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Effectiveness of different classes of molecular marker for classifying and revealing variation in rice (*Oryza sativa*) germplasm

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Summary

We have examined the effectiveness of similar numbers of markers from four molecular marker systems (AFLP, isozymes, ISSR and RAPD) for revealing genetic diversity and discriminating between infraspecific groups of *Oryza sativa* germplasm. Each marker system classifies the germplasm into three major groups (most effectively with isozymes and AFLPs), but with differences (primarily with ISSR) between the precise classifications generated. However, at the highest levels of genetic similarity there was only partial agreement as to relationships between individual accessions when different markers were used. When variance was partitioned among and within the three subspecific groups, although the differences were not significant, greater variation was found among than within groups using AFLP and isozymes, with the reverse for RAPD and ISSR. Measurement of polymorphism using average heterozygosity and effective number of alleles gave similar results for each marker system. These results are discussed in relation to various genetic resources conservation activities, and the advisability of extrapolating to other sets of germplasm particularly of other crop species.

Introduction

The accurate identification of plant material in a gene bank is essential for effective germplasm characterisation: without such information breeders have no means of selecting appropriate material for entry into breeding programmes. While such identification may be undertaken using traditional field characters, in rice for example, this is not always possible or indeed accurate. The structure of the genetic diversity is also important when considering the development of 'core collections' (Jackson et al., 1998) in a genetic resources context. Further, without determining diversity reliably it would not be possible to identify molecular marker/quantitative trait associations which have been shown to be useful in the process of germplasm evaluation (Virk et al., 1996).

We have previously shown that identification of conserved rice germplasm at the species level can be effective using RAPD (Williams et al., 1990) markers (Martin et al., 1996). Historically in Asian rice (*Oryza sativa*), intraspecific classification into varying numbers of groups has been achieved using combinations of morphological, serological and hybrid fertility information (Kato et al., 1928), intervarietal hybrid sterility (Terao & Mizushima, 1942), and morphogeographical (Matsuo, 1952; Morinaga, 1954; Oka, 1958) data. Subsequently, the indica, japonica and javanica terminology for subgroups has been used extensively by plant breeders (Chang, 1976), and this has most recently been followed by the very successful adoption of six isozyme groups to describe in a biologically meaningful and robust way, the bulk of the primary gene pool of Asian rice (Glaszmann, 1987). It is of particular importance to breeders that unambiguous identification of indica (group I) and japonica (group VI) rices can be achieved, as these are currently the focus of plant breeders' attention for crossing and

for the development of the 'new plant type' (Khush, 1995; Peng et al., 1994), despite the fact that effecting hybridization and genetic recombination between these two types may not be easy.

Breeders attempting to utilize the considerable variation represented by these groups face increasing difficulty in classifying germplasm because of the inefficiency involved in the use of morphological characters. For example, the lines Azucena and PR 304 have been classified as indica using morphological characters, whereas they behave as japonica types in crossing studies (Gurdev Khush, personal communication). Because of this breeders have regularly used isozymes to help make the necessary identifications (Glaszmann, 1987, 1988).

Recently, a range of DNA-based markers have been employed for the study of plant diversity (Newbury & Ford-Lloyd, 1997) and each method has its own benefits and constraints. This applies to rice, where the infraspecific *O. sativa* groups can now be identified using RAPD (Virk et al., 1995), as well as other molecular marker strategies including RFLP (Wang & Tanksley, 1989; Zhang et al., 1992; Zheng et al., 1994), ISSR (Parsons et al., 1997; Zietkiewicz; et al., 1994), AFLP (Vos et al., 1994; Zhu et al., 1998) and microsatellites (Wu & Tanksley, 1993). RFLP-based markers are often considered more reliable than those based on the use of arbitrary PCR primers. For example, RAPD is often criticised for unreliability, particularly in terms of lack of reproducibility and transferability. Additionally, with fingerprinting strategies such as RAPD and AFLP, reliability could be affected by a lack of allelism between co-migrating bands since no information on sequence homology or marker inheritance is available. However, valuable studies such as those of Rouppe van der Voort et al. (1997) have indicated that the vast majority of co-migrating AFLP markers from different potato genotypes represent alleles at the same locus. This was shown localizing 89% of the markers to similar map locations. Where markers did not appear to map to similar locations, close re-examination of the autoradiograms showed subtle mobility differences for half of the apparently non-allelic markers (presumably due to differences in base composition of amplified fragments). The authors suggest that at least some of the other apparently non-allelic markers may be explained by structural chromosome differences between potato genotypes. Similar conclusions are drawn for rice by Nandi et al. (1997).

