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Genetic differentiation of wild relatives of rice as assessed by RFLP analysis

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Abstract To study genetic diversity and relationships of wild relatives of rice, 58 accessions of *Oryza rufipogon*, *Oryza nivara*, *Oryza sativa* f. *spontanea* and the cultivated *Oryza sativa*, representing a wide range of their distribution, were analyzed using the restriction fragment length polymorphism (RFLP) technique. All 30-used RFLP probes detected polymorphisms among the *Oryza* accessions, with an average of 3.8 polymorphic fragments per probe. Considerable genetic diversity was scored among the *Oryza* accessions with a similarity coefficient ranging from 0.28 to 0.93; but the cluster analysis of the accessions did not show an apparent grouping based on the species classification, instead they were scattered randomly in different groups. Noticeably, the *Oryza* accessions from the same geographic region, or near-by geographic regions, tended to be clustered in the same groups. The *indica* rice varieties showed relatively high genetic diversity and were scattered in different groups of their wild relatives, but the *japonica* varieties showed a relatively low variation and formed an independent group. It is concluded from the molecular analytical result that: (1) the four *Oryza* taxa have a remarkably close relationship and their independent species status need to be carefully reviewed; (2) geographic isolation has played a significant role in the differentiation of the *Oryza* accessions; therefore, a wide geographic range needs to be covered in collecting wild rice germplasm for *ex situ* conservation; and (3) the conventional conclusion of *indica* rice being directly domesticated from its ancestral wild species, and *japonica* rice being derived from *indica*, gains support from our data.

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Introduction

The Asian cultivated rice (*Oryza sativa* L.) is the world's most important staple food that supports nearly one-half of the globe population. As an old crop with about 11,500 years of domestication (Normile 1997), *O. sativa* has had significant genetic differentiation among varietal types and therefore two subspecies, i.e. subsp. *indica* and subsp. *japonica*, representing two partially isolated gene pools, are recognized (Oka 1988). Considerable variation between the two subspecies at both morphological and molecular levels is observed and considered to be associated with their adaptability to different environments (Glaszmann 1987; Oka 1988; Wang et al. 1992). There are two different hypotheses regarding the origin of the two subspecies. Most authors believed that subsp. *indica* was domesticated primarily from its wild relatives in South or Southeast Asia, whereas subsp. *japonica* was an adaptive type to the high elevation and high latitude derived from subsp. *indica* (Chang 1976; Oka 1988). On the other hand, some authors suggested that the differentiation into *indica* and *japonica* types already occurred in the wild species before their domestication. As a consequence, the *indica* type of wild rice developed into subsp. *indica* and the *japonica* wild rice into subsp. *japonica* during the domestication process (Second 1982; Wang and Sun 1996).

Regarding the direct ancestor of *O. sativa*, there were also different hypotheses long being debated (Sampath and Govindaswami 1958; Chang 1976; Sano et al. 1980; Morishima et al. 1992). These authors all had strong experimental supports to their respective hypotheses, although none of these hypotheses is completely accepted by all scientists over the world. Nevertheless, it might be more important to understand the true genetic relationships and diversity of the cultivated rice, and its existing wild relatives, rather than debating on which specific wild relative

Table 1 *Oryza* materials used in this study with information on their identification and origin

