Complexity of indica-japonica varietal differentiation in Bangladesh rice landraces revealed by microsatellite markers

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To understand the genetic diversity and indica-japonica differentiation in Bangladesh rice varieties, a total of 151 accessions of rice varieties mostly Bangladesh traditional varieties including Aus, Boro, broadcast Aman, transplant Aman and Rayada varietal groups were genotyped using 47 rice nuclear SSRs. As a result, three distinct groups were detected by cluster analysis, corresponding to indica, Aus and japonica rice. Among deepwater rice varieties analyzed some having particular morphological features that mainly corresponded to the japonica varietal group. Some small seeded and aromatic varieties from Bangladesh also corresponded to the japonica varietal group. This research for the first time establishes that the japonica varietal group is a prominent component of traditional varieties in Bangladesh, particularly in deepwater areas.

Key Words: Bangladesh, deepwater rice, Simple Sequence Repeat (SSR), diversity, indica-japonica differentiation.

Introduction

Asian cultivated rice (\textit{Oryza sativa} L.) was domesticated approximately 8,000 years ago in China (Fuller 2011) and since then rice has evolved different ecotypes in many areas of the world. Now half of the world’s population relies on rice as a staple food (International Rice Genome Sequencing Project 2005).

Rice genetic resources, including local varieties and wild relatives, are essential for rice breeding. Currently the International Rice Genebank (IRRI) holds more than 113,000 accessions of rice from most areas where rice is cultivated, including modern and traditional varieties and wild relatives of rice. Of them, 14,931 accessions (as of 2012/09/02) are from Bangladesh. Traditional cultivated rice ecotypes in Bangladesh are differentiated according the cropping system, season and geography. The main cropping seasons are called Aus (early summer), Aman (autumn consisting of transplanted and broadcast varieties), Boro (winter). In addition there are a few locally adapted varietal types recognized from particular areas such as Rayada and Ashina (Alim 1974, Ando 1987, Morishima \textit{et al.} 1990). Although rice varieties of Bangladesh have been analyzed using isozymes, RFLP and SSR markers (Garris \textit{et al.} 2005, Glaszmann 1987, Wang and Tanksley 1989) the complexity of Bangladesh deepwater rice varieties is not very well understood.

The objectives of the present study are: 1) to analyze a comprehensive set of traditional landrace rice varieties from Bangladesh for their intra- and inter-population genetic structure, 2) to determine the extent of variation that exists in the deepwater rice varieties from Bangladesh. The results of this study will provide a better understanding of the variation of Bangladesh’s rice varieties and also facilitate their effective use.

Materials and Methods

\textit{Plant material}

A total of 151 rice accessions, mostly Bangladesh landraces were used as the materials (Supplemental Table 1). The Bangladesh landraces were classified as broadcast Aman (B-Aman), transplant Aman (T-Aman), Aus, Boro and Rayada based on passport data. The B-Aman varieties were further divided into typical B-Aman (B1 type) and special deepwater B-Aman (B2 type). Among B-Aman varieties there is a group of varieties with a specific set of
morphological characteristics that include light green-leaf color, few basal tillers, aerial roots from nodes, extreme tallness and tillering from higher nodes after lodging (HW Cai personal observation). These varieties are distinguished in this paper as special B-Aman (B2).

In addition to germplasm from Bangladesh, six Chinese typical japonica and indica cultivars from Zhejiang and Guangxi province carrying the specific esterase isozyme allele Est10-4 (Cai et al. 2003) which is predominant in common wild rice (O. rufipogon) were used in this study. Some typical indica, japonica varieties and common wild rice from other countries were also used as check materials. Accessions were all from an acquired set of rice stocks maintained by the National Center for Evaluation of Agricultural Wild Plants (Rice) in China Agricultural University, Beijing, China.

Genomic DNA extraction and SSR genotyping
DNA was extracted from fresh leaves of one plant of each accession using the CTAB method (Murray and Thompson 1980). A total of 47 nuclear SSRs distributed throughout the 12 rice chromosomes (2–6 markers on each chromosome) were employed to analyze population structure (Supplemental Table 2). For PCR amplification, a total of 20 µl reaction mixture consisting of 1.0 unit of Taq polymerase, 2.0 µl of 10 x Taq DNA polymerase buffer, 0.4 µl of dNTPs, 0.5 µM of each primer and 40 ng of total DNA was used. PCR amplification was performed in a thermal cycler programmed with 35 cycles each of 30 s at 95°C for denaturation, 30 s at 55°C for annealing and 1 min at 72°C for extension. PCR products were run on 8% polyacrylamide denaturing gel, the bands were detected using the silver staining methods (Panaud et al. 1996). To determine the size of alleles, six check samples (Nipponbare, Kasalath, GC2, T65, T65g/g and W1944) were directly compared with all the samples analysed.