Whereas the reliability of any marker system can be effectively assessed using segregating populations to determine band heritability, when studying diversity in germplasm there is often no baseline or control with which a comparison of techniques can be made. Nevertheless, there are three kinds of method that are often employed to address various questions pertaining to the assessment of variation. Firstly the degree of relatedness is commonly studied by one or more clustering strategies (dendrograms generated from similarity or genetic distance matrices) to obtain an overall pattern of variation as well as the degree of relatedness among accessions. Pairwise values of similarity constituting the genetic similarity matrix however, can give a more detailed picture of the degree of relationship. Secondly, the overall level of polymorphism amongst accessions is generally estimated from the following statistics: mean heterozygosity (Hav), the sum of effective number of alleles (SENA), and average gene diversity. Thirdly, where a set of accessions can be further subdivided into small groups on a predetermined criterion, analysis of molecular variance (AMOVA) is used to test and quantify between and within group variation.

Given that in rice there is now a biologically meaningful, robust infraspecific classification which is derived using a range of independent criteria, namely crossability, morphological and isozyme data, this provides a sound base with which comparisons of molecular techniques applied to the above assessments can be made. Similar studies have previously often focused on the practical issues of choosing marker types (cost, technical and safety issues etc). Very little attention has been given to experimental comparison of estimates of genetic variation and diversity. Where they have, such investigations have suffered from an important fundamental limitation: the estimates made have been based upon a variable number of marker loci and hence their efficiency could not be the same (Lu et al., 1996; Milbourne et al., 1997; Powell et al., 1996; Russell et al., 1997). This in turn has made their comparison biased towards those estimates which are computed from greater numbers of markers.

In this paper we have set out to determine the relative effectiveness of very similar numbers of markers generated from four different molecular marker systems for the identification of, and discrimination between, three of the infraspecific groups of *O. sativa* drawn from a wide range of rice diversity. Additionally, we have assessed their ability to reveal 'genetic diversity' within the group of accessions under

consideration, and discuss their relative values for germplasm conservation management. This has been undertaken in the context of the need for effective infraspecific taxonomic identification in the work of gene banks, with particular reference to the collection of more than 104,000 germplasm samples held in the International Rice Genebank at IRRI.

Materials and methods

Forty two accessions previously characterised as groups I, II and VI, were obtained from the International Rice Genebank at IRRI. Four molecular marker strategies were employed in order to give, in each case approximately the same number of markers (37–41), namely isozymes (37), AFLP (41), RAPD (40) and ISSR (inter-simple sequence repeat, 38), and the details for each technique were as follows.

Isozymes

Ten coleoptiles were used for enzyme extraction and the isozyme variation was surveyed using 22 loci following Glaszmann et al. (1988) namely: *Enp-1, Est-5, Got-1, Got-3, Mdh-1, Mdh-2, Mdh-3, Pgi-1, Pgi-2, Amp-1, Amp-2, Amp-3, Amp-4, Sdh-1, Icd-1, Cat-1, Est-1, Est-2, Est-9, Adh-1, Pgd-1* and *Pgd-2*.

AFLP

DNA was isolated from leaf samples pooled from 10 seedlings (Virk et al., 1995; Garland et al., 1999) following a modified version of CTAB method of Murray & Thomson (1980). The AFLP procedure was modified from Vos et al. (1995). The genomic DNA was double digested with *Pst* I and *Mse* I restriction enzymes. *Pst* I and *Mse* I adapters were ligated to the restricted fragments. The sequence complexity of the mixture was reduced by selecting biotinylated DNA fragments using streptavidin coated beads. Non-selective amplification of the fragments was achieved by using the following primer combination: GATGAGTCCTGAGTAA (M-00) and AGACTGCGTACATGCAG (P-00). Selective amplification was performed using M-00+AG and P-00+GAT (end labelled with ^{33}P-γATP) primers. The amplification conditions and thermal profiles are described in detail elsewhere (Zhu et al., 1998). The amplified products were separated on a 5% polyacrylamide gel containing 7 M urea and visualised by autoradiography.

