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A. P. Resurreccion · C. P. Villareal · A. Parco · G. Second
B. O. Juliano

Classification of cultivated rices into indica and japonica types by the isozyme, RFLP and two milled-rice methods

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Abstract Four methods for classifying cultivated rices (*Oryza sativa* L.) (including IR varieties) into indica and japonica types – waxy gene product in endosperm starch, glutelin α_3 molecular weight in milled rice, RFLP polymorphism at the *Wx* locus and Glaszmann's isozyme method – were compared. On the basis of the two endosperm traits and the RFLP method Glaszmann's group 1 (indica) was classified as mainly indica and intermediate groups 2, 3 and 4 as exclusively indica. However, the endosperm traits classified Glaszmann's group 5 as mainly indica, while the RFLP method classified it as japonica. The RFLP waxy gene probe was closest to the isozyme method in classifying group 6 as japonicas; the waxy gene product gave mainly indica reaction even in group 6, and the glutelin α_3 method was intermediate. All IR rices were classified as being indica on the basis of Wx gene product and by Glaszmann's method, but a few were classified as japonica by the glutelin α_3 method and by the RFLP waxy gene probe.

Key words *Oryza sativa* L. · Waxy gene product
Glutelin α_3 molecular weight · Isozyme type
RFLP waxy gene probe

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A. P. Resurreccion · C. P. Villareal · A. Parco · G. Second[1] ·
B. O. Juliano (✉)[2]
Plant Breeding, Genetics, and Biochemistry Division,
The International Rice Research Institute,
P. O. Box 933, 1099 Manila,
Philippines

Present addresses:
[1] Centro National de Recursos Geneticos e Biotechnologia, Sain, CEP 70770-Brasilia DF, Brasil
[2] Institute of Chemistry, and Philippine Rice Research Institute, Division of Rice Chemistry and Food Science, University of the Philippines at Los Baños, 4031 College, Laguna, Philippines

Introduction

The classification of cultivated rice on the basis of seedling isozymes has placed indica (tropical) rice in group 1 and japonica (temperate and tropical) rice in group 6 (Glaszmann 1987). This method of categorization requires seed germination and is not applicable to milled rice.

At the waxy locus (*Wx*), the Wx^a allele predominates in indica rice and the Wx^b allele prevails in japonica rices (Sano et al. 1986). Indica rice has three times as much waxy gene product in endosperm starch as japonica rice at the same apparent amylose content (Villareal and Juliano 1989). Differentiation is possible only on non-waxy starch since the granule-bound waxy gene product is absent in waxy starch. Expression seems to be affected by location and season (Villareal and Juliano 1989).

Glutelin is the major storage protein of rice endosperm, constituting about 65% of total endosperm protein (Ogawa et al. 1987). Kagawa et al. (1988) reported that japonica rices show the glutelin α_3 band [molecular weight (MW) 37 kDa] upon sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with a low concentration of bisacrylamide crosslinker. This band was found to be absent in some indica rices. We verified these results and found that at a higher electric current (75 vs 30 mA) indicas showed a lower-mobility glutelin α_3 band (MW >37 kDa, close to glutelin α_2) than what is present in japonicas (IRRI 1991). Thus, the glutelin α_3 band overlaps with the glutelin α_2 band in indica rices. In many varieties, classification based on the glutelin α_3 band coincided with the waxy gene product and Glaszmann's isozyme classification.

Mapped restriction fragment length polymorphism (RFLP) probes differentiate between indica and japonica rices (Wang and Tanksley 1989, Zhang et al. 1992). As part of a broad survey of mapped RFLP in cultivated rice, RFLP at the waxy locus was found to correspond to a single fragment per plant, with three different sizes among the plants – two frequent and one rare (Second et al. in preparation).

This paper presents a comparative classification of cultivated rice into indica and japonica rices based on the isozyme, RFLP polymorphism at the *Wx* locus and the two endosperm traits.

Materials and methods

Rough rice samples of different varieties were obtained from the International Rice Germplasm Center (IRGC) and from the Plant Breeding, Genetics, and Biochemistry Division, IRRI. Samples for the preliminary study consisted of 74 cultivated rices (*Oryza sativa* L.) representing various Glaszmann (1987) groups grown at IRRI and originating from the USA, Thailand, Madagascar, Taiwan, Colombia, Korea and Vietnam, and 5 wild rices (2 *O. nivara* and 1 each of *O. perennis, O. punctata* and *O. ridleyi*). A set of 212 representative IRGC world collection rices and 26 IR rices were then analyzed. Information on the entries' Glaszmann (1987) isozyme classification was obtained from the IRRI Isozyme Laboratory.

