# The use of RAPD markers to facilitate the identification of *Oryza* species within a germplasm collection

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## Summary

RAPD analysis was carried out using 93 accessions held within the *Oryza* collection in the Genetic Resource Center at IRRI. These accessions had been designated as *O. meridionalis*, *O. glumaepatula*, *O. nivara* or *O. rufipogon* on the basis of the identification of the original collector although in some cases these had been subjected to subsequent taxonomic revision. Following numerical analysis of the RAPD data, we propose that five of the forty accessions designated as *O. meridionalis* and four of the 22 accessions designated as *O. glumaepatula* have been mis-identified. The relationship between accessions designated as *O. nivara* and *O. rufipogon* is complex, although it appears that some mis-identification has also occurred for these two taxa. The results indicate that RAPD technology can be used as a fast and accurate method to assist in the validation of the identification of wild *Oryza* species.

## Introduction

The potential use of wild relatives of crop species in plant breeding programmes has been one of the main reasons for conserving such material in germplasm collections. These collections are often difficult to manage because of their size. One of the problems that faces managers of collections which include wild relatives of a crop species is the accurate identification of collected material. Accessions are usually identified by the original collector and, partly because the material may not have been in ideal condition for the identification to the species level, the initial designation may not always be reliable. Within a large germplasm collection, it is not usually possible to grow and properly characterise all accessions to ensure that the initial identification is correct. Problems with identification are exacerbated when the taxonomy of a group is complex or confused, and becomes even more difficult if taxonomic revisions occur, as is the case within the genus *Oryza*. The collection of rice held in the Genetic Resource Center at IRRI holds more than 80,000 accessions of the genus *Oryza*. Whilst most of these are *Oryza sativa* L. (Asian

rice) there are also relatively large collections of other *Oryza* species with very interesting traits which can be included in breeding programmes. For example, *O. nivara* confers the gene for resistance to grassy stunt virus disease (Ling et al., 1970; Chang et al., 1975). A programme of morphometric characterisation of these accessions is under way, but this is time consuming. There are doubts about the accuracy of the species identification for some groups of *Oryza* accessions.

In recent years, molecular genetic techniques have provided a valuable tool for the study of germplasm. In particular, RAPD technology has been extensively used for studies of diversity in plant germplasm, as in *Musa* by Howell et al. (1994), *Brassica oleracea* (Kresovich et al. 1992), *Sorghum bicolor* (Tao et al., 1993), *Allium* (Wilkie et al., 1993), *Avena sterilis* (Heun et al., 1994), *Coffea* (Orozco-Castillo et al., 1994) and *Lens* (Sharma et al., 1995). Some studies have been focused on the classification and study of diversity of Asian *Oryza* (*O. sativa*) (Fukuoka et al., 1992; Yu & Nguyen, 1994; Mackill, 1995; Virk et al., 1995a). Work with rice has indicated that the pattern of variation revealed by RAPD analysis agrees well with

the pattern of diversity revealed by isozyme (Second, 1985; Glaszmann, 1987, 1988) and RFLP studies (Nakano et al., 1992; Wang et al., 1992). RAPD analysis has also been used to designate duplicate accessions of *O. sativa* (Virk et al., 1995b). Finally, associations between RAPD markers and performance for quantitative traits have been identified using a diverse set of *O. sativa* germplasm; this has allowed accurate predictions of the performance of accessions in the field using marker data and multiple regression models (Virk et al., 1996).

In this study, we have used RAPD technology to examine four wild species related to *O. sativa*, all of which are so-called A-genome species (*O. sativa* complex): *Oryza meridionalis* Ng, *O. glumaepatula* Steud., *O. rufipogon* Griff., and *O. nivara* Sharma et Shastry. For a long time, all four species were considered to be different forms of the same species: *O. perennis* Moench, most recently renamed *O. rufipogon* (Tateoka, 1963). After studies by Ng et al. (1981) some of the Australian germplasm was re-classified as a new species *O. meridionalis*. The situation of the other three species is not so clear. *O. glumaepatula* occurs in South America, and has been considered the American form of *O. rufipogon* (Tateoka, 1963). In fact, some authors still classify it as *O. rufipogon* (Second, 1985; Vaughan, 1994). The differentiation between *O. rufipogon* and *O. nivara* is even more complicated. *O. nivara* was recognized as a new species by Sharma and Shastry (1965) mainly based on the annual form of *O. nivara* and the perennial form of *O. rufipogon*, as well as other different characteristics. But, as has happened with *O. glumaepatula*, some authors consider these to be two forms of the same species (Second, 1985; Wang et al., 1992).