RAPD

DNA was extracted from 20 mg fresh leaf material taken from 10 randomly selected 2–3 week old seedlings (Virk et al., 1995). For the RAPD analysis, the total reaction volume was 25 μl containing 5 ng DNA, 200 μM of each dNTP, 0.4 μM decanucleotide primer, 1 U *Taq* polymerase, 2.25 mM $MgCl_2$ and 1 $\times$ PCR buffer. The amplification was performed in a thermocycler (Hybaid-Omnigene) programmed as follows: cycle 1, 2 min at 95 °C; cycles 2–3, 30 sec at 95 °C, 1 min at 37 °C and 2 min at 72 °C; cycles 4–5, 30 sec at 94 °C, 1 min at 37 °C and 2 min at 72 °C; cycles 6–46, 30 sec at 94 °C, 1 min at 35 °C and 2 min at 72 °C; cycle 47, 5 min at 72 °C. Ten μl of the amplified products were subjected to electrophoresis on a 1.4% agarose gel cast in 1X TBE and run in 0.5X TBE at 200 V for 2.5–3.0 h. The electronic image of the ethidium bromide-stained gel was captured using a Flowgen IS500 Imaging System and the bands were scored from the image displayed on the monitor. Four Operon primers used in the RAPD analysis were C-03, C-08, C-10 and C-14. All the reactions were repeated at least twice to monitor the reproducibility of banding patterns.

ISSR

Five primers representing five repeats of trinucleotides, anchored at 3′ end by dinucleotides were synthesized (Alta Bioscience, University of Birmingham). Their sequence was as follows, $(AAG)_5GC$, $(AAG)_5TG$, $(AGC)_5GG$, $(AGC)_5GA$ and $(GGC)_5TA$. The DNA template used for ISSR-PCR was the same as that used for RAPD analysis (above). The PCR reaction mix was also essentially similar to that of RAPD analysis with minor modifications (Parsons et al., 1997). The primer concentration and annealing temperature in the thermal profile varied from primer to primer and are given below:

Primer	Concentration	Annealing temperature (°C)
$(AAG)_5GC$	0.8 μM	39 °C
$(AAG)_5TG$	0.8 μM	39 °C
$(AGC)_5GG$	0.8 μM	52 °C
$(AGC)_5GA$	0.4 μM	52 °C
$(GGC)_5TA$	0.4 μM	59 °C