Statistical analysis
Genetic distances were estimated using Nei’s distance (Nei 1972) and phylogenetic tree construction was based on the UPGMA method implemented in PowerMarker v3.25 (Liu and Muse 2005, http://statgen.ncsu.edu/powermarker/). PowerMarker was also used to calculate the average number of alleles, gene diversity and polymorphism information content (PIC) values. In addition, population structure was evaluated with the model-based program STRUCTURE (Pritchard et al. 2000, http://pritch.bsd.uchicago.edu/structure.html). The analysis had a burn-in length of 10,000 iterations and a run length of 100,000 iterations. The admixture and correlated allele frequencies were determined. The graphical display of the STRUCTURE result was generated using Distruct software (Rosenberg 2002, http://www.stanford.edu/group/rosenberglab/distruct.html).

Table 1. Genetic parameters of each group

| Group                  | Sample Size | Major Allele Frequency | Allele No | Gene Diversity | PIC      | Fst     |
|------------------------|-------------|------------------------|-----------|----------------|----------|---------|
| Aus                    | 16          | 0.57 (0.25–1.00)       | 3.21 (1–7)| 0.53 (0.00–0.84)| 0.46 (0.00–0.82)| 0.09 (–0.46–1.00) |
| Boro                   | 18          | 0.61 (0.33–1.00)       | 3.26 (1–6)| 0.51 (0.00–0.77)| 0.45 (0.00–0.74)| 0.12 (–0.62–1.00) |
| Aman*                  | 10          | 0.60 (0.27–1.00)       | 3.06 (1–7)| 0.49 (0.00–0.83)| 0.43 (0.00–0.80)| 0.17 (–0.64–1.00) |
| B1 type of B-Aman      | 15          | 0.61 (0.27–1.00)       | 3.21 (1–7)| 0.51 (0.00–0.84)| 0.44 (0.00–0.82)| 0.12 (–0.29–0.68) |
| B2 type of B-Aman      | 20          | 0.66 (0.25–0.95)       | 3.26 (2–7)| 0.46 (0.10–0.81)| 0.41 (0.10–0.78)| 0.13 (–0.31–0.66) |
| T-Aman                 | 57          | 0.60 (0.27–0.93)       | 3.91 (2–8)| 0.53 (0.13–0.81)| 0.47 (0.12–0.78)| 0.13 (–0.13–0.45) |
| indica Control         | 3           | 0.68 (0.33–1.00)       | 1.96 (1–3)| 0.39 (0.00–0.67)| 0.31 (0.00–0.59)| 0.05 (–1.48–1.00) |
| japonica Control       | 9           | 0.80 (0.44–1.00)       | 1.81 (1–4)| 0.25 (0.00–0.69)| 0.21 (0.00–0.64)| 0.54 (–0.22–1.00) |
| O. rufipogon Control   | 2           | 0.65 (0.50–1.00)       | 1.70 (1–2)| 0.35 (0.00–0.50)| 0.26 (0.00–0.38)| –0.14 (–2.72–1.00) |

* Cultivation type is unknown.
wild rice checks (−0.02). It was also found that high $F_{st}$ values were obtained between $japonica$ checks with other groups (Table 2).

An unrooted UPGMA tree of 151 accessions was constructed using Nei’s distances (Nei 1972) (Fig. 1). All 151 accessions can be divided into three main groups, group 1 corresponds to $indica$ including most T-Aman (41/57), some B1 type of B-Aman (10/20), some Boro (12/20) and two indica checks, group 2 corresponds to Aus ecotype including most Aus (10/16), some T-Aman (4/57) and some B2 type of B-Aman (4/20) accessions. Group 3 corresponds to $japonica$ including all $japonica$ checks, most B2 type of B-Aman (13/20) and some Boro (4/18). Wild rice accessions W1944 seemed independent from the three groups and the other wild rice CH45 clustered in the Aus group (Table 3). A similar result was found when using Aman varieties only (Supplemental Fig. 1).

