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Interrelationship of cultivated rices *Oryza sativa* and *O. glaberrima* with wild *O. perennis* complex

Analysis of Fraction 1 protein and some repeated DNA sequences

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Summary. Phylogenetic relationship of the cultivated rices *Oryza sativa* and *O. glaberrima* with the *O. perennis* complex, distributed on the three continents of Asia, Africa and America, and *O. australiensis* has been studied using Fraction 1 protein and two repeated DNA sequences as markers. Fraction 1 protein isolated from the leaf tissue of accessions of different species was subjected to isoelectric focusing. All the species studied have similar nuclear-encoded small subunit polypeptides and chloroplast-encoded large subunit polypeptides, except two of the *O. perennis* accessions from South America and *O. australiensis,* which have a different pattern for the chloroplast subunit. Two DNA sequences were isolated from Eco R1 restriction endonuclease digests of total DNA from *O. sativa.* One of the sequences has been characterized as highly repeated satellite DNA, and the other one as a moderately repeated DNA sequence. These sequences were used as probes in DNA/DNA hybridization with restriction endonuclease digested DNA from some accessions of the different species. Those accessions that are divergent for large subunit polypeptides of Fraction 1 protein (*O. australiensis* and two of the four South American *O. perennis* accessions) also lack the satellite DNA and have a different hybridization pattern with the moderately repeated sequence. All other accessions, irrespective of their geographical origin, are similar. We propose that various accessions of *O. perennis* from Africa and Asia are closely related to *O. sativa* and *O. glaberrima*, and that the dispersal of cultivated and *O. perennis* rices to different continents may be quite recent. The American *O. perennis* is a heterogeneous group. Some of the accessions ascribed to this group are closely related to the Asian and African *O. perennis,* while others have diverged.

Key words: *Oryza sativa* – Wild rices – Fraction 1 protein – Repeated DNA – Phylogeny

Introduction

Oryza sativa is the major cultivated rice on the Asian continent; *O. glaberrima* is cultivated in Western Africa. No cultivated rice occurs naturally in the South American continent.

Considerable disagreement exists in the literature about the evolution of the cultivated rices and their relationship with the *O. perennis* complex, which is distributed in South East Asia, Southern China, South America and Africa (Nayar 1973; Khush 1975; Chang 1976a). *O. perennis* is a name of doubtful validity and the complex is supposed to include species named *O. rufipogon* and *O. nivara* in Asia, *O. longistaminata* and *O. barthii* or *O. breviligulata* in Africa, and *O. rufipogon* or *O. glumaepatula* in South America (Tateoka 1963; Chang 1976b). While the name *O. perennis* could be abandoned on the basis of wrong taxonomic identification of the original specimen, the problem of understanding the interrelationship of the *O. perennis* complex with cultivated rices remains.

Widely divergent views have been expressed on the phylogenetics and evolution of cultivated rices and wild *O. perennis* populations. On one extreme is the proposal by Chang (1976a) that *O. glaberrima* and *O. sativa* have originated independently from *O. perennis* complex species in the African and Asian continents. He suggests that the putative ancestor of the wild rices on the two continents was present on the Gondwana lands and that the Asian, African and American populations were separated at the time of continental drift. At the other extreme is Nayar's (1973) suggestion that *O. glaberrima* evolved from *O. sativa* that was introduced into Africa around the 10th century AD, and that as a semi-isolated peripheral population it has rapidly diverged from *O. sativa.* In the absence of any pertinent fossil record or substantial archaeological data, the study of rice evolution in a temporal sense is almost impossible. In a spatial sense interspecific relationships of cultivated rices and closely related *O. perennis*

have been studied on the basis of morphological characters (Chatterjee 1948; Oka 1964; Sampath 1962; Tateoka 1963) and crossability (Henderson 1964; Morinaga 1964; Oka 1964). Second (1982) has studied the relationships of *O. sativa* and *O. glaberrima* with related weedy and wild forms from Africa for up to 40 isozyme loci.

