Genetic Differentiation of Asian Cultivated Rice as Revealed by RFLP Analysis

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Abstract

Restriction Fragment Length Polymorphism (RFLP) was studied in 135 accessions of Asian cultivated rice, *Oryza sativa* L., of which 123 were landraces collected mainly from Asia. For Southern hybridization, 56 clones of rice genomic library and one maize cDNA clone were selected as DNA probes. Various kinds of polymorphism were detected, and almost all the cultivars could be distinguished from each other. Cluster analysis (CA) and principal component analysis (PCA) applied to the RFLP data revealed two major groups of landraces, with only a few cultivars intermediate between the groups. The groups, designated as clusters I and II based on the cluster analysis, were considered to correspond with the conventional varietal groups (Indica and Japonica), because they showed differences in phenol color reactions, *Est3* genotypes and responses to low temperature. Cluster I contained the cultivars collected from Southeast Asia, South Asia and their vicinity, while cluster II consisted of those from Southeast Asia and the temperate zone of Asia. Their distribution areas overlapped in Southeastern Asia. Several minor groups were recognized in each major group. Most of the smaller groups showed a particular geographical distribution, indicating that indigenous landraces had evolved in different areas over a long period of time.

Introduction

It is generally recognized that the evaluation of plant genetic resources is important and necessary for their utilization. Morphological characteristics, resistance and tolerance to pests and diseases, and quality traits are investigated in the primary, secondary and tertiary evaluation processes in different crops worldwide. These investigations are useful for analyzing genetic variations of a certain crop together with the geographical distribution. It is, however, difficult to evaluate a large number of accessions collected from various areas sometimes at one site where the conditions may be different from the original area. If local cultivars of a certain crop could be classified into several landrace groups which show a particular geographical distribution, such a classification may contribute to a better understanding and characterization of crop genetic resources.

Asian cultivated rice, *Oryza sativa* L., which is one of the most important cereals, is an ideal material for research on genetic variation and phylogenetic differentiation, since many researchers have studied various genetic characters to classify landrace groups, to investigate phylogenetic differentiation and to provide basic information for breeding. Hereafter, I would like to present our studies on the intraspecific differentiation of rice as revealed by restriction fragment length polymorphism (RFLP) (Kawase *et al.*, 1991).

RFLP describes the differences in the length of DNA fragments formed through the digestion with a restriction endonuclease. Recently, the use of RFLP has became very popular for linkage studies or gene mapping and for estimating of genetic differences. Since variation is detected directly at the DNA level,

RFLP can provide important information about genetic diversity and phylogenetic differentiation.

Materials and methods

A total of 135 accessions of *O. sativa* were used (Table 1). Among them, 123 were local cultivars collected from various areas of Asia, while 12 were improved ones. All the accessions were introduced and maintained at the National Institute of Agrobiological Resources (NIAR) in Tsukuba. Phenol color reaction, chilling injury, esterase isozymes and morphological characteristics of the accessions were investigated in the Laboratory of Plant Germplasm Introduction, NIAR (precise data are unpublished).

Germinating seeds were sown in small plastic pots filled with sterilized soil in a green house. The total DNA of each accession was extracted from the leaves of 4-week-old seedlings using the cetyltrimethyl-ammonium bromide method (Murray and Thompson, 1980).

RFLP was detected in 57 DNA clones which were selected from those used as markers for linkage mapping of rice (Saito *et al.*, 1991). Insert DNA fragments from the clones were labeled with ^{32}P-dCTP using the random primer method, and were used as probes for Southern hybridization (Southern, 1975).

Plant DNA from each accession was digested with either *Bam*HI, *Bgl*II, *Eco*RV or *Hin*dIII. The resulting DNA fragments were resolved by 0.8% agarose gel electrophoresis, denatured, transferred to nylon membrane filters (Southern, 1975). The DNA fragments immobilized on the filters were hybridized with a labeled probe and detected by autoradiography on X-ray films.