Table 2. Pairwise $F_{st}$ indicating the difference between each group

|        | Aman | Aus | B1 | Boro | $japonica$ | T-Aman |
|--------|------|-----|----|------|------------|--------|
| Aus    | 0.08 |     |    |      |            |        |
| B1     | 0.05 | 0.09|    |      |            |        |
| Boro   | 0.07 | 0.11| 0.00|      |            |        |
| B2     | 0.10 | 0.13| 0.12| 0.13  |            |        |
| $indica$ | 0.03 | 0.11| −0.02| 0.01 | 0.09       |        |
| $japonica$ | 0.48 | 0.35| 0.35| 0.37 | 0.30       | 0.40   |
| T-Aman | 0.06 | 0.11| 0.00| 0.03 | 0.10       | 0.01   | 0.32   |
| Wild rice | 0.11| 0.10| 0.03| 0.07 | 0.14       | −0.02  | 0.44   | 0.06   |

B1-B1 type of B-Aman; B2-B2 type of B-Aman; T-aman: Transplanted Aman.

Genetic structure

To further determine genetic differentiation and population structure, the probability (Ln likelihood) of the numbers of clusters (K), which ranged from 2 to 7, was estimated.
using STRUCTURE software. The values of ln likelihood increased as the number of K increased, suggesting that data on these samples were highly structured. However, consistent results among independent runs were obtained when the number of clusters (K) was 3. When K = 3, most accessions can be classified into three groups, which corresponded to indica (73), Aus (27) and japonica (41) (Fig. 2 and Supplementary Fig. 2). Of the 151 accessions analyzed, 141 were clearly assigned to a single group, where >90% of their inferred ancestry derived from one of the model-based populations, however, 10 accessions (6.6%) were classified as having admixed ancestry. The 10 accessions consisting of 1 wild rice accession belong to indica-japonica-aus admixed group, 1 indica from China (Zhejiang province) belong to indica-japonica admixed group, 2 Boro belong to aus-japonica admixed group and 6 accessions (1 wild rice, 1 Aus, 4 Aman) belong to indica-aus admixed group. The STRUCTURE-based and Powermarker-based clustering results were very similar.

The ecotype based genetic structure were also analyzed, the results showed that suitable K values were 3 for B-Aman and T-Aman, three sub-populations correspond to indica, japonica and Aus, indicating the clear genetic structure in these two ecotypes. In contrast, the suitable K values were 4 and 6 for the Aus and Boro, because of the smaller number of varieties for Aus (16) and Boro (18). It seems no clear genetic structure present either in Aus or Boro ecotypes.

### Table 3. Cluster results based on variety groups

| Variety group (Initials) | (indica) | (Aus) | (japonica) | Unknown group | Total |
|-------------------------|---------|-------|------------|--------------|-------|
| Aus (U)                 | 3       | 10    | 3          | 16           |
| Boro (O)                | 12      | 2     | 4          | 18           |
| B1 type of B-Aman (B1)  | 10      | 2     | 3          | 15           |
| B2 type of B-Aman (B2)  | 3       | 4     | 13         | 20           |
| T-Aman (T)              | 41      | 14    | 12         | 57           |
| Aman (A)                | 5       | 5     | 10         |              |
| Rayada (R)              |         | 1     | 1          |              |
| indica (I)              | 2       | 1     | 3          |              |
| japonica (J)            |         | 9     | 9          |              |
| O. rufipogon (W)        | 1       | 1     | 2          |              |
| Total                   | 76      | 28    | 45         | 2            | 151   |