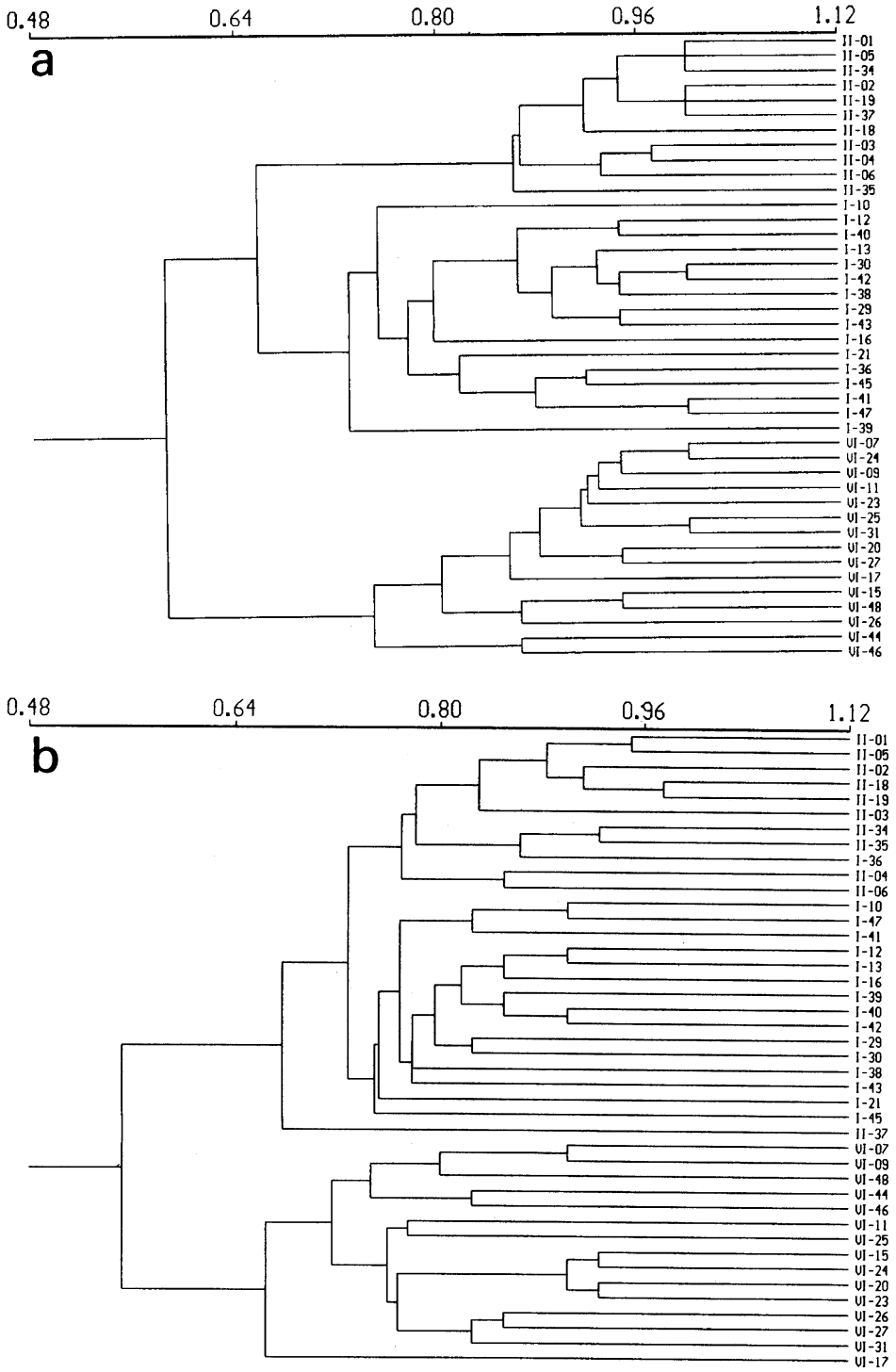

Figure 1. Dendrograms resulting from Cluster Analyses using the Simple Matching Coefficient and UPGMA: 1a – isozymes; 1b – RAPD; 1c – ISSR; 1d – AFLP.