Species	Code	Acc. No.	Origin
O. rufipogon	RF-BA-1	103404	Bangladesh, Cox’s Bazaar
	RF-BA-2	105891	Bangladesh, Kustia, Mirpeur
	RF-BA-3	105901	Bangladesh, Sylhet, Srimongle
	RF-CA-1	105738	Cambodia, Takeo, Bar En
	RF-CA-2	106325	Cambodia, Prey Veng, Preah Sdach
	RF-CA-3	106336	Cambodia, Siem Reap, Siem Reap
	RF-CH-1	100916	China, Guangxi
	RF-CH-2	104624	China, Guangxi
	RF-CH-3	9Y	China, Jiangxi
	RF-CH-4	547Y	China, Hunan
	RF-CH-5	3120Y	China, Fujian
	RF-CH-6	6237Y	China, Guangdong
	RF-CH-7	7025Y	China, Guangxi
	RF-CH-8	7035Y	China, Hunan
	RF-IN-1	105325	India, Kerala, Malamphuza
	RF-IN-2	106103	India, West Bengal, Medilipur
	RF-MA-1	106036	Malaysia, Kelantan, Kota Bharu
	RF-MA-2	106037	Malaysia, Kelantan, Tumpat
	RF-MY-1	106379	Myanmar, Ayeyawady, Kyaugkone
	RF-PNG-1	106262	Papua New Guinea, West Province, Lake Murray
	RF-PNG-2	106267	Papua New Guinea, West Province, Lake Murray
	RF-PNG-3	106272	Papua New Guinea, West Province, Lake Murray
	RF-PNG-4	106275	Papua New Guinea, West Province, Lake Murray
	RF-PNG-5	106281	Papua New Guinea, West Province, Lake Murray
	RF-PNG-6	106289	Papua New Guinea, West Province, Lake Murray
	RF-SL-1	105424	Sri Lanka, Kalutura, Mahawela
	RF-TH-1	100219	Thailand, Konkhen, Chumpae
O. nivara	NI-BA-1	105880	Bangladesh, Jhenaidah, Shadar
	NI-CA-1	105716	Cambodia, Kandal, Prey Kampu
	NI-CA-2	105740	Cambodia, Kompong Chhnang, Long Vey
	NI-CH-1	103824	China, Guangdong, Boluo,
	NI-IN-1	104705	India, Maharashtra, Goa
	NI-IN-2	106111	India, West Bengal, Hoara
	NI-MY-1	100197	Myanmar
	NI-MY-2	106396	Myanmar, Bago, Minhla
	NI-NE-1	105706	Nepal, Dang, Dubichaur Ward
	NI-SL-1	105444	Sri Lanka, Hambantota, Ruhuna
	NI-TH-1	105391	Thailand, Central Thailand, Chai Nat
	NI-TH-2	105763	Thailand, North Thailand, Buri Ram
	NI-TH-3	105867	Thailand, Northeast Thailand, Muang
O. spontanea	SP-BA-1	105869	Bangladesh, Kustia, Mirpur
	SP-IN-1	102172	India, Uttar Pradesh, Palhana
	SP-IN-2	104700	India, Karnataka, Kholapur
	SP-IN-3	105311	India, Kerala, Allepey
	SP-IN-4	106064	India, Bihar, Ranchi
	SP-IN-5	106137	India, Westbengal, Barddhaman
	SP-MY-1	106367	Myanmar, Ayeyawady, West Pathein
	SP-MY-2	106378	Myanmar, Ayeyawada, Kyungkone
	SP-MY-3	106401	Myanmar, Bago, Shwetaung
	SP-TH-1	105778	Thailand, Northeast Thailand, Nang Rong
O. sativa (*japonica* type)	SA-JAP-1	79	China, Taiwan
(*japonica* type)	SA-JAP-2	36818	Japan
(*japonica* type)	SA-JAP-3	58302	Korea
(*japonica* type)	SA-JAP-4	ZSG	China, Jiangsu
(*indica* type)	SA-IND-1	53433	Philippines
(*indica* type)	SA-IND-2	30416	Philippines
(*indica* type)	SA-IND-3	NTH	China, Jiangxi
(*indica* type)	SA-IND-4	NJ11	China, Jiangsu

species is responsible for the origin of cultivated rice. A better understanding of the genetic diversity pattern and relationships of these close relatives of rice will facilitate more-effective conservation and utilization of the wild rice germplasm for the improvement of rice varieties.

Oryza rufipogon is a perennial diploid species containing the AA genome, widely distributed from south China (up to Jiangxi Province) and South Asia, through Southeast Asia and Malay Petpalogo down to the northern part of Australia. It is often found in swamps, lakes and along canals with deep water. *Oryza nivara* is a diploid and annual taxon with the AA genome, and is mainly found in South and Southeast Asia. It occurs in seasonally dry/wet areas, in ponds, swamps and the vicinity of rice fields with shallow water. *O. sativa* f. *spontanea* (or sometimes referred as *Oryza perennis*) is a weedy type and considered to have its origin mainly from hybridization between *O. sativa* and the above two wild rice species, apart from its other genesis. It is usually found in the areas where the cultivated rice and its wild relatives coexist. These wild relatives are very important genetic resources in rice breeding. For example, the male sterility gene (MS) which greatly promoted the hybrid rice breeding and production was first found in *O. rufipogon* in Southern China and transferred to cultivated rice (Yuan 1993), and one population of *O. nivara* from India provided the world's only germplasm resistant to the grassy stunt virus of rice (Khush 1977).