The variation between each pair of cultivars was expressed as the proportion of probes that showed

Table 1   Rice cultivars used

Region	No. of cultivars	Code name
Japan	5	JP 1 to JP 5
Northern part of China	2	NC 1, NC 2
Southern part of China	4	SC 1 to SC 4
Yunnan Province, China	10	YN 1 to YN 10
Philippines	5	PH 1 to PH 5
Vietnam	9	VT 1 to VT 9
Thailand	6	TA 1 to TA 6
Myanmar (Burma)	8	MY 1 to MY 8
Laos	10	LA 1 to LA 10
Malaysia	10	MA 1 to LA 10
Indonesia	10	IN 1 to IN 10
Nepal	10	NP 1 to NP 10
Bhutan	3	BT 1 to BT 3
Assam, India	2	AS 1, AS 2
Bangladesh	10	BA 1 to BA 10
India	8	ID 1 to ID 8
Northern part of Pakistan	5	PK 1 to PK 5
Sri Lanka	5	SL 1 to SL 5
Iran	2	IR 1, IR 2
Afghanistan	1	AN 1
CIS (ex USSR)	2	US 1, US 2
Italy	2	IT 1, IT 2
Africa	1	AF 1
North America	3	NA 1 to NA 3
South America	2	SA 1, SA 2
Total	135	

different RFLP profiles. Cluster analysis (CA) based on the unweighted pair group method with arithmetic means, and principal component analysis (PCA) were used to investigate intraspecific variation and to classify the cultivars.

Results

1 Variation of RFLP

Various kinds of polymorphism were detected by Southern analysis of the DNA: dimorphism (detected in 18 of the 57 clones) trimorphism (20), tetramorphism (9), pentamorphism (1), hexamorphism (7), heptamorphism (1), and tertradecamorphism (1). Almost all the 135 accessions used in the study could be distinguished from each other by RFLP except for 2 accessions (YN 2 and YN 5) collected from Yunnan Province of China.

Twenty-five cultivars showed intravarietal heterogeneity for at least one RFLP clone. In such a case, the most frequent genotype estimated was used to represent the accession for CA and PCA.

2 Landrace group classification

The PCA revealed two major groups of landraces in the scatter diagram (Fig. 1). The first and the second principal components contributed to 44.6% and 5.5% of the total variance, respectively. The two groups were distinguishable mainly by the first principal component. They clearly corresponded to the two large clusters, tentatively designated as clusters I and II in the dendrogram drawn by CA (Fig. 2).

The accessions in cluster I included those from Malaysia, Vietnam, Bangladesh, India, Yunnan Province of China, Sri Lanka, Myanmar (Burma), southern part of China, Thailand and Nepal. Cluster II included cultivars from Indonesia, Laos, Japan, Nepal, northern part of Pakistan, Yunnan Province of China and Philippines.

Cluster I consisted of smaller clusters designated as Ia, Ib, Ic and Ie. The accessions in Ia, Ib, Ic and Id overlapped in the PCA scatter diagram, from which those in Ie were clearly apart. Cluster II contained
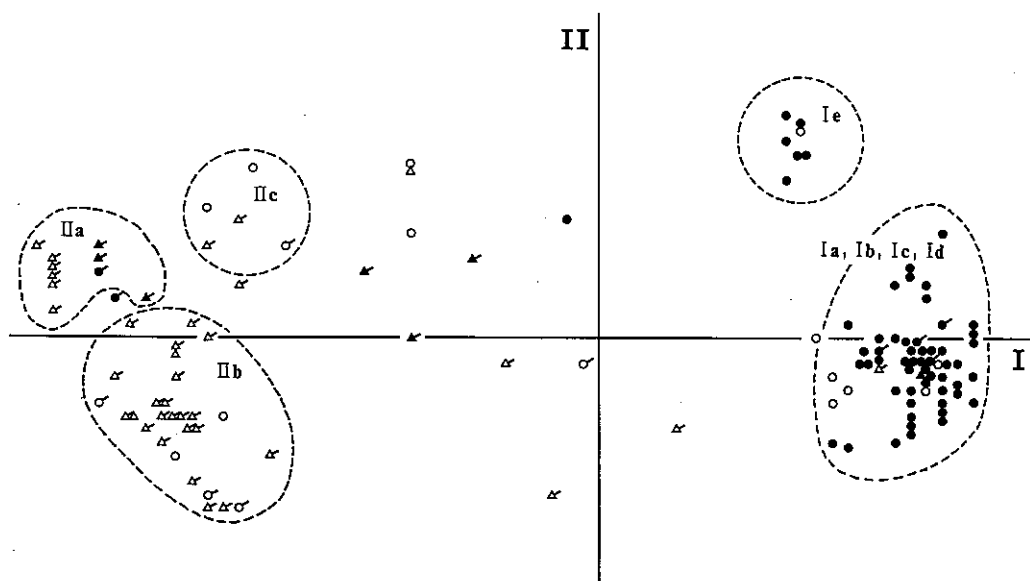