We have taken a number of accessions listed as *O. perennis* from Asia, Africa and South America, *O. nivara* from Asia and cultivated *O. sativa* and *O. glaberrima* (Table 1), and studied their interrelationship using Fraction 1 protein (Ribulose bisphosphate carboxylase oxygenase) and two repeated DNA sequences as markers. We have also included *O. australiensis,* which is more readily accepted as a distinct species, in our study.

Fraction 1 protein has been used extensively as a marker for studying the evolution of polyploid species (Wildman 1979). The protein is composed of a chloroplast-encoded large subunit and a nuclear-encoded small subunit. We have used Fraction 1 as it is a fairly conserved marker (when compared with some other enzyme loci), and isoelectric focusing of this protein gives information on both the nuclear and the chloroplast genomes.

A large proportion of the plant genome is made up of repeated DNA sequences (Ranjekar et al. 1978; Flavell 1982; Evans et al. 1983), the bulk of which are not transcribed or translated. While there is considerable disagreement concerning the function (if any) of repeated DNA sequences (Doolittle 1982; Dover et al. 1982), isolated repeated DNA sequences may be used as markers for studying phylogenetic relationships between the nuclear genomes of related species. We have characterized a tandemly repeated (satellite) DNA sequence isolated from the genome of *O. sativa* by restriction endonuclease digestion and agarose gel electrophoresis of total plant DNA. The satellite sequence has been used as a hybridization probe for the DNA of different accessions of *Oryza* used in this study. A moderately repeated sequence, isolated in a similar way to the satellite DNA, has also been used.

Materials and methods

Seed and plant growth

All the accessions of *Oryza sativa, O. glaberrima, O. nivara, O. perennis* and *O. australiensis* used in this study (Table 1) were acquired from the International Rice Research Institute, Los Banos, Philippines. Seeds were germinated in petri dishes and small seedlings were transferred to a half compost (Eff compost, Guildford, U.K.), half soil mixture in the greenhouse (25 ± 3 °C; 16 h light, 8 h dark; light intensity 8,000 lux maximum at the pot level). Plants were lightly watered for 2 weeks and then transferred to paddy conditions in the same greenhouse. Protein and DNA were extracted from plants that were about 2 months old.

Isoelectric focusing of Fraction 1 protein

Isoelectric focusing of the subunits of Faction 1 protein was carried out following the method of Cammerts and Jacobs (1980). Leupeptin (Sigma) was added to the protein extraction buffer at a concentration of 10 μM, following Johal and Chollet (1983). Polyacrylamide gel electrophoresis of total leaf proteins was carried out using a vertical electrophoresis kit (Shandon) at 10 mA/gel for 12 h. Isoelectric focusing was carried out on 1.4 mm thick polyacrylamide horizontal gels (LKB Multiphor system, Sweden) containing 8 M urea and 1.5% Ampholine (2 parts pH 5–8, 1 part pH 3–9.5, LKB) using 1 M sodium hydroxide as catholyte and 1 M phosphoric acid as anolyte. Isoelectric focusing gels were polymerized with ammonium persulphate. The maximum current at any time was 10 mA and the final voltage was maintained at 1,400 V for 6 h. The gels were fixed in a solution containing 3.5% w/v sulphosalicylic acid (Sigma) and 12% trichloroacetic acid (BDH). Protein was visualized by staining for 10 min at 60 °C in a solution of 0.15% PAGE blue 83 (BDH) in 25% v/v ethanol and 8% v/v acetic acid. Destaining was carried out in the above solution lacking PAGE blue 83.

DNA isolation, restriction and DNA/DNA hybridisation

Fresh leaf tissue was ground in liquid nitrogen, first in a Waring blender, and then briefly using a pestle and mortar. To each gram of leaf tissue was added 1 ml of 0.1 M sodium chloride, 0.025 M disodium EDTA (pH 8.0) 2% sodium dodecyl sulphate, 0.1% diethyl pyrocarbonate, 0.2 ml of 6 M sodium perchlorate, 1 ml of phenol (equilibrated with 0.2 M Tris HCl, pH 7.4) and 1 ml of chloroform/isoamyl alcohol (23 : 1). The mixture was shaken in the cold for 45 min, prior to centrifugation for 10 min at 4,000 g. The aqueous layer was re-extracted with chloroform/isoamyl alcohol until no further debris remained at the interface following centrifugation. DNA was precipitated by the addition of 2 volumes of cold ethanol, and spooled into a small volume of a solution containing 10 mM Tris, 1 mM EDTA (pH 8), and further purified by two rounds of caesium chloride density gradient centrifugation in the presence of ethidium bromide. Ethidium bromide was removed by repeated isopropanol extraction, and caesium chloride by dialysis against a solution containing 10 mM Tris, 1 mM EDTA (pH 8). DNA samples were stored at −20 °C.