Molecular markers provide a powerful tool for such genetic diversity studies and evolutionary relationship detection. Restriction fragment length polymorphism (RFLP) has been intensively used for the investigation of genetic diversity and relationships of crop species and their wild relatives. As a result, genetic diversity and evolutionary patterns have been illustrated in many crops, such as wheat, barley and rice (Wang et al. 1992; Zhang et al. 1992; Salvo-Garrido et al. 2001; Sun et al. 2001). The objective of the present study was to investigate genetic diversity and relationships of the wild *Oryza* relatives and their geographic distribution pattern using the RFLP technique, in addition to discussion on the origin of *indica* and *japonica* rice.

Materials and methods

Materials

A total of 58 rice accessions representing four taxa of the genus *Oryza*, i.e. *O. rufipogon*, *O. nivara*, *O. sativa* f. *spontanea* (*O. spontanea* for short) and *O. sativa*, were used in this study. Twenty seven accessions of *O. rufipogon*, 13 accessions of *O. nivara*, and 10 accessions of *O. spontanea*, representing a wide range of geographic distribution, were included in the experiment. Eight *O. sativa* varieties, four each representing typical *indica* and *japonica* subspecies (or types), were also used (Table 1). The rice accessions were kindly donated by the International Rice Genebank of the International Rice Research Institute (IRRI), and the Rice Genebank of the China National Rice Research Institute (CNRRI).

DNA extraction and RFLP analysis

Leaf tissues of the rice materials were collected for DNA extraction from seedlings of one individual, representing each accession planted in a screen-house. Total DNA extraction, restriction endonuclease digestion, electrophoresis, Southern blotting, hybridization and autoradiography were carried out following the methods described previously by Zheng et al. (1990) and Lu and Zheng (1992). Thirty rice clones were selected based on our previous work on phylogenetic analysis of the cultivated *O. sativa* (Zheng et al. 1994; Qian et al. 1995), which are distributed over different chromosomes of the cultivated rice (see Table 2). Most clones (RG clones) are random genomic clones from Cornell University. The clone G318 was a gift from Dr. Uchimiya of the Institute of Applied Microbiology, the University of Tokyo, Japan, and mapped on chromosome 12 (Uchimiya, personal communication). Three restriction endonucleases *Eco*RI, *Eco*RV and *Hin*dIII were employed for digestion, each of which was used for a particular clone (see Table 2).

Table 2 Numbers of polymorphic fragments detected by the probes used in the study

Probe	Chromosome	Enzyme	Number of polymorphic fragments		
			Total	Wild rice	Cultivated rice
RG13	5	*Hin*dIII	5	5	2
RG64	6	*Eco*RV	5	5	3
RG81	12	*Eco*RI	7	7	3
RG101	1	*Hin*dIII	5	5	3
RG108	8	*Hin*dIII	2	2	0
RG139	2	*Eco*RI	2	2	0
RG157	2	*Hin*dIII	4	4	2
RG171	2	*Eco*RI	3	3	3
RG182	5	*Eco*RI	7	7	0
RG214	4	*Eco*RV	6	6	2
RG222	1	*Eco*RV	3	3	0
RG227	3	*Eco*RV	3	3	2
RG256	2	*Eco*RV	2	2	2
P286	?	*Hin*dIII	2	0	2
RG303	11	*Eco*RI	3	3	2
G318	12	*Hin*dIII	2	2	1
RG348	3	*Eco*RI	1	1	1
RG351	7	*Eco*RI	6	6	2
RG358	9	*Eco*RI	1	1	1
RG375	4	*Eco*RI	1	1	1
RG435	5	*Hin*dIII	7	7	2
RG462	1	*Eco*RV	3	1	2
RG532	1	*Hin*dIII	4	4	2
RG543	12	*Hin*dIII	3	3	2
RG553	9	*Hin*dIII	6	6	2
RG620	4	*Eco*RV	10	10	4
RG634	2	*Eco*RV	6	5	1
RG667	9	*Eco*RI	3	3	3
RG684	–	*Hin*dIII	2	2	2
RG869	12	*Hin*dIII	1	1	1
Average			3.8	3.7	1.8