Fig. 1   A scatter diagram obtained from principal component analysis of RFLP of 123 landraces of rice (modified from Kawase et al., 1991). The horizontal and the axes show the first and the second principal components, respectively. A circle indicates an accession with $Est\,3^2$ allele, and a triangle indicates that with $Est\,3^1$. A black painted symbol indicates an accession with a Ph allele, while an open one indicates that with a ph allele. A symbol with a bar indicates an accession which was tolerant to chilling injury
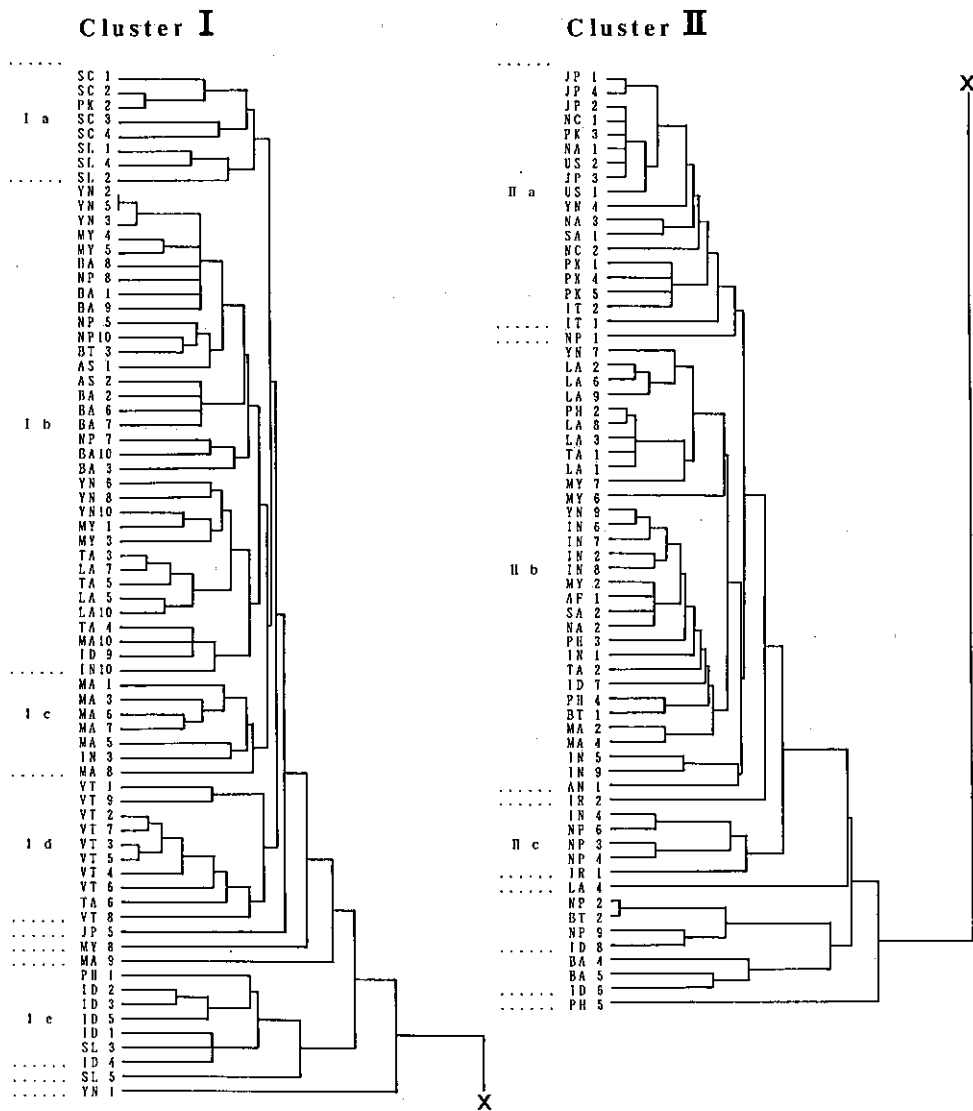
Fig. 2　A dendrogram indicating the relationships among 135 rice cultivars based on the cluster analysis of RFLP (Kawase *et al.*, 1991). Clusters I and II are linked between X and X'