Rough rice was dehulled in a Satake-type THU 35 dehuller, milled in a Kett Pearlest micromill or a test tube mill and ground in a Udy cyclone mill with a 60-mesh sieve. Starch granules were rid of surface proteins by the repeated extraction of milled rice flour with SDS buffer (Villareal and Juliano 1989). The starch preparation (20 mg) was gelatinized in a boiling water bath for 10 min in SDS buffer to extract the waxy gene protein. Protein extract (100 ul) was then electrophoresed through a 10% SDS-polyacrylamide vertical slab gel. The 60 kDa waxy gene protein band, made visible by Coomassie blue stain, was assessed either qualitatively by sight or measured quantitatively by densitometry at 610 nm against a standard sample of known protein content. Apparent amylose content (AC) was determined by iodine colorimetry (Juliano et al. 1981) and classified as waxy (0–3%), low (8–20%), intermediate (20–25%) and high (25–33%), on a milled rice dry basis. Waxy gene product was determined from the regression plot of waxy gene protein with AC for indica and japonica milled rices (Villareal and Juliano 1989).

Milled rice flour (50 mg) was stirred for 1 h in 1.5 ml 0.062 *M* TRIS-HCl pH 6.8 buffer with 2% SDS and 5% 2-mercaptoethanol and centrifuged at 8,000 *g* for 10 min at 20 °C. The crude glutelin extract (200 l) was mixed with five sucrose granules and a drop of 0.1% bromphenol blue, and 5–10 ul of this solution was loaded onto a 14% SDS-polyacrylamide vertical slab gel (30% acrylamide: 0.135% BIS)(Kagawa et al. 1988). SDS-PAGE was adapted from Laemmli's (1970) standard method. A current of 75 mA was employed to resolve glutelin α_3 in indica rices: (MW of 37 kDa for japonica rices and >37 kDa for indica rices).

The RFLP study was conducted on Southern blots from total genomic DNA extracted from leaves and restricted with *Eco*RV (Second et al. in preparation). The maize waxy gene (McCouch et al. 1988) was used as a probe. A single band appeared in inbred varieties and corresponded to all or part of the waxy gene plus the flanking sequences. Three RFLP bands were distinguished among different varieties in the present study; two were frequent, with a MW of 16.3 kbp (japonica) and 8.9 kbp (indica).

Results and discussion

Preliminary study

The results of the preliminary study on selected cultivated varieties showed a very good correspondence between waxy gene product in starch and glutelin α_3 MW traits only in groups 2 and 4; the varieties behaved like indicas (Table 1). Group 1 was heterogeneous for the glutelin α_3 MW trait but still predominantly Wx^a or indica for the waxy gene product.

On the basis of waxy gene product, group 5 was close to japonica; in contrast, on the basis of the glutelin α_3 MW trait (Table 1) it was classified as predominantly indica. The majority of the varieties in group 6 were japonicas on the basis of glutelin α_3 MW and waxy gene product. The 23 entries unclassified by the isozyme method were divided equally into indica and japonica groups on the basis of the two endosperm traits. However, only 12 of the 23 had the same classification by both endosperm traits. The wild rices showed a predominantly indica reaction.

The samples which behaved like those of Kagawa et al. (1988) were 'Fujisaka 5' and 'Milyang 23' (japonica glutelin α_3 MW) and 'Badshabhog' (indica glutelin α_3 MW). The results obtained with 'Labelle', 'IR24' and 'Century Patna 231' were different in the two laboratories. The IR36-based EM5 *sugary* mutant and EM16 *amylose extender* (*ae*) mutant of 'Kinmaze' and the 2064 *ae* mutant of 'Sasanishiki' all had a japonica glutelin α_3 MW, as did the EM129 *ae* mutant of 'Kinmaze'. 'Malagkit Sungsong', a Philippine waxy rice, had the japonica glutelin α_3 band as did indica/japonica hybrid derivative 'Mahsuri' (mutant) from Malaysia and 'Moroberekan'.

Representative world IRGC collection

The classification of 212 representative IRGC world collection samples showed that varieties belonging to group 1 of Glaszmann (1987) had predominantly a 8.9 kbp (indica) RFLP allele and the Wx^a allele; no clearcut classification using glutelin α_3 MW was achieved, particularly among intermediate- and high-amylose rices (Table 2). Group 1 had only 4 rices with the 16.3 kbp (japonica) RFLP allele – these were mainly waxy and low-amylose rices. All 3 waxy rices showed the 16.3 kbp RFLP allele, but only 1 showed the japonica glutelin α_3 band. 'Khao Dawk Mali 105' had the 16.3 kbp RFLP allele and the Wx^b allele; on the basis of glutelin α_3 MW it was an indica. Glutelin α_3 MW was 37 kDa (japonica) in 14 varieties carrying the Wx^a allele, including 'IR5'. The glutelin α_3 MW method showed the least number of indica types. Two varieties classified by the endosperm traits as being indica – 'ASD1' (26% AC) and 'Peh-Kuh-Tsao-Tu' (24% AC) – had the rare 20 kbp RFLP band instead of the 8.9 kbp band.