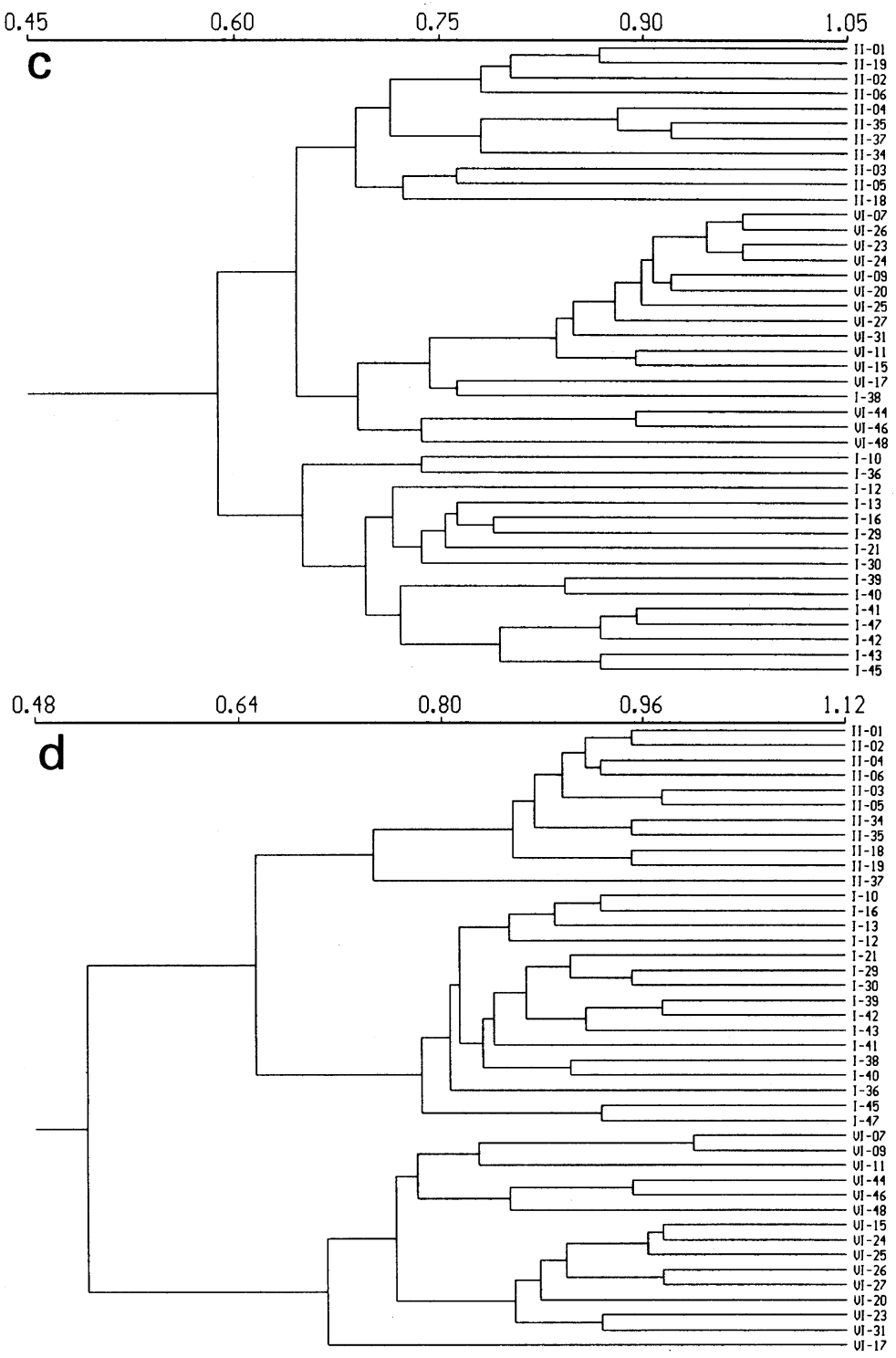

Figure 1. Continued.

Data analysis

For RAPD, ISSR and AFLP, amplification products were scored as present (1) or absent (0). This was also the case for isozyme loci since virtually all the material studied was homozygous. Indices of similarity were calculated using simple matching coefficient to estimate relationships between accessions. The unweighted pair-group method with arithmetic means (UPGMA) was used for clustering to produce dendrograms. Computation was performed using NTSYS-pc (Rohlf, 1992). This procedure was undertaken separately for each of RAPD, ISSR, AFLP and isozyme data.

The Mantel test of significance (Mantel, 1967) was used to compare each pair of similarity matrices produced above. In addition, for each similarity matrix produced, the average similarity was calculated for all pairwise comparisons within each of the three infraspecific groups, and for all pairwise comparisons between groups. The similarity coefficients follow the rule of binomial proportions (Ellis et al., 1997) and in order to perform a standard analysis of variance and comparison of groups and marker types the data were transformed according to arcsin $\sqrt{}$(percentage) (Steel & Torrie, 1981). Student's t test was performed in order to determine the level of significance of differences obtained. Also, the rank correlations were calculated using similarity coefficient comparisons of accessions within and among groups.

Analysis of molecular variance (AMOVA: Excoffier et al., 1992) was undertaken (using Arlequin) to determine the way that variation was partitioned within and among groups by each molecular marker system, and the significance of the differences between the methods was determined using *chi* square. The same software package was used to calculate the average expected heterozygosity (H_{av}), the sum of the expected number of alleles (SENA) and the average gene diversity (Nei, 1987) for each of the molecular marker data sets.