The major objective of this work was not to question the taxonomic organisation of these A genome species but to assess the utility of RAPD technology for aiding the identification of accessions which have an anomalous taxonomic status. This has been carried out using *Oryza* germplasm designated as four wild species and held within the Genetic Resources Center at IRRI.

## Materials and methods

### Plant material

Forty accessions designated as *O. meridionalis*, fourteen accessions of *O. rufipogon*, fourteen of *O. nivara*, three hybrids of *O. nivara* and twenty two accessions of *O. glumaepatula* (Table 1) were obtained from the Genetic Resources Center (GRC) at the International Rice Research Institute (IRRI). *O. meridionalis* material was grown at IRRI in pots in a screenhouse. Fresh leaf samples were collected, wrapped in moist paper, and sent to Birmingham, UK. Plants of *O. glumaepatula*, *O. nivara* and *O. rufipogon* were grown in Birmingham. Seeds were maintained in an oven at a constant temperature of 50 °C for one week to break their dormancy. They were then de-husked and surface-sterilized with 70% ethanol for 10 min and rinsed three times in sterile distilled water. Germination was carried out on moist filter paper in Petri dishes incubated at 30 °C under a 12 h light photoperiod. When seedlings approached 1–2 cm in length, they were transferred to 5.5 cm jiffy pots with compost and placed in an environmental growth chamber at 27 °C.

### DNA isolation

Initially, DNA was extracted from single individuals of four accessions (*O. nivara* 105895, *O. rufipogon* 106278 and 105942, and *O. glumaepatula* 101960) to assess the diversity within accessions of these wild species. The number of individuals tested varied between 12 and 21. For the main part of this work, DNA was extracted from bulked material of each accession. For *O. meridionalis* material, each accession was represented by 2–8 leaves from 1–4 plants. For the other three species, 20 mg of leaf material from two week old seedlings from ten plants per accession was used. In both cases, the DNA isolation was performed following the method of Virk et al. (1995a). DNA concentrations were estimated by subjecting samples to electrophoresis on 0.8% agarose gels in TBE buffer, staining with ethidium bromide, and visual assessment of band intensities compared with lambda DNA standards.

### PCR and electrophoresis

For the study of diversity within germplasm designated as *O. meridionalis*, three decanucleotides of arbitrary sequence obtained from Operon Technologies Inc. (Alameda CA, USA) were used: OPC-3, OPC-6 and OPK-11. In the later analysis, including samples of all four species, the same decanucleotides were used along with another six from the same company: OPH-1, OPH-2, OPF-13, OPC-8, OPC-10, and OPC-14.

*Table 1.*

| Species | Code | IRRI Accession Number | Country of Origin |
|---|---|---|---|
| *Oryza meridionalis* | | | |
| | 1m | 101145 | Australia |
| | 2m | 101146 | Australia |
| | 3m | 101147 | Australia |
| | 4m | 101148 | Australia |
| | 5m | 101411 | Australia |
| | 6m | 101466 | Australia |
| | 7m | 103304 | Australia |
| | 8m | 103317 | Australia |
| | 9m | 103319 | Australia |
| | 10m | 103320 | Australia |
| | 11m | 103321 | Australia |
| | 12m | 103322 | Australia |
| | 13m | 104085 | Australia |
| | 14m | 104086 | Australia |
| | 15m | 104089 | Australia |
| | 16m | 104092 | Australia |
| | 17m | 104093 | Australia |
| | 18m | 104498 | Australia |
| | 19m | 105279 | Australia |
| | 20m | 105281 | Australia |
| | 21m | 105282 | Australia |
| | 22m | 105283 | Australia |
| | 23m | 105286 | Australia |
| | 24m | 105287 | Australia |
| | 25m | 105288 | Australia |
| | 26m | 105289 | Australia |
| | 27m | 105290 | Australia |
| | 28m | 105291 | Australia |
| | 29m | 105283 | Australia |
| | 30m | 105295 | Australia |
| | 31m | 105298 | Australia |
| | 32m | 105299 | Australia |
| | 33m | 105300 | Australia |
| | 34m | 105301 | Australia |
| | 35m | 105302 | Australia |
| | 36m | 105303 | Australia |
| | 37m | 105304 | Australia |
| | 38m | 105305 | Australia |
| | 39m | 105306 | Australia |
| | 40m | 105598 | Australia |
| *Oryza glumaepatula* | | | |
| | 1g | 100184 | Cuba |
| | 7g | 100924 | Brazil |
| | 9g | 100961 | Cuba |
| | 11g | 100969 | Surinam |
| | 12g | 100970 | Brazil |
| | 13g | 100971 | Brazil |
| | 14g | 101960 | Brazil |
| | 20g | 103810 | Venezuala |
| | 22g | 103812 | Venezuala |