Data encoding and analysis

Hybridization patterns were scored for each probe. Polymorphic fragments from each probe detected among the 58 accessions were used to make comparisons between accessions, where each polymorphic fragment was treated as a unit character that was scored as either 1 (for presence) or 0 (for absence). The ratios of shared DNA fragments and similarity coefficients between accessions were quantified according to Nei (1987, formula 5.53–5.55). A dendrogram showing genetic relationships of the 58 accessions was constructed using the unweighted pair-group method with an arithmetic mean (UPGMA) (Sokal and Michener 1958). The analysis was carried out using the NTSYS-pc analytical software (Rohlf 1998).

Results

All the probes used in the experiment detected polymorphisms among the *Oryza* accessions, and the numbers of polymorphic fragments for each probe varied significantly from 1 to 10. The average number of detected polymorphic fragments was 3.8 per probe, although polymorphic fragments were not equally distributed among the *Oryza* accessions included. This suggests abundant variation in the *Oryza* accessions at the molecular level. The majority of the polymorphic fragments were retained in the wild rice accessions, in which the average number of detected polymorphic fragments was 3.7 per probe. Only less than half of the polymorphic fragments were scored in the cultivated rice accessions, with an average of 1.8 fragments per probe (Table 2).

A total of 115 polymorphic fragments were detected from the *Oryza* accessions and all the polymorphic fragments were used for the genetic analysis. Genetic diversity was considerably high among the accessions used in this study, with the similarity coefficient ranging from 0.28 to 0.93. A dendrogram was generated based on the similarity coefficient calculated from the RFLP variation of the 58 *Oryza* accessions (Fig. 1). The dendrogram illustrated a clear genetic variation pattern of the included *Oryza* accessions. As a summary, a few points need to be emphasized regarding the genetic variation. First, the variation pattern and grouping of the *Oryza* accessions was not in association with the species classification, although the variation of the accessions was easily detectable. In other words, the accessions belonging to the same species did not show a tendency of being clustered together in the same group; instead they were scattered randomly in different groups. Second, there was a clear trend that the *Oryza* accessions from the same geographic region or near-by geographical regions tended to stay together in the same groups. As indicated in Fig. 1, the *Oryza* accessions from the Southeast Asian countries, such as Cambodia, Thailand, Myanmar and Malaysia, tended to be included in a large group, with only few exceptions. Accessions from the South Asian countries, such as Bangladesh, India, Nepal and Sri Lanka, were clustered into two independent groups with a relatively large variation. One of the South Asian groups includes accessions from India and Bangladesh, and another from India, Nepal and Sri Lanka, indicating further differentiation of rice germplasm in the large geographical region of South Asia. Accessions from Papua New Guinea were clustered in a distinct group with small differentiation, indicating the unique genetic status of wild rice in this region. The Chinese accessions showed significant differentiation compared with samples from other countries. The majority of these accessions were found in an independent cluster, apart from two samples that were found in the Southeastern Asian group and one in a unique group. Thirdly, the four *indica* rice varieties showed relatively high genetic diversity and were scattered among different groups of their wild relatives, with one variety (SA-IND-2) being clustered between the Chinese and South Asian groups, and the other three (SA-IND-2, SA-IND-3 and SA-IND-4) in the Southeast Asian group. By contrast, the four *japonica* varieties showed a relatively low variation (particularly among SA-JAP-1, SA-JAP-2 and SA-JAP-3) and were included in an unambiguous group, quite deviant from all other *Oryza* accessions (Fig. 1).