Results

Seven isozyme loci namely, *Enp-1, Est-5, Got-1, Got-3, Mdh-1, Mdh-2* and *Mdh-3* showed no variation, while the remaining 15 loci (*Pgi-1, Pgi-2 Amp-1, Amp-2, Amp-3, Amp-4, Sdh-1, Icd-1, Cat-1, Est-1, Est-2, Est-9, Adh-1, Pgd-1* and *Pgd-2*) revealed 37 alleles among 42 accessions.

Table 1. Comparisons of similarity matrices derived from different data sets using the Mantel test. Values are given for r, the product-moment correlation. Values greater than 0.5 are statistically significant at the 1% level

	RAPD	AFLP	ISSR
AFLP	0.77		
ISSR	0.22	0.42	
ISO	0.57	0.72	0.46

Dendrograms resulting from cluster analysis of isozyme, RAPD, ISSR and AFLP data obtained from 42 accessions of rice are presented in Figure 1. The analysis of the isozyme data, which includes data from loci which are commonly used to reflect the group structure in *O. sativa* (Glaszmann, 1987) shows a clear separation of the accessions into the three groups, with the greatest separation of groups one and two from group six (Figure 1a). In several cases however, the data fail to separate accessions within some small groups.

The analyses of RAPD, ISSR and AFLP data all allow discrimination between all 42 accessions. Compared to the isozyme analysis (Figure 1a), the RAPD analysis (Figure 1b) leads to the displacement of a single group I accession (Acc 36). A second group II accession (Acc 37) appears to be only distantly related to the remaining group II accessions. As with the isozyme classification, the RAPD analysis reveals a closer similarity between groups I and II compared to group VI.

Analysis of the ISSR data (Figure 1c) misplaces one group I accession (Acc 38) compared to the isozyme classification, but otherwise the accessions are once again separated into the same three groups. However, in contrast to the other classifications, the ISSR analysis indicates that group II accessions are more similar to group VI than to group I.

The AFLP data analysis (Figure 1d) is identical to the isozyme analysis in terms of assignment of accessions to groups, as well as revealing the closer association between accessions of groups I and II compared to VI.

The comparisons made so far have centred upon the ability of marker types to provide classification into three groups. However, at higher levels of similarity there is much less agreement among data sets when visual comparisons are made. For instance, while

Table 2. Significance of rank correlations calculated on pairwise similarities (simple matching coefficient) within and between crossability groups

	AFLP	ISO	ISSR
Within group I			
RAPD	NS	NS	NS
AFLP		NS	*
ISO			NS
Within group II			
RAPD	*	*	NS
AFLP		NS	NS
ISO			NS
Within group VI			
RAPD	*	NS	*
AFLP		NS	*
ISO			*
Between groups I & II			
RAPD	NS	NS	NS
AFLP		NS	NS
ISO			NS
Between groups I & VI			
RAPD	*	NS	NS
AFLP		NS	NS
ISO			NS
Between groups II & VI			
RAPD	*	NS	NS
AFLP		NS	NS
ISO			*
Between groups I, II & VI			
RAPD	*	*	*
AFLP		*	*
ISO			*

* Significant difference in rank; NS – not significant.

Table 3. Average similarities (%) (top figure) calculated using the simple matching coefficient and arcsin $\sqrt{}$percentage transformed data (lower figure)

Group	Isozyme	RAPD	ISSR	AFLP
I	80.5	77.3	70.3	83.7
	64.5 ± 0.6	61.7 ± 0.4	57.2 ± 0.4	66.5 ± 0.4
II	90.3	80.0	72.7	86.4
	73.3 ± 1.0	63.9 ± 0.8	58.8 ± 0.7	68.9 ± 0.7
VI	83.3	74.1	79.6	79.9
	66.7 ± 0.7	59.7 ± 0.5	63.9 ± 0.7	64.1 ± 0.7
I & II	65.8	72.4	54.5	66.4
	54.3 ± 0.4	58.4 ± 0.3	47.6 ± 0.3	54.7 ± 0.2
I & VI	58.1	55.5	62.3	49.3
	49.8 ± 0.4	48.2 ± 0.3	52.2 ± 0.3	44.6 ± 0.2
II & VI	58.9	54.9	65.0	47.2
	50.3 ± 0.4	47.9 ± 0.4	53.8 ± 0.3	43.4 ± 0.3
I, II & VI	60.7	60.4	60.7	53.9
	51.3 ± 0.2	51.2 ± 0.3	51.3 ± 0.2	47.3 ± 0.2
Overall	68.1	65.7	65.1	63.3
	56.4 ± 0.3	54.5 ± 0.3	54.1 ± 0.2	53.4 ± 0.4

accession 37 is the most atypical group II accession according to both AFLP and RAPD, with ISSR it is more than 90% similar to accession 35, and with isozymes it is indistinguishable from two other accessions.