DNA was digested with restriction endonucleases under conditions recommended by the suppliers (Bethesda Research Laboratories). Electrophoresis was performed in 1% vertical agarose gels, using Tris acetate EDTA buffer (0.04 M Tris, 5 mM sodium acetate, 1 mM disodium EDTA, pH 7.9).

Fragment isolation, nick translation and DNA/DNA hybridisation were carried out essentially as described by Evans et al. (1983).

Results

Analysis of Fraction 1 protein

When electrophoretically purified Fraction 1 protein bands were subjected to isoelectric focusing, the chloroplast-encoded large subunit polypeptides showed similar banding patterns for *O. sativa* (acc 20220, Al-Nan-Tsao 39, IR36, Tetep, V20B – indica types; CI 8970-S, Taipei 309 – japonica types), *O. glaberrima* (101135, 101855), *O. nivara* (101524, 103407), Asian

Table 1. A list of the *Oryza* species, their accession numbers and distribution, used in this study

International Rice Research Institute (IRRI) accessions no.	Species name[a] as used in this text	Also classified as	Classification by key of Chang (1976 b)	Country, place and continent of origin
101,410	*O. australiensis*		*O. australiensis*	Darwin, Australia
100,901	*O. perennis*		*O. rufipogon*	Samalkot, India, Asia
103,818	*O. perennis*		*O. rufipogon*	Mainland China, Asia
103,840	*O. perennis*		*O. rufipogon*	Thailand, Asia
101,524	*O. nivara*	*O. rufipogon* (annual)	*O. nivara*	India, Asia
103,407	*O. nivara*	*O. rufipogon* (annual)	*O. nivara*	Sri Lanka, Asia
20,220	*O. sativa*		*O. sativa*	Assam, India, Asia
	O. sativa (Var Al – Nan-Tsao 39, IR36, Tetep, V20B – indica types; CI 8970-S, Taipei 309 – japonica types)		*O. sativa*	
100,135	*O. glaberrima*		*O. glaberrima*	Guinea, Africa
101,855	*O. glaberrima*		*O. glaberrima*	Upper Volta, Africa
100,117	*O. perennis*		*O. barthii*	Guinea, Africa
101,202	*O. perennis*		*O. barthii*	Nigeria, Africa
101,254	*O. perennis*		*O. barthii*	Upper Volta, Africa
103,885	*O. perennis*	*O. barthii*	*O. longistaminata*	Tanzania, Africa
104,062	*O. perennis*	*O. barthii*	*O. longistaminata*	Nigeria, Africa
104,153	*O. perennis*	*O. barthii*	*O. longistaminata*	Cameroon, Africa
100,924	*O. perennis*	*O. glumaepatula*	*O. rufipogon*	Amazon River, Brazil, S. America
100,968	*O. perennis*	*O. glumaepatula*	*O. rufipogon*	Paramaribo, Surinam, S. America
100,970	*O. perennis*	*O. glumaepatula*	*O. rufipogon*	Amazonas, Brazil, S. America
103,810	*O. perennis*	*O. glumaepatula*	*O. rufipogon*	Venezuela, S. America