Entries belonging to the isozyme groups 2, 3 and 4 were consistently classified as indica by all three methods (Table 2). Group 5 was classified as predominantly japonica on the basis of the RFLP allele at the waxy locus, but was still considered to be predominantly indica on the basis of waxy gene product and glutelin α_3 MW traits. In group 5, 14 varieties with the 16.3 kbp RFLP band had the Wx^a allele and were categorized as japonica by glutelin α_3 MW, like many tropical group 6 varieties.

In group 6, a total of 26 entries of the 55 analyzed for both the RFLP waxy allele and waxy gene product had the japonica 16.3 kbp RFLP band and Wx^b alleles. Of these, 13 had low amylose content while the other 13 had inter-

Table 1 Classifications of 74 cultivated rices and five wild rices using three methods[a]

Glaszmann's variety group (1)	Sample (*n*)	Glutelin α_3 MW (2)		Wx gene product (3)		Frequency of agreement between methods			
		>37 kDa	37 kDa	Wx^a	Wx^b	(1) & (3)	(1) & (2)	(2) & (3)	(1), (2) & (3)
Cultivated rice									
1 (indica)	13[b]	6	7	9	2	9	6	7	3
2	7	7	0	7	0	0	0	7	0
3	2	1	1	2	0	0	0	1	0
4	3	3	0	3	0	0	0	3	0
5	7	6	1	2	5	0	0	3	0
6 (japonica)	19[c]	7	12	4	11	11	12	9	6
Unclassified	23	12	11	11	12	–	–	12	–
Wild rices	5	4	1	5	0	–	–	4	–
Total	79	46	33	43	30	20	18	46	9

[a] Glutelin α_3 MW: >37 kDa, indica; 37 kDa japonica. Wx gene product: Wx^a indica, Wx^b japonica
[b] Two waxy rices
[c] Four waxy rices

Table 2 Comparative classification of representative IRGC world collection rices by four methods

Glaszmann & amylose group[a]	Number	RFLP waxy allele[b]		Waxy gene product[b]		Glutelin α_3 MW[c]	
		8.9 kbp	16.3 kbp	Wx^a	Wx^b	>37 kDa	37 kDa
Group 1							
Waxy	3	0	3	0	0	2	1
Low AC	1	0	1	0	1	1	0
Intermediate AC	24	18	0	22	2	18	6
High AC	38	31	0	38	0	26	12
(Subtotal)	(66)	(49)	(4)	(60)	(3)	(47)	(19)
Group 2							
Intermediate AC	8	6	0	8	0	8	0
High AC	20	14	0	20	0	20	0
Group 3							
Intermediate AC	1	1	0	1	0	1	0
High AC	5	3	0	5	0	5	0
Group 4							
High AC	5	4	0	5	0	5	0
Group 5							
Low AC	21	0	13	21	0	18	3
Intermediate AC	6	1	2	6	0	5	1
Group 6							
Waxy	13	0	10	0	0	3	10
Low AC	41	2	22	23	18	11	30
Intermediate AC	25	3	17	20	5	13	12
High AC	1	1	0	1	0	1	0
(Subtotal)	(80)	(6)	(49)	(44)	(23)	(28)	(52)
Total	212	84	68	170	26	137	75

[a] Apparent amylose (AC) type: waxy, 0–3%, low, 8–20%; intermediate, 20–25%; high, 25–33%
[b] RFLP Wx allele: 8.9 kbp indica, 16.3 kbp, japonica. Wx gene product: Wx^a, indica, Wx^b, japonica. Not all of the 212 entries were characterized. For RFLP, 2 had the 20-kbp band only and 58 were not classified. Waxy entries (n=16) had negligible waxy gene product
[c] Indica >37 kDa; japonica 37 kDa

mediate amylose content. Most of these 26 are from tropical areas; the temperate japonicas generally have the indica 8.9-kbp RFLP allele and the Wx^a allele. Only 6 rice varieties of group 6 had the indica 8.9 kbp RFLP band (Table 2) and Wx^a allele; these were 'Baber', 'Caawa'/'Fortuna 6-103-15', 'Dawasan' (red), 'Jumali', 'Pratao' and 'Ruthal' (Acc. IRGC 31525). All 13 waxy rices had the 16.3-kbp RFLP allele. Many nonwaxy entries had the Wx^a allele, but were japonicas on the basis of the glutelin α_3 MW trait.