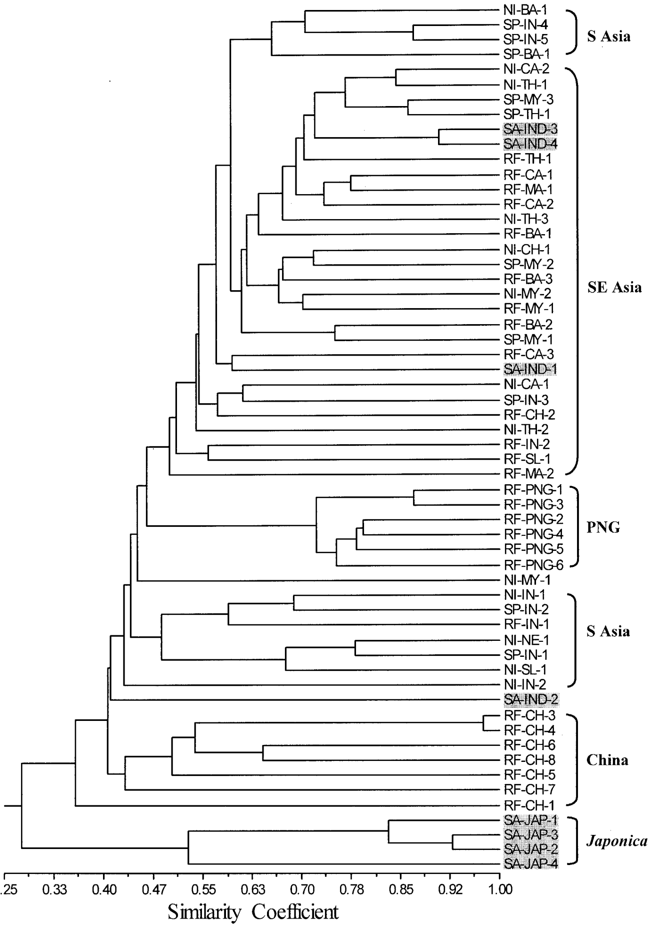

Fig. 1 Dendrogram generated from cluster analysis of RLFP data of *O. rufipogon* (*RF*), *O. nivara* (*NI*), *O. sativa* f. *spontanea* (*SP*) and *O. sativa* (*SA*). Strong correlation between genetic differentiation and geographic distribution of the *Oryza* species is illustrated. *O. sativa* accessions are *shaded* on the dendrogram. For species and accession code please refer to Table 1

Discussion

The Asian cultivated rice is supposedly domesticated from its wild ancestral species, *O. rufipogon* (Sampath and Govindaswami 1958; Oka 1974), *O. nivara* (Chang 1976) or the intermediate types between *O. rufipogon* and *O. nivara* (Sano et al. 1980). No matter which hypothesis is more appropriate for illustrating the origin and true evolutionary process of the cultivated rice, all previous studies consistently supported the conclusion that *O. sativa*, *O. rufipogon*, *O. nivara* and the intermediate type of *O. spontanea* have remarkably close relationships. This provides a strong basis for the easy utilization of the wild relatives in the rice breeding program through a sexual approach.

In this study, a total of 115 polymorphic RFLP markers were detected from the 58 accessions, representing a wide range of collections of the wild and cultivated rice species. The cluster analysis of the RFLP markers indicated great genetic diversity among these accessions, particularly in the wild relatives of rice, with similarity coefficients ranging from 0.28 to 0.93, generally supporting the estimation for genetic variation in these rice species in previous studies (Morishima et al. 1992; Martin et al. 1997; Sun et al. 2001). This result confirms that the abundant diversity found in the wild relatives of rice still remains of great importance to broaden the genetic background of cultivated rice varieties through breeding practices. However, the cluster analysis of these accessions did not show apparent groupings of three (or four) entities; in other words, there is no strong species-associated classification for the included materials. Accessions of the four taxa are distributed randomly among each other, indicating their close genetic relationships and the ambiguous species boundary at the molecular level. This result adds weight to the arguments that the traditional classification of the above taxa into three independent species is somehow questionable (Vaughan 1994; Lu 1999), although morphological differentiation of the extreme forms of the traditionally defined species is distinct. In fact, spontaneous outcrossing among *O. rufipogon*, *O. nivara* and *O. sativa* was frequently recorded in the field, provided that these species have sympatric distribution. This phenomenon also strongly supports the con-specific status of the three taxa, although *O. rufipogon* is a perennial and *O. nivara* is an annual taxon, and they prefer relatively different habitats. Data from previous artificial hybridization also suggested that these species did not have an apparent reproductive isolation, and that they should be considered as the same biological species (Lu et al. 2000). Therefore, an appropriate taxonomic treatment of these taxa needs to be undertaken.

