. those involving Yatsyn) or very close (e.g. Blazer – Repell). The implication is that these molecular markers, while not yet suited to certain operations in the registration of new varieties, offer a promising method for examining possible EDV relationships, subject to establishing standardised protocols and statistical techniques.

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