*Table 1.* Continued

| Species | Code | IRRI Accession Number | Country of Origin |
|---|---|---|---|
| | 26g | 104387 | Brazil |
| | 32g | 105465 | French Guiana |
| | 36g | 105663 | Brazil |
| | 37g | 105665 | Brazil |
| | 38g | 105666 | Brazil |
| | 39g | 105667 | Brazil |
| | 40g | 105668 | Brazil |
| | 41g | 105670 | Brazil |
| | 42g | 105672 | Brazil |
| | 43g | 105686 | Brazil |
| | 44g | 105687 | Brazil |
| | 45g | 105688 | Brazil |
| | 46g | 105689 | Brazil |
| *Oryza nivara* | | | |
| | 4n | 100593 | Taiwan (ROC) |
| | 16n | 101967 | India |
| | 19n | 103407 | Sri Lanka |
| | 23n | 103821 | China (PROC) |
| | 24n | 103830 | Bangladesh |
| | 31n | 105431 | Sri Lanka |
| | 49n | 105704 | Nepal |
| | 51n | 105721 | Cambodia |
| | 54n | 105834 | Thailand |
| | 56n | 105895 | Bangladesh |
| | 61n | 106148 | Laos |
| | 62n | 106154 | Laos |
| | 65n | 106185 | India |
| | 68n | 106345 | Myanmar |
| *Oryza rufipogon* | | | |
| | 3r | 100588 | Taiwan (ROC) |
| | 5r | 100678 | Taiwan (ROC) |
| | 27r | 104714 | Thailand |
| | 28r | 105214 | Sri Lanka |
| | 30r | 105402 | China (PROC) |
| | 50r | 105720 | Cambodia |
| | 52r | 105759 | Thailand |
| | 55r | 105890 | Bangladesh |
| | 57r | 105942 | Thailand |
| | 60r | 106135 | India |
| | 63r | 106161 | Laos |
| | 64r | 106168 | Vietnam |
| | 66r | 106275 | Papua New Guinea |
| | 67r | 106278 | Papua New Guinea |
| 'Hybrids' | | | |
| *O. rufipogon x O. nivara* | | | |
| | h15 | 101965 | India |
| | h17 | 102468 | Bangladesh |
| *O. nivara x O. spontanea* | | | |
| | h53 | 105815 | Thailand |

The DNA amplification reactions were performed in a volume of 25 $\mu$l containing 5 ng template DNA, 0.4 $\mu$M concentration of a single decanucleotide, 200 $\mu$M of each dNTP, 2.5 mM magnesium chloride and 1 unit Taq polymerase in the ammonium buffer provided by the manufacturer of the enzyme. The reaction mixture was overlaid with two drops of mineral oil. Amplification was achieved in a Hybaid Omnigene thermocycler programmed as follows: one cycle 2 min at 94 °C; two cycles of 30 sec at 94 °C, 1 min at 37 °C and 2 min at 72 °C; two cycles of 30 sec at 94 °C, 1 min at 35 °C and 2 min at 72 °C; 41 cycles of 30 sec at 93 °C, 1 min at 35 °C and 2 min at 72 °C, followed by one cycle of 5 min at 72 °C. Aliquots of 10 $\mu$l of amplification products were loaded onto 1.4% (w/v) agarose gels for electrophoresis in 1xTBE buffer (Sambrook et al., 1989), followed by staining with ethidium bromide, and viewed and photographed under U.V. light. Molecular weights were estimated by reference to a 1 Kb DNA ladder (Gibco-BRL). All the amplifications were repeated at least twice, and only bands reproducible in several runs were considered for analysis. DNA samples from certain accessions were used as standards and amplified along with new batches samples in order to allow accurate comparisons of relative band mobilities.