It is noteworthy that the differentiation of the *Oryza* accessions included in this study showed an apparent geographic pattern. In other words, the accessions from the same geographic regions tend to be clustered in the same groups, regardless of their species classification. This strongly indicates that geographic isolation plays a significant role in the differentiation of these *Oryza* species. Our previous hybridization study also showed that samples from the same or near-by regions had a higher compatibility than those from different regions (Naredo et al. 1997, 1998). The finding of the geographic differentiation pattern of *Oryza* accessions has a significant implication for the conservation and utilization of rice germplasm, in terms of decision-making and sampling strategies. We therefore recommend to cover different geographic regions when collecting wild rice germplasm for *ex situ* conservation, which will ensure the capture of the maximum genetic diversity of the wild *Oryza* relative species.

It is a common understanding that the cultivated rice has differentiated into *indica* and *japonica* during the domestication and selection process (Morishima et al. 1992; Zhang 1992). The majority of rice scientists believe that the *indica-japonica* differentiation is due to the adaptive evolution of rice varieties to different ecological environments. Those varieties, that adapted to the colder environment at a higher elevation or higher latitude, developed from the original *indica* into the *japonica* type (Oka 1988). Recently, through genetic diversity studies of *O. sativa* and its wild relatives, several authors however proposed the hypothesis that the differentiation of the "*indica*" and "*japonica*" types already occurred in their wild ancestral species, *O. rufipogon*. As a consequence, the cultivated *indica* and *japonica* rice originated separately from the "*indica*" and "*japonica*" wild rice (Second 1982; Wang and Sun 1996).

Data from this study showed, as in many other studies, a much greater variation of the *indica* rice varieties than the *japonica* rice varieties, even though the *japonica* varieties were collected from a slightly wider geographic coverage than the *indica* varieties. Results from the cluster analysis further demonstrated that the *indica* varieties were scattered among the wild and weedy rice accessions randomly, but the *japonica* varieties were grouped together independently from all other rice accessions included in this study. This result supports the conventional conclusion that the *indica* type of cultivated rice was directly domesticated from its ancestral wild species and the *japonica* type was differentiated later from the *indica* type (Chang 1976). We believe that the *japonica* rice was a derivative type adapting to its unique environments at high elevations and latitudes, although the origin of cultivated rice could have occurred at more than one site. After its derivation, the *japonica* rice disseminated to more extensive regions and adapted to broader ecological conditions.

The hypothesis of independent origins of the cultivated *indica* and *japonica* rice from their respective "*indica*" and "*japonica*" wild types was only based on the detection of *indica*- and *japonica*-associated isozyme or molecular markers in different *O. rufipogon* populations (including *O. nivara*) (Second 1982; Wang and Sun 1996). However, the authors of the previous studies ignored the very important fact that all the current wild rice accessions used in various studies were usually collected in habitats in the vicinity of cultivated rice fields. We would argue that introgression between cultivated rice and its wild relatives has occurred relatively frequently during thousands of years of the co-evolutionary process in the wild and cultivated rice. The exchange and accumulation of genetic materials between different types of cultivated rice and their wild relatives distributed in the same areas should be responsible for the occurrence of the different "types" of wild rice populations, in which *indica*- and *japonica*-related loci were detected by the molecular markers.

In fact, spontaneous hybridization and outcrossing between wild and cultivated rice strains have been reported in many locations (Sano et al. 1980; Oka 1988). Most of the wild *O. rufipogon* used in analyses were usually collected from niches where cultivated rice varieties were grown nearby, and it is very difficult nowadays to find the true type of *O. rufipogon* populations that occur out of contact with the cultivated rice in many countries, due to extensive rice cultivation. In addition, from the evolutionary point of view, there is no kinetic basis for such tropical and aquatic species as *O. rufipogon* to differentiate into the "*indica*" and "*japonica*" types. Therefore, the hypothesis of *indica* and *japonica* rice varieties having independent origins from different types of wild rice does not have sound evidence, unless the pure *O. rufipogon* populations that have no introgression with cultivated rice are used in analyses.

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