[a] The accessions were supplied by IRRI under this species name

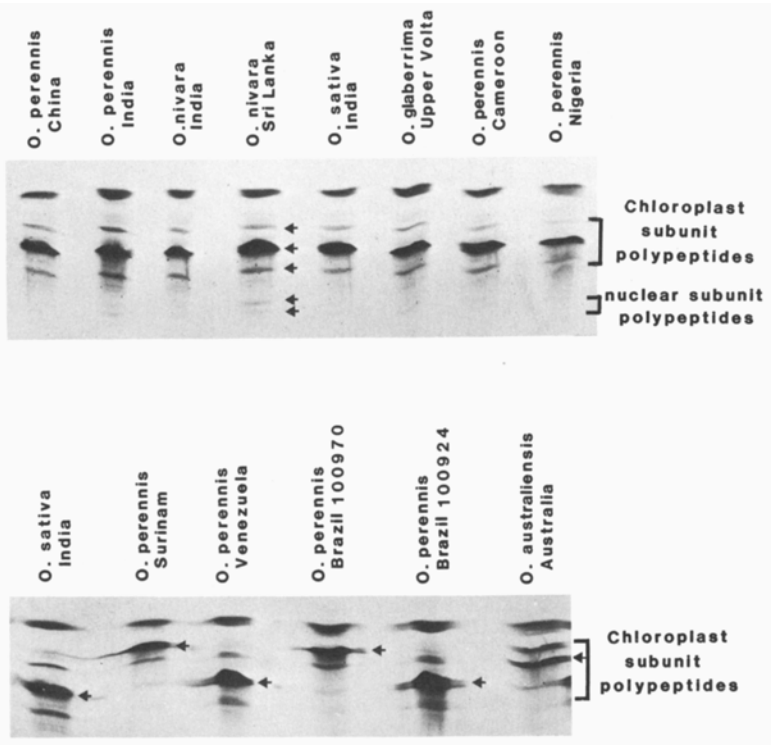

Fig. 1. Isoelectric focusing of Fraction 1 protein of some Asian and African cultivated and wild rice accessions. The chloroplast-encoded large subunit has three bands; the middle one being the most conspicuous. All the bands of *O. nivara* have been marked by *arrows.* The isoelectric focusing pattern in all accessions is similar

Fig. 2. Isoelectric focusing pattern of the large subunit of Fraction 1 protein of *O. sativa,* American *O. perennis* accessions and *O. australiensis.* Two of the four American *O. perennis* accessions and *O. australiensis* have different isoelectric focusing pattern. The middle bands of all the accessions have been marked with *arrows*

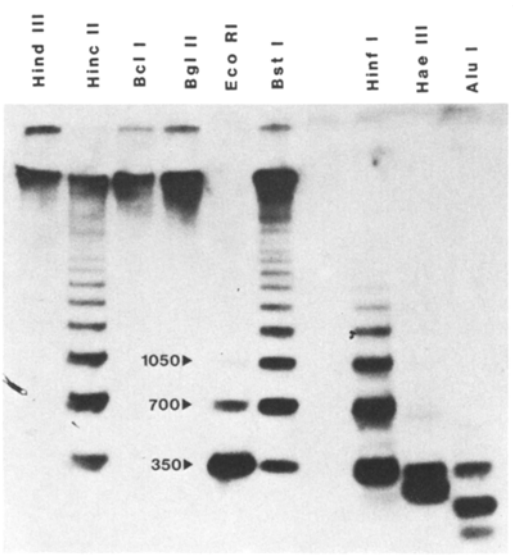

Fig. 3. Hybridisation of ^{32}P-labelled RI 350 probe to *O. sativa* DNA digested with various restriction endonucleases. Approximately 5 µg of *O. sativa* DNA was digested per track. Fragment lengths (base pairs) are indicated

O. perennis (100901, 103818, 103840) and African *O. perennis* (100117, 101202, 101254, 103885, 104062, 104153) – (Fig. 1). In all accessions there were two bands for the nuclear-encoded small subunit (Fig. 1). It is not clear whether the presence of two bands is a focusing artifact or it indicates that the small subunit is composed of two polypeptides. Nevertheless, the pattern is similar in all the above mentioned accessions.

The four accessions of American *O. perennis* (100968, 103810, 100970, 100924) and *O. australiensis* (101410) had small subunit isoelectric focusing patterns that were similar to that of *O. sativa.* Two of the American *O. perennis* (103810, 100924) were similar to *O. sativa* for their large subunit band pattern (Fig. 2). The isoelectric focusing pattern of large subunit polypeptides was different for two of the American *O. perennis* accessions (100968, 100970) and *O. australiensis* (Fig. 2).