*Data analysis*

For the analysis, only polymorphic amplification products were considered. Specific amplification products were scored as present (1) or absent (0). The simple matching coefficient was employed to create the similarity matrix in order to construct a dendrogram by the UPGMA method (Rohlf, 1992).

**Results**

In a preliminary experiment, amplifications of DNA from at least twelve individual plants from four accessions of different wild species (see Material and Methods), revealed that there was variation within accessions (data not shown). The frequency of polymorphic bands within accessions was usually in the range 0.03–0.08. Our previous studies with *O. sativa* accessions demonstrated that a representative set of amplification products for an accession could be obtained by pooling leaf material from individuals before DNA extraction (Virk et al., 1995a,b). This procedure was followed with the wild *Oryza* species in all later experiments.

Of the four species considered in this work, the one most clearly accepted as a different species, and with a lower degree of similarity to the other members of the *sativa* complex is *O. meridionalis*. For this reason, germplasm designated as *O. meridionalis* was first subjected to RAPD analysis in order to assess the degree of homogeneity within the material. Following amplification with three decanucleotides a total of 26 reliable polymorphic bands were scored for 40 *O. meridionalis* accessions. The dendrogram obtained from these data is shown in Figure 1. Several sets of accessions are not separated from each other on the basis of the use of 26 marker bands (e.g. accessions 29m and 35m, and 1m, 2m, 3m and 4m). Later studies on these sets of potentially duplicate accessions used a further eight primers, and resulted in the production of more than 100 bands per accession (not shown). In no case did any two accessions share all the scored bands. Accessions 1m and 2m differed in only one band out of 108, with other pairs of potentially duplicate accessions differing by 4–7% of the markers.

Figure 1 shows a clear division between two main clusters of accessions. Whilst one of them contains most of the accessions considered, the other one, with less than 30% similarity with the main group, contains five accessions (5m, 22m, 23m, 28m and 36m). The major division between these five and the other accessions of *O. meridionalis* suggests that the identification of some of this material is not correct. To check the possible misidentification of this material, these five accessions (5m, 22m, 23m, 28m and 36m) plus two *O. meridionalis* holotypes (accessions 2m and 3m) were subjected to further, more intensive, RAPD analysis along with other *Oryza* material to which the suspect five *O. meridionalis* accessions may be closely related.

In this second study, the seven above mentioned accessions designated as *O. meridionalis* plus a total of 53 accessions designated as *O. glumaepatula*, *O. nivara*, or *O. rufipogon* and the three hybrids of *O. nivara* (Table 1) were analysed using nine oligonucleotide primers. From these amplifications, 81 reliable polymorphic bands were scored, and the cluster analysis obtained with these data is shown in Figure 2. The first group of accessions separated in the dendrogram corresponds to the two holotype accessions of *O. meridionalis* indicating that this species is substantially different to the other 58 accessions examined. However, the position within the dendrogram of the five suspect accessions shows that these accessions are more closely related to *O. rufipogon* than to true *O. meridionalis*.
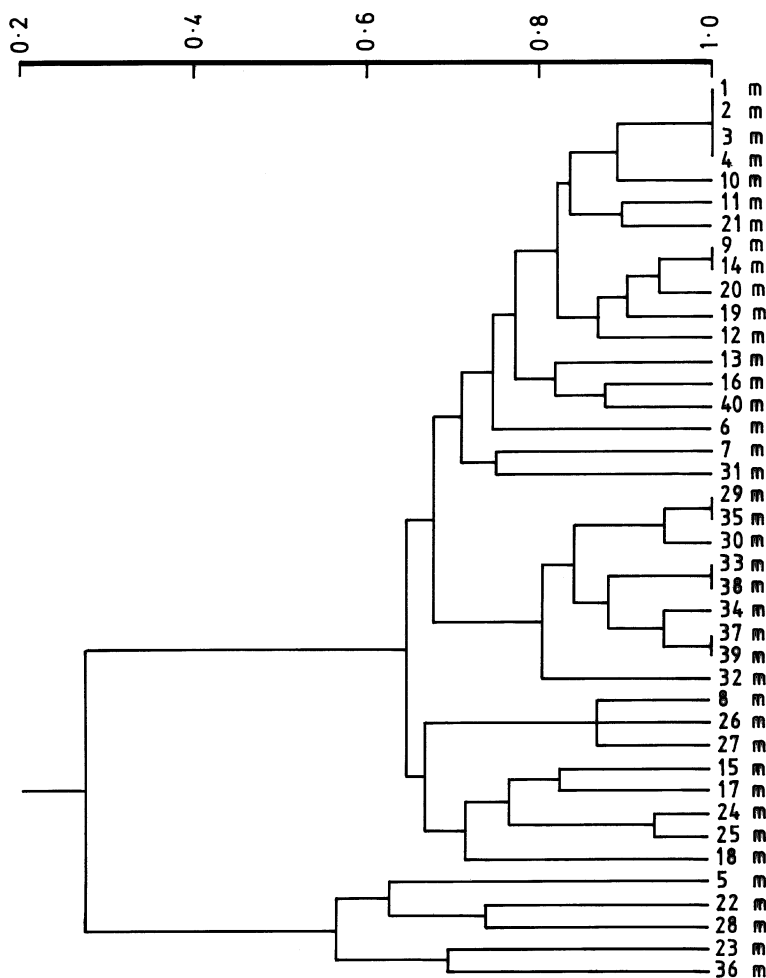
*Figure 1.* Dendrogram generated by clustering using UPGMA analysis computed from pairwaise comparisons of RAPD bands of 40 accessions designated as *O. meridionalis*.