smaller clusters, IIa, IIb and IIc, which were distributed separately in the scatter diagram.

Discussion

Brief history of studies on varietal classification of rice Kato *et al.* (1928) studied intraspecific hybrid sterility of rice and found two groups of cultivars, which they designated as subspecies *indica* and *japonica*. Terao and Midzusima (1939), however, reported a continuous variation in hybird sterility among cultivars and suggested the presence of another group distributed on Java Island and its vicinity. Matsuo (1952) propsed three different groups designated as A, B, and C types based on several morphological and agronomical characters. Oka (1953 a, b) surveyed variations in several physiological, biochemical and morphological characters, performed genetic analysis of hybird sterility, and classified rice varieties into continental and insular types, subdividing the latter into tropical insular and temperate insular types. Morinaga (1954) and chang (1976) recognized three groups. The three groups of rice are generally referred to as Indica, Javanica and Japonica types. Genetic variations at isozyme loci were examined to obtain a

precise classification (Nakagahra, 1978; Glaszmann, 1986). Ogawa *et al*. (1990) classified rice cultivars into 4 groups and 4 subgroups based on the difference in the responses to 4 races of bacterial blight pathogen. Japonica and Javanica types fell into one group, while Indica type cultivars were classified into several groups.

1 RFLP detection in rice genetic resources

Almost all the accessions used could be clearly distinguished from each other by RFLP. Two Chinese cultivars (YN 2 and YN 5), which showed an identical profile for all the 57 probes, exhibited a similarity in plant morphology and esterase isozymes, but differed in the phenol color reaction.

Genetic heterogeneity was noted in 25 accessions. The genetic variation within the cultivars used should have been reduced, because the rice materials collected and introduced to Japan were subdivided into different accessions when different morphological forms were found admixed, and each accession was cultivated on a small scale more than once for maintenance and multiplication. This heterogeneity implies the presence of a considerable genetic variation in a landrace population. It also suggests that a population of a rice landrace consists of mixed genotypes, and that we should be more careful in collecting plant materials of rice genetic resources during explorations. RFLP analysis may also be a useful tool for studies on genetic characterization as well as phylogenetic differentiation in cultivated crops compared with morphological characters which can be used as a direct means for artificial selection. The RFLP technique for the detection of DNA variation could be used for the identification of accessions in Gene Bank management if the method could be simplified and/or automatized.