All accessions were run at least three times: their banding patterns were found to be constant and as described above.

Use of repetitive DNA hybridization probes

Digestion of *O. sativa* DNA with restriction endonucleases liberates several families of repeated DNA sequences that may be observed as bands by ethidium bromide staining of the DNA after agarose gel electrophoresis. In the present study we isolated two such bands from EcoRI-digested *O. sativa* total DNA, having lengths of approximately 350 bp and 830 bp, designated RI 350 and RI 830, respectively.

RI 350 sequences were nick translated and used to probe Southern blots of various digests of *O. sativa*

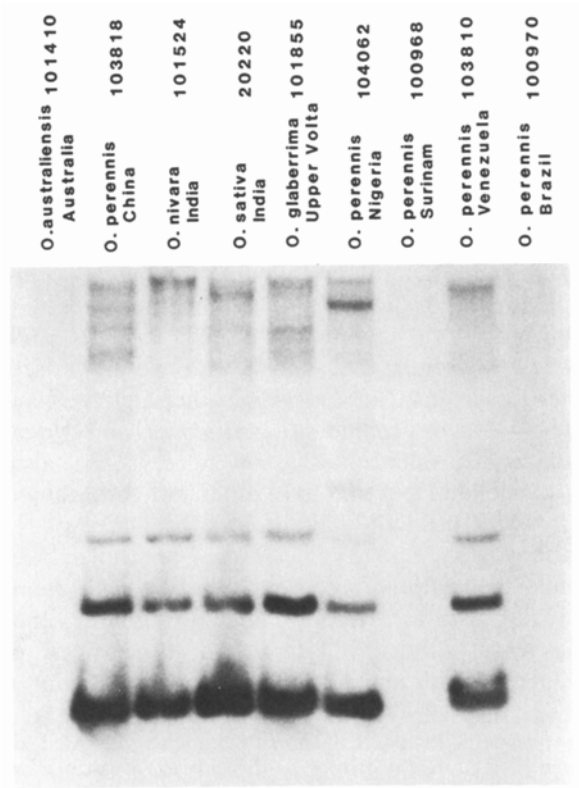

Fig. 4. Hybridisation of ^{32}P-labelled RI 350 probe to EcoRI-digested DNAs from nine *Oryza* accessions. The same amount of DNA was loaded in each case; accessions 101410, 100968 and 100970 show virtually no homology to this probe

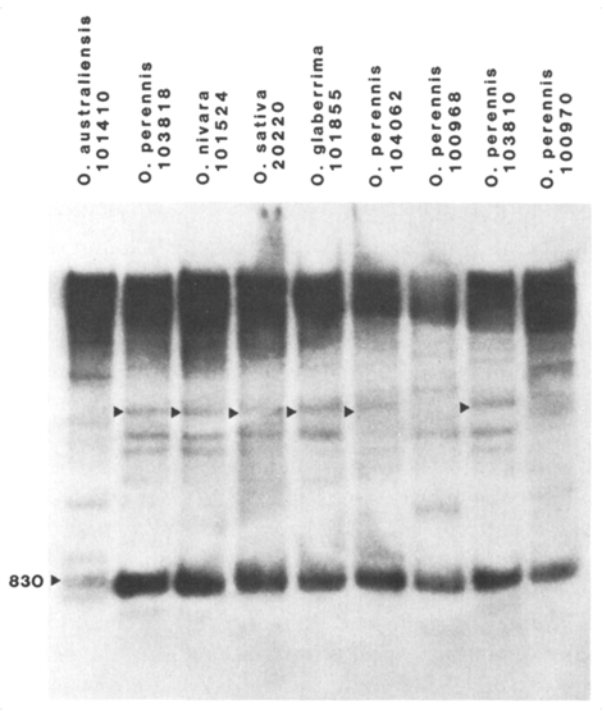

Fig. 5. Hybridisation of ^{32}P-labelled RI 830 probe to EcoRI-digested DNAs from nine *Oryza* accessions. A major band of hybridisation is seen at 830 bp in all accessions (somewhat reduced in 101410). Other bands are clearly visible, including one higher molecular-weight band that is shared by six accessions (*arrowed*)