Most of the accessions designated as *O. glumaepatula* were clearly clustered in a well-defined group (Figure 2); amplification products produced from 13 of these accessions are shown in Figure 3. However, the results strongly suggest that four accessions (1g, 14g, 20g and 26g) have been mis-identified. The differentiation between *O. nivara* and *O. rufipogon* in the dendrogram is not very clear, with samples from the two species falling within a complex cluster along with the suspect *O. meridionalis* and *O. glumaepatula* accessions. However, within this complex group, it is possible to distinguish two different clusters (A and B) and a group of accessions which do not cluster either with A or B and are defined in the dendrogram as group C (Figure 2). Cluster B is composed of most of the *O. rufipogon* accessions (except the accession 5r), the sus-

pect five accessions of *O. meridionalis*, one accession previously designated as *O. glumaepatula*, and only one sample designated as *O. nivara* (4n). The other main cluster (A) is formed by many of the accessions of *O. nivara*, the three putative hybrids, only one sample designated as *O. rufipogon* and two of the samples designated as *O. glumaepatula* (20g and 26g). The very loose group C comprised one accession designated as *O. glumaepatula* (14g) and four accessions designated as *O. nivara* (19n, 31n, 68n and 49n). However, these accessions show very low similarity levels to one another.
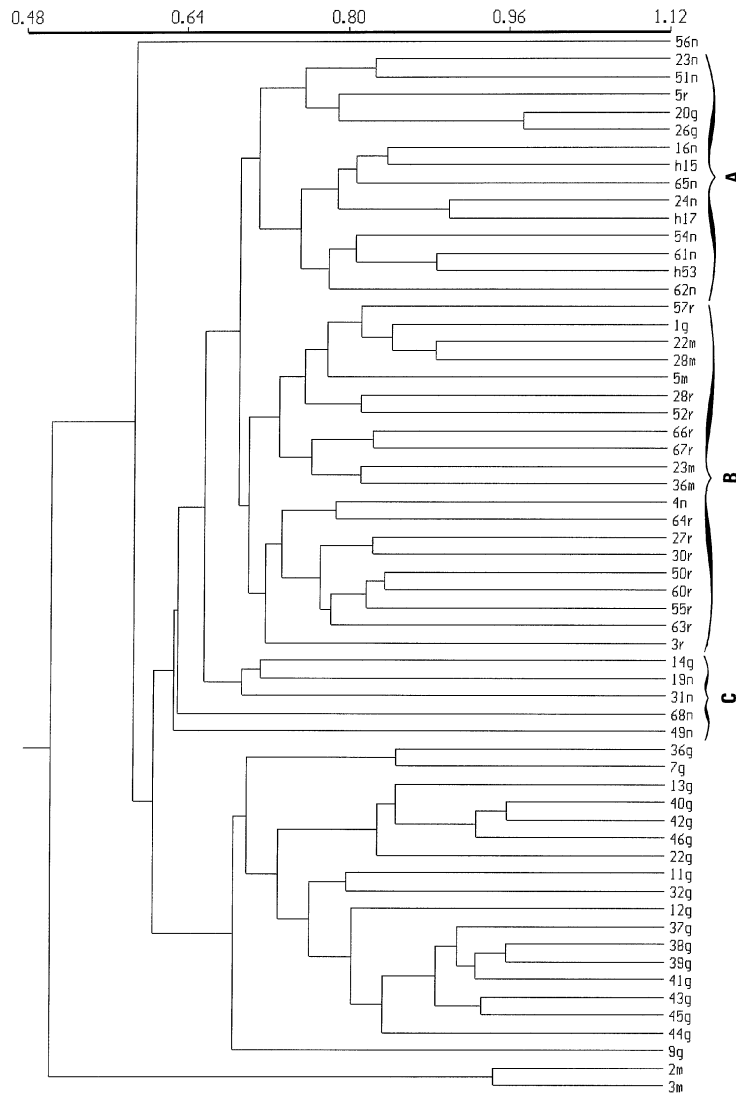
*Figure 2.* Dendrogram generated by clustering using UPGMA analysis computed from pairwaise comparisons of RAPD bands of 53 accessions variously designated as *O. meridionalis* (m), *O. glumaepatula* (g), *O. nivara* (n), *O. rufipogon* (r) or hybrids (h).