2 Intraspecific differentiation in rice

PCA and CA revealed the presence of two distinct groups of cultivars, namely clusters I and II. It appeared that the accessions in cluster I and those in cluster II showed different genetic characters such as phenol color reaction, esterase isozymes and chilling injury (Fig. 1). Most of the landraces in cluster I showed a positive phenol color reaction (having Ph allele), had $Est\,3^2$ and were sensitive to chilling injury. In contrast, most of those in cluster II showed a negative phenol color reaction (ph), had $Est\,3^1$ and were tolerant to chilling injury. Consequently, the major landrace groups classified as clusters I and II are considered to correspond well to the conventional varietal groups, Indica type and Japonica (including Javanica) type, respectively. These results are in agreement with the observation made by Oka (1988) that the Indica and Japonica types differ in many characters and genes, but there is no evidence for the entity of so-called Javanica type as the same rank as Indica and Javanica and that Javanica may be regarded as a tropical subgroup of the Japonica type. The concept of two distinct groups of rice cultivars genetically differentiated is supported by isolation mechanisms such as hybrid sterility (Oka, 1953 a) and gametophyte genes (Nakagahra, 1972). Those groups overlap geographically in Southeast Asia, where only a few landraces were found to be genetically intermediate between them (Fig. 1).

Several smaller groups were recognized within the major groups (Fig. 2). The cultivars in cluster Ie were distinct from other accessions in cluster I. Most of the smaller groups were distributed in particular regions. For example, the cultivars in cluster Ia were collected mainly from the southern part of China and Sri Lanka, Ic from Malaysia, Id from Vietnam, Ie from India, and IIc from Nepal. A few smaller clusters were distributed in rather wider areas. Cluster Ib comprised landraces of areas extending from Southeast Asia to the northern part of the Indian Subcontinent. Cluster IIa comprised those distributed in the temperate zone of Eurasia including Japan. Most of the accessions in cluster IIb were collected from Southeast Asia. Small indigenous landrace groups are considered to have evolved for a long time in their own specific areas and their geographical distribution sometimes overlaps.

Further comparative analysis of RFLP, morphological, biochemical and physiological characters, and other genetic characters using a large number of landraces, and if necessary wild relatives should be performed to clarify the genetic differentiation of Asian cultivated rice.

It is concluded that investigations on DNA polymorphism may become a new field of evaluation of plant genetic resources in addition to the conventional evaluation procedures based on morphological characters, resistance and tolerance, quality traits, etc. Consequently, such investigations can provide important information on genetic variations and phylogenetic differentiation of plant genetic resources in

combination with geographical distribution.

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Discussion

Valkoun, J. (ICARDA): You used RFLP to gain information on the DNA structure and composition of your crop, did you use other DNA techniques such as RAPD or PCR for molecular characterization and what was the correlation with the RFLP technique?

Answer: I did not use these methods. PCR technique could be useful for characterizaton although several problems should be solved including non-specific amplification, design of the primers and/or

annealing temperature, purity of template DNA, etc.

Zahoor Ahmad (Pakistan): Most of the scientists are using RFLP for studies on genetic variability. Aren't morphological characters being overlooked?

Answer: Morphological characters are of course very important. However, RFLP can be detected directly at the DNA level and is free from gene expression. It is a new approach for the characterization of plant genetic resources. Both conventional characters and DNA polymorphism should be combined for more detailed classification.

Songkran Chitrakon (Thailnad): Can the use of RFLP markers help discard duplicate samples in case no differences are revealed by this technique?

Answer: DNA polymorphism such as RFLP and PCR variation should be studied in future to determine the purity and identity of accessions.

Zahor Ahmad (Pakistan): If accessions show exactly the same banding patterns when you apply RFLP with one or two probes, would you discard such duplicates from a germplasm collection?

Answer: Fingerprinting methods using a limited number of probes, particularly with repeated sequences are also useful for the classification. It is, however, difficult to decide whether accessions should be discarded based on DNA polymorphism only.

Morishima, H. (Japan): Isozyme analysis revealed 6 varietal groups including 2 major (indica and japonica) and 4 minor groups. Do small groups in the 2-dimensional space of PCA analysis from RFLP data correspond to the minor groups of isozyme variation?

Answer: We did not study isozymes except for esterases in the rice cultivars used. Detailed study is necessary to solve this problem.