DNA: in many digests the probe hybridised with a series of bands corresponding to multimeric lengths of a basic 350 bp repeat (Fig. 3). The results are precisely in agreement with those expected for a tandemly-repeated sequence, or satellite DNA, with a repeating unit of 350 bp. The fact that the satellite was virtually undigested by Hind III, Bcl I and Bgl II suggests that the basic repeat length does not include the recognition sites for those enzymes. On the other hand, most repeats include at least one site for EcoRI, Hae III and Alu I. With Hinc II and Bst I, only a fraction of the satellite is cut to produce a "ladder" of fragments, showing that only a subset of all repeats carry these sites. The results show that this plant satellite has a structure very similar to those of many animal satellite DNAs (Horz and Zachau 1979; Singer 1982).

RI 350 sequences were hybridized with EcoRI-digested DNAs from eight different *Oryza* accessions (Fig. 4). Of these, five showed patterns essentially identical to that seen with *O. sativa* DNA. *O. australiensis* (101410), however, and two *O. perennis* lines (100970 from Brazil, and 100968 from Surinam) lacked detectable sequence homology to the RI 350 probe under the conditions used here. Other *O. perennis* accessions, from Venezuela (103810), Nigeria (104062) and China (103818) shared the *O. sativa* pattern. Some high molecular weight bands are also visible in Fig. 4; it is not yet clear whether these are truly homologous to the satellite sequences, or simply to other DNA fragments contaminating the probe. The use of cloned satellite DNA probes will resolve this question.

When RI 830 sequences were nick translated and used to probe EcoRI digests of DNA from the various accessions, hybridisation was to a smear of fragments, with certain prominent bands (Fig. 5). Such a pattern is expected for a sequence that is interspersed with single copy sequences at many sites, and with repeated sequences at others (see Evans et al. (1983) for a discussion). All accessions show strong hybridisation to a band at 830 bp, with the exception of *O. australiensis* which has several weakly-hybridising bands at around that position. *O. glaberrima, O. nivara,* and three *O. perennis* accessions – from China (103818), Nigeria (104062) and Venezuela (103810) – share at least one strong higher molecular weight band with *O. sativa* (arrowed in Fig. 5). The other *O. perennis* accessions, from Surinam (100968) and Brazil (100970), showed different sets of bands at higher molecular weights.

Discussion

We propose that phylogenetic relationships can best be studied by structural analysis at different hierarchical levels (morphological, protein, DNA sequences etc). By quantifying divergence at these different levels and by superimposing the patterns upon one another, one can produce the most rational phylogenetic scheme. The results reported here are a preliminary study of rice phylogenetics using Fraction 1 protein marker and two repeated DNA probes, one of which is a satellite DNA and the other a moderately repeated DNA sequence. The Fraction 1 nuclear subunit polypeptide banding pattern is similar for all of the *Oryza* accessions used in this study (Table 1), but the chloroplast encoded large subunit polypeptide bands have different isoelectric focusing patterns for *O. australiensis* and two of the South American *O. perennis* accessions (100968, 100970) in comparison to all the others. Further analysis (Pental, unpublished) has shown that Fraction 1 profiles of eighteen species recognised within the genus *Oryza* (Chang 1976 b) have different profiles from those described here for the various accessions of *O. sativa, O. glaberrima* and *O. perennis.* On the basis of this analysis we propose that *O. sativa, O. glaberrima,* and *O. perennis* in Africa and Asia and at least some of the *O. perennis* accessions from America are more closely related to one another than they are to other species of *Oryza* present on each continent. As the genus *Oryza* has a number of polyploid species (Chang 1976 c), and there is considerable controversy on the genomic composition of different species (Nayar 1973), the Fraction 1 protein marker can be further used for a broad classification of *Oryza* genomes.