**Discussion**

In this study, a total number of 93 accessions held within the *Oryza* collection at IRRI have been examined. These had been classified as representing four wild species largely on the basis of the identification made by the original collectors, although in some cases this has been changed because of taxonomic revisions. In a previous study of cultivated Asian rice (*O. sativa*) germplasm, the use of molecular data for the recognition of duplicate accessions was described (Virk et al., 1995b). It is clear from the results obtained here that

none of the 93 wild species accessions examined can be regarded as duplicates.

The main objective of this study was to assess the usefulness of RAPD technology for the identification of species within a germplasm collection. This was achieved by Transue et al. (1994) using *Amaranthus* species. As with the *Oryza* species considered here, accurate classification of *Amaranthus* genetic resources on the basis of morphology is difficult. However, these workers were able to classify *Amaranthus* accessions into three groups corresponding to morphological species identification. Furthermore, they
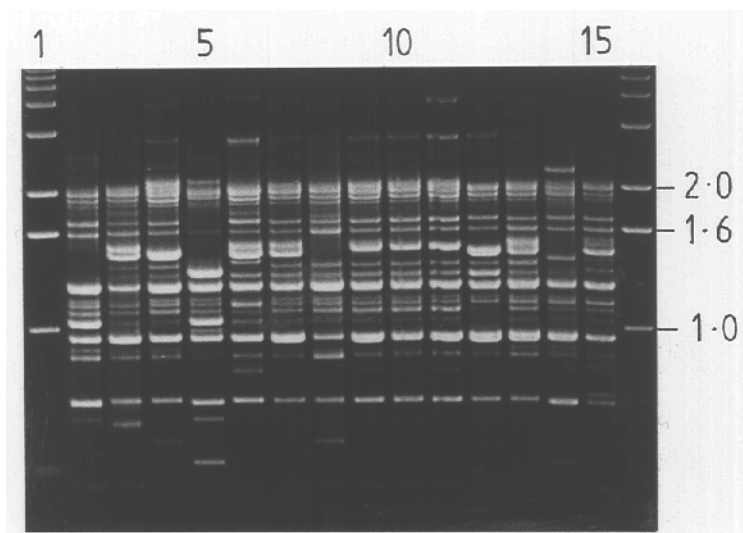
*Figure 3.* Amplification products following PCR primed by decanucleotide OPK-11 and supported by template DNA from *Oryza glumaepatula* accessions (lane 2–13) 1g, 11g, 12g, 14g, 32g, 37g, 38g, 39g, 41g, 43g, 44g, 45g; *O. rufipogon* accession 3r (lane 14); *O. glumaepatula* accession 9g (lane 15). Lanes 1 and 16 contain molecular weight markers some of which are indicated in kbp.

were able to identify one accession that had been mis-classified.