A more objective comparison of patterns of similarity generated using the different data sets was provided by the use of the Mantel test to compare each pair of similarity matrices derived from the data sets. The test gives an indication of how any pair of similarity matrices correspond to each other in terms of the order of their individual elements. The product-moment correlations (Mantel, 1967) are given for each comparison in Table 1. From this analysis it can be seen that the AFLP and RAPD data have a very close relationship, and to a lesser extent (but still significant) the AFLP and RAPD with the isozyme data. The ISSR data show a non-significant (<0.5) correlation with RAPD, AFLP and isozyme data.

A further assessment of relationships at high levels of similarity was made by calculating the rank correlations of similarity coefficients of pairwise comparisons within each of the three groups, and then among members of the different groups (Table 2). If an assessment is made from Table 2 of rank from all of the 42 possible comparisons of techniques and groups (within and among), then it is found that 16 are significantly different. More specifically, the rank orders using both AFLP and RAPD are significantly different in 9 cases, 8 cases for ISSR and 6 for isozymes. Further, RAPD and AFLP rank orders differ significantly in 5 cases, whereas AFLP and isozymes only differ significantly in 1 case.

The overall average similarity was calculated using the similarity matrix for each data set, together with that for each of the groups, and all pairwise comparisons between each pair of groups. The results are shown in Table 3. An analysis of variance (data not shown) of the arcsin $\sqrt{}$percentage transformed similarity coefficient data (Table 3) showed that most marker types and isozyme groups differed among themselves, and their interaction was also statistically significant. Sixteen accessions belonging to isozyme group I showed the maximum diversity (SI = 77.96%) while 11 group II accessions revealed the

Table 4. Partitioning of variance within and between crossability groups derived from the Analysis of Molecular Variance (AMOVA), for data derived from different markers

	RAPD	AFLP	ISO	ISSR
Percent variance:				
among	41.66	63.32	59.67	34.53
within	58.34	36.68	40.33	65.47
Total variance:	7.93	9.47	7.32	7.47

Table 5. Polymorphism revealed in the four data sets, using average heterozygosity (H_{av}) and effective number of alleles (SENA)

Polymorphism	Isozymes	RAPD	ISSR	AFLP
H_{av}	0.31 (0.18)	0.33 (0.13)	0.34 (0.16)	0.36 (0.15)
SENA	20.00	22.33	22.52	25.82

minimum diversity (SI = 82.38%). In general, ISSR marker types unveiled the maximum molecular variation (SI = 74.28%) while on the other hand isozyme and AFLP marker types fared worse for diversity estimates (SI = 83.49 and 82.83 respectively). It is also noteworthy that RAPD markers revealed maximum molecular variation among japonica types while the overall trend favours ISSR (Table 3).

The analysis of molecular variation (AMOVA) revealed apparent differences in the partitioning of the variation within and among groups accomplished by the different marker systems (Table 4). However, a chi square analysis revealed the effects of the large variances involved, and critically demonstrated that the apparent differences were in fact not significant.

The mean expected heterozygosity (H_{av}), and the sum of effective number of alleles (SENA) as estimates of polymorphism were calculated for each data set (Table 5) together with the average gene diversity (Table 6). The results presented show that there are generally no differences among marker types. This is particularly the case with gene diversity where the standard deviations are particularly large.