DNAs of *O. sativa, O. nivara, O. glaberrima* and *O. perennis* accessions from Asia (103818), Africa (104062) and one of the three accessions of *O. perennis* from South America (103810) show strong hybridization to the RI 350 satellite DNA probe isolated from *O. sativa.* On the other hand, two of the South American *O. perennis* accessions (100968, 100970) and *O. australiensis* do not show any hybridization with this satellite DNA. Gillespie et al. (1982) have advocated the use of satellite DNA as a marker for studying phylogenetic relationships, while others (Miklos 1982) have criticized the use of satellite DNA as a marker on the basis that it could be highly variable within a species or even a population. However, in our results the accessions either have satellite DNA – in which case the pattern is similar for the monomer, dimer and trimer form of the satellite sequence – or they completely lack the satellite. This all or none response makes this sequence a useful marker for studying *Oryza* phylogenetics. The study can be extended to another hierarchical level by studying the variability in nucleotide sequence of homologous sequences from different accessions of *Oryza* species which hybridise strongly with the *O. sativa* satellite DNA probe.

The moderately repeated DNA sequence isolated from *O. sativa* (RI 830) shows similar hybridization patterns to DNAs from *O. sativa, O. glaberrima, O. nivara, O. perennis* from Asia and Africa and to one of the *O. perennis* accessions (103810) from South America. *O. australiensis* and two of the *O. perennis* accessions (100968, 100970) show different hybridization.

Our results of Fraction 1 protein analysis and DNA/DNA hybridization analysis with two probes are in good agreement. Those accessions that are divergent for the chloroplast genome – *O. australiensis* and two of the four South American *O. perennis* accessions – also lack the satellite DNA and have different hybridization patterns with the RI 830 probe. All other accessions, irrespective of their geographical origin, are similar. We conclude that various accessions of *O. perennis* from Africa and Asia are closely related to *O. sativa* and *O. glaberrima.* Thus the dispersal of these rices to different continents may be quite recent.

Our results do not support Chang's (1976 a) proposal that *O. perennis* populations on the three continents separated at the time of continental drift. It is difficult to visualize how populations of *O. sativa* and *O. glaberrima* that have been isolated for almost the entire geological history of the Angiosperms (Daghlian 1981) can be so similar as to intercross (Chu et al. 1969) and in mixed strands are morphologically indistinguishable except for the difference in ligule length of *O. glaberrima* and *O. sativa* (Oka and Chang 1964). Chang's (1976 a) proposal could be valid only if there has been a remarkable degree of stasis in the morphological evolution of the *O. perennis* complex. In that case, *O. perennis* accessions from different continents should have shown divergence for the protein and DNA markers that we have used in this study. The African cultivated rice, *O. glaberrima,* crosses with *O. sativa* under natural (Chu et al. 1969; Nayar 1973) and artificial conditions (Oka 1964), suggesting a close relationship between the two. The results of the present study point to the same conclusion.

At the morphological level the difficulty of separating annual and perennial wild, weed and cultivated rices in South East Asia is well documented (Nayar 1973; Oka 1964). *O. rufipogon* (*O. perennis*), *O. nivara* and *O. sativa* form an interbreeding complex in large areas of South East Asia. These 'species' are not diverged enough to warrant species level designations. On the basis of close morphological resemblance between American and Asian *O. perennis,* Tateoka (1963) has suggested that they be merged under *O. rufipogon,* now used mostly for Asian *O. perennis.* Sampath (1964) agrees with this revision, although Henderson (1964) points out that, while the hybrids of *O. sativa* and Asian form of *O. perennis* are fertile, those between *O. sativa* and American *O. perennis* are sterile. This indicates considerable genomic divergence between American and Asian *O. perennis.* Our conclusion is that, for Fraction 1 protein and repeated DNA sequences, some of the American *O. perennis* are closely related to Asian *O. perennis,* while others have diverged. More accessions of American *O. perennis* need to be examined with the markers used in this study. It is possible that *O. perennis* accessions such as acc. 103810 will readily cross with *O. sativa* to produce

fertile F1 progeny, while the diverged accessions like 100970 will form sterile F1 progeny. Some of the American *O. perennis* accessions closely related to *O. sativa* may well turn out to be useful in plant breeding programmes.

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