Our initial work was on germplasm that had been classified as *O. meridionalis*. Of the forty accessions available, two accessions (2m and 3m) were holotype material with which the others could be compared. In the initial test (Figure 1) it was clear that whilst 35 of the accessions clustered with the two holotype samples, five accessions that had been designated as *O. meridionalis* were not, in fact, very closely related to the others. Further investigations were carried out including the holotype *O. meridionalis* accessions and the five suspect accessions, along with germplasm classified as three other *Oryza* species. On the basis of the RAPD data, there is a clear distinction between the true *O. meridionalis* accessions and all the other germplasm used here (Figure 2). This supports the classification of *O. meridionalis* as a different *Oryza* species, and is in agreement with other work (Wang et al., 1992). The five suspect *O. meridionalis* accessions were much more closely related to material which had been designated as *O. rufipogon* . We therefore suggest that these accessions (5m, 22m, 23m, 28m and 36m) were not correctly identified when initially introduced into the collection, although the possibility must exist that mis-handling has occurred when processing the material during conservation activities.

Among the accessions used for comparison with the *O. meridionalis* germplasm were 22 accessions that had been classified as *O. glumaepatula*. Eighteen of these accessions formed a distinct cluster well separated from accessions of other species. Although we do not have type material available for this work, the RAPD data support the classification of this *Oryza* material as a different species. The dissimilarity of four of the 22 accessions designated as *O. glumaepatula* from the rest, along with their close similarity to accessions designated as *O. rufipogon* suggest that these four accessions have also been mis-identified.

It is usual to use key characters to distinguish between closely related species. Vaughan (1989) initially suggested that a lack of extravaginal branching and a semi-erect habit can be used to distinguish *O. glumaepatula* from *O. rufipogon*; however, later the same author stated that no good key characters can be found which can distinguish between these two species (Vaughan, 1994). This difficulty has led workers at IRRI to use a range of quantitative characters to compare accessions designated as these taxa. Their results support our observation that four accessions designated as *O. glumaepatula* (1g, 14g, 20g and 26g) are distinct from a homogenous group of the other *O. glumaepatula* accessions and that they fall within either *O. nivara* or *O. rufipogon* using morphological characters (Naredo et al., pers. commun.). One of the anomalous accessions has also been used in a crossability study by the same group and did not form fertile hybrids with other *O. glumaepatula* material.

The use of RAPD to classify material designated as *O. rufipogon* or *O. nivara* proved more difficult. These species are generally considered to be closely related; the original distinction was largely on the basis of annual/perennial habit. Vaughan (1989) refers to *O. nivara*, *O. rufipogon* and *O. sativa* as forming a large species complex. Taxonomists have differed in their acceptance of the name *O. nivara* to represent a separate species (Chang, 1976; Duistermaat, 1987). In our work, one accession (56n) is clearly separated from the other *O. nivara* and *O. rufipogon* accessions based upon RAPD data. Further RAPD analyses on this accession (not shown) indicate that this accession has a much closer similarity to *O. sativa* than to the two wild species. With regard to the remainder of the material designated as *O. nivara* and *O. rufipogon*, the clear separation shown between clusters A and B in the dendrogram based on RAPD data suggests that there should be a taxonomic sub-division within this material. Clusters A and B correspond to the existing identification of accessions as *O. nivara* and *O. rufipogon* respectively. Accessions 4n and 5r are inconsistent with this, but this could be because of mis-identification. However, four of the accessions classified as *O. nivara* fall within group C which contains diverse material that is markedly different from accessions in clusters A and B. It is possible that these accessions, along with the one originally classified as *O. glumaepatula*, that are also well-separated from the other clusters, represent intermediate forms resulting from hybridisation between wild species and *O. sativa* as described by Vaughan (1989). Regarding the three accessions which had been designated as putative hybrids, all were found to be closely related to accessions of *O. nivara* (Figure 2), always with more than 80% similarity. Although two of them had been classified as hybrids between *O. nivara* and *O. rufipogon* (h15 and h17), there is no evidence for this from our results.

This study indicates that, where genuine *Oryza* species differences exist, RAPD technology can assist in identification to support the management of germplasm collections. We have identified five accessions designated as *O. meridionalis* (5m, 22m, 23m, 28m and 36m) four as *O. glumaepatula* (1g, 14g, 20g and 26g), one as *O. rufipogon* (5r) and six of *O. nivara* (56n, 4n, 49n, 68n, 31n and 19n) for which the species identification is anomalous when compared to the RAPD data. We propose that these accessions should receive further study to confirm their identity before being passed on to plant breeders because of the possible confusion and technical difficulties that would arise from the use of mis-identified material.

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