Of the 4 varieties included in both the present study and in that of Kagawa et al. (1988) 3 were consistent with re-

Table 3 Indica-japonica classification of IR varieties[a] by four methods

Variety	Apparent amylose (%)	RFLP Wx allele MW (kbp)	Waxy gene product	Glutelin α_3 MW (kDa)
IR5	25.8	8.9	Wx^a	37
IR8	25.5	8.9	Wx^a	>37
IR20	18.6[b]	16.3	Wx^a	37
IR22	24.4[b]	8.9	Wx^a	>37
IR28	26.5	8.9	Wx^a	>37
IR29	2.7	16.3	–	>37
IR30	26.9	8.9	Wx^a	37
IR32	26.6	8.9	Wx^a	>37
IR34	28.8	8.9	Wx^a	>37
IR36	23.4[b]	8.9	Wx^a	>37
IR38	26.4	8.9	Wx^a	>37
IR40	27.2	8.9	Wx^a	>37
IR42	28.4	8.9	Wx^a	>37
IR43	17.4	16.3	Wx^a	>37
IR44	27.8	8.9	Wx^a	>37
IR45	27.8	8.9	Wx^a	>37
IR50	26.1	8.9	Wx^a	>37
IR52	28.0	8.9	Wx^a	>37
IR54	27.5	8.9	Wx^a	>37
IR56	26.0	8.9	Wx^a	>37
IR62	26.8	8.9	Wx^a	37
IR64	16.4[b]	8.9	Wx^a	37
IR65	2.4	16.3	–	>37
IR66	24.0[b]	8.9	Wx^a	37
IR72	26.2	16.3	Wx^a	>37
IR74	26.6	8.9	Wx^a	37

[a] All belong to group 1 by Glaszmann's (1987) classification system. RFLP Wx allele: 8.9 kbp indica, 16.3 kbp, japonica. Wx gene product: Wx^a indica, Wx^b japonica. Glutelin α_3 MW: >37 kDa, indica, 37 kDa, japonica

[b] Unusually low values for apparent amylose content

spect to glutelin α_3 MW – in 'Aichi Asahi' and 'Kamenoo' as japonica and 'Taichung Native 1' as indica. Kagawa et al. (1988) found the fourth, 'Tetep' to have japonica glutelin α_3 MW; while our study classified it as indica.

IR varieties

The 26 IR varieties tested were classified as indica by Glaszmann's (1987) method and showed predominantly the indica 8.9-kbp RFLP allele (Table 3). The 2 waxy rices, 'IR29' and 'IR65', and non-waxy 'IR20', 'IR43' and 'IR72' had the 16.3-kbp RFLP allele. All 24 non-waxy rices had the Wx^a allele, whereas only 19 showed the indica glutelin α_3 MW and 7 the japonica glutelin α_3 MW. Of these, only 'IR20' was classified as japonica by the RFLP probe. Earlier studies at IRRI (1991) showed the presence of japonica glutelin α_3 MW in 'IR24' and 'IR64' but not in 'IR36'.

Amylose content of the samples (particularly in 'IR20', 'IR36', and 'IR64') was lower than what was usually obtained (Table 3). 'IR64' showed intermediate amylose (20–25%). The high-amylose (>25%) rices with less than 25% amylose were 'IR20', 'IR22', 'IR36' and 'IR66'.

Conclusions

The results of our study reveal that there is a close agreement among classification methods based on RFLP at the waxy locus, the waxy gene product in endosperm starch and Glaszmann's (1987) classification as far as isozyme groups 1–4, including IR varieties, are concerned. The glutelin α_3 MW data were less discriminating, even for japonica or group 6, in contrast to the conclusions of Kagawa et al. (1988) on Japanese rice varieties. The majority of the varieties of group 1 had higher MW indica glutelin α_3 (>37 kDa), thereby supporting the observation that many indicas do not show the normal 37-kD band observed in japonica rice (Kagawa et al. 1988).

Sano et al. (1986) suggested that the Wx^b allele might have been selected for through differences in grain quality during domestication of Japanese rice. This allele is characteristic of the temperate japonicas only. Tropical japonicas and isozyme group 5 varieties, on the other hand, often have the Wx^a allele associated with the 16.3-kbp RFLP band. Wx^b, however, is associated with the 16.3 kbp RFLP band in some tropical japonicas, such as 'Azucena' in the Philippines, 'Rathal' in Sri Lanka, 'IRAT 13' and 'Bico Branco' in Brazil, 'Gogo Lempuk' in Indonesia, etc., as well as in a high-quality indica variety, 'Khao Dawk Mali 105' from Thailand. This pattern of variation may be the outcome of reciprocal introgression between indicas and japonicas, as shown by an extensive survey of mapped RFLP loci (Second et al. in preparation).

It should be noted that RFLP characterization could be conducted from DNA extracted from milled rice. Rather than do Southern blot hybridization with a probe, RFLP characterization could also be done from polymerase chain reaction-based RFLP using primers from the waxy gene (Williams et al. 1991). This would allow a straightforward test for milled rice. We are at present investigating this possibility.

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