Table 6. Average gene diversity (Nei) calculated for each crossability group using different molecular markers (standard deviation in brackets)

Group	RAPD	AFLP	ISO	ISSR
I	0.23 (0.12)	0.16 (0.09)	0.19 (0.11)	0.30 (0.16)
II	0.20 (0.11)	0.14 (0.08)	0.10 (0.06)	0.27 (0.15)
VI	0.26 (0.14)	0.20 (0.11)	0.17 (0.09)	0.20 (0.11)

Discussion

In this study, we have focused upon the relative efficiencies with which marker data sets, produced using four different techniques, can be used to define genetic relationships within a very diverse set of 42 accessions of *Oryza sativa*. Other workers have compared the effectiveness of different marker systems for revealing patterns of relationships with varying results. A study on winter wheat cultivars could not reveal a common pattern of relationships using three different marker systems (Bohn et al., 1999); similar patterns of relationships were revealed by three out of four marker systems when applied to maize inbred lines (Pejic et al., 1998); in barley, classification patterns were similar when using RFLP and AFLP but not SSR (Russell et al., 1997); for soybean genetic relationships were similar when using four different marker systems, but only when including both wild and cultivated germplasm, and not when restricting the study to cultivated only (Powell et al., 1996). In our study, we have found that each of the marker techniques tested can more or less effectively discriminate between the accepted intraspecific *O. sativa* groups. This is clear from the dendrograms resulting from the cluster analyses, and the subsequent similarity matrix comparisons. There are however, differences between the marker techniques in terms of classification. AFLP and isozyme markers classify all accessions according to their original designation, but ISSR and RAPD 'misclassify' either one or two accessions respectively.

With the exception of ISSR each marker technique is able to illustrate the generally accepted situation (Glaszmann, 1987) that groups I and II are more closely related to each other than they are to group VI. This probably accounts for the fact that the Mantel test indicates a lower correlation between the ISSR and each of the other markers. The seemingly anomalous ISSR classification of groups has been identified previously (Parsons et al., 1997), and is related to an increased number of centromeric markers which heavily influence the classification.

At the highest levels of similarity, there is only partial agreement with regard to the relationships of accessions defined by the different markers. This lack

of agreement is emphasised when the rank correlations are examined and raises a note of caution in terms of some of the possible applications of marker technology, particularly with regard to identification of duplicate or very similar accessions.

The second approach to the discrimination between groups by the different markers involved the calculation of average genetic similarity (Powell et al., 1996; Bowcock et al., 1994). High levels of average similarity within groups, and lower averages when two groups are combined can give an idea of the marker's ability to discriminate between groups. This discrimination was most effective with isozymes and AFLPs, with AFLPs being most effective at discriminating all groups. However, while RAPD might appear least effective in this discrimination, this is primarily due to RAPD revealing a fairly high level of similarity between groups I and II. This result is not surprising in that groups I and II are generally thought to be closely related.

The partitioning of the variance among and within the three crossability groups using AMOVA gave the indication that for AFLP and isozyme data there was greater variation among than within groups, and that the reverse was true for both RAPD and ISSR data. This situation supports the previous conclusion that AFLP and isozymes are both more efficient at discriminating between groups. However, this conclusion is tenuous given that the differences in the partitioning of the variance were found not to be significant.

Given that the measurement of polymorphism using the average heterozygosity and sum of effective number of alleles indicates there is essentially no difference between the marker techniques used, what overall conclusions can be drawn from the different comparisons of the marker techniques used to study molecular variation in rice? Clearly, estimates of diversity based upon allele frequencies can be obtained with a reasonable degree of confidence using any of the techniques applied. Discrimination of groups at the subspecific level using cluster analysis can also be achieved quite effectively with any of the techniques, but more effectively with AFLP and isozymes. There needs to be caution over the choice of technique in determining the relationships between these groups using cluster analysis however; RAPD, AFLP and isozymes support the accepted view of the relationships between groups, but ISSR does not. Also, at the highest levels of similarity, relationships between individual accessions vary depending on the technique used, and further caution is needed in drawing conclusions about some of the pairwise relationships. Other than the fact that isozymes are least able to discriminate all accessions, it would not be possible to say that one technique or other is more effective in this respect. A specific caution must be, not in the reliance on one marker technique or other, but in drawing conclusions about how the variation in the system is partitioned using AMOVA, and whether apparent differences are, or are not significant. While from our study it is possible to draw conclusions about the effectiveness of different marker systems for analysing diversity and genetic relationships in rice, it would not be appropriate to extrapolate directly to other crops.

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