**Fig. 2.** The genetic structure of Bangladesh local varieties.

### Discussion

**Indica-japonica differentiation**

It has generally been accepted that rice has two genetically divergent subspecies, indica and japonica, based on the hybrid sterility (Kato *et al.* 1928, Terao and Mizushima 1942), a number of physiological and morphological traits such as drought tolerance, potassium chloride resistance, phenol reaction (Oka 1958), isozymes (Glaszmann 1987, Second 1982) and molecular marker analyses (Cheng *et al.* 2003, Garriss *et al.* 2005, Sun *et al.* 2002, Wang and Tanksley 1989). Glaszmann (1987) identified six varietal groups using 1688 landraces of rice collected from Asia, including 99 Bangladesh varieties, by surveying 15 polymorphic loci for 8 enzymes, of which the two major groups corresponded to the indica (Group I) and japonica (Group VI) and other groups were characterized by different varieties including Aus (Group II), Ashina (Group III), Rayada (Group IV) and Basmati and Sadri (Group V). In that study Bangladesh was recognized as having very high diversity of rice germplasm with varieties in 5 of the 6 isozyme groups (I–V) but not Group VI, japonica (Glaszmann 1987). Garriss *et al.* (2005) used 69 nuclear SSRs and two chloroplast loci to analyze genetic structure and diversity of 234 cultivated rice accessions. Most accessions were classified into five groups, which corresponded to indica, Aus, aromatic (Basmati), temperate japonica and tropical japonica. Of the 15 accessions from Bangladesh used by Garriss *et al.* (2005) seven were classified as indica, four as aus, one aromatic and two as tropical japonica. One variety was an admixture of aus and indica.

In this study, the Bangladesh local varieties clustered into three groups corresponded to indica, Aus and japonica group. Of the five ecotypes from Bangladesh, Aus and most Boro can be classified into the Aus group, most T-Aman and some Boro can be classified into indica group, and most B2 type of B-Aman, some Boro and Aus can be classified into japonica group. One Rayada (CH153) and one aromatic (CH26) accession was classified into the japonica group. These results were almost same to that reported by Garriss *et al.* (2005), although a larger number of Bangladesh varieties have been used in this study.

Allele 4 of the isozyme Est10 is the predominant allele found in wild rice in China (Cai *et al.* 2003). Most Chinese wild rice has characteristics associated with japonica rice (Sun *et al.* 2002, Yuan *et al.* 1992b). In this study, the five Chinese japonica varieties carrying specific esterase allele Est10-4 were grouped with typical japonica checks (Nipponbare, T65, T65glg) and one Chinese indica (CH147) carrying this specific esterase allele was not assigned to any of the three groups. Among the traditional varieties of rice from Bangladesh this allele was found in 17 of the accessions in the japonica group, 10 in the indica group and 4 in the Aus group and 23 of these varieties were B2 type of B-Aman. We have checked 1270 rice local varieties from most rice cultivation areas for this Est10 locus before this study.
and found the allele 4 of the isozyme Est10 were only detected from a few Chinese varieties from Guangxi, Jiangxi and Zhejiang province and some deepwater rice including B-Aman and Rayada from Bangladesh (Cai HW, unpublished data). The results found in this study suggested that the B2 type of B-Aman is somewhat similar to common wild rice, and these materials together with the Chinese varieties carrying specific esterase allele Est10-4 may belong to primary types of the rice domestication process.

Based on the results of the overall AMOVA, the highest value was not due to the differences among groups, but due to the differences within groups. The higher variation values were from within populations including T-Aman (30.4%), B2 type of B-Aman (18.9%), Boro (10.5%), Aus (9.6%) and B1 type of B-Aman (8.6%) compared with only 15.0% of the variation among groups, indicating these varieties groups were genetically divergent and complex.

The specificity of deepwater rice and varieties with very small grain

The Aman group in Bangladesh included three types: T-Aman and B-Aman and a special B-Aman (B2 type) which can elongate the internodes rapidly in response to the rising water level (Cai and Morishima 2000). Hakoda et al. (1990) analyzed allozyme variation of a large group of accessions from Bangladesh (495) and reported high average genetic diversity of varieties from the Ganges and Irrawaddy deltas compared with Chao Phraya and Mekong deltas. It was reported that varieties from the Ganges and Irrawaddy deltas had deepwater varieties similar to japonica type (Hakoda et al. 1990). Another study of various traits including internode elongation, grain characteristics and acid phosphatase isozyme genotype of deepwater rice from Bangladesh and deltas of Southeast Asian countries showed that many Bangladesh deepwater rice were of a different ecotype than those from Southeast Asia (Inouye 1987). Cai and Morishima (2000) also found that some of the cultivars grown in deepwater areas in Bangladesh consisted of a japonica-like group and an unclassified unique group (mostly B-Aman and Rayada) in addition to a major indica group based on isozyme analysis. Results here show that these special B-Aman varieties including 9 varieties that are the same as those used by Inouye (1987) were mainly classified into the japonica group. Results here agreed with the results of Hakoda et al. (1990), Inouye (1987) and Cai and Morishima (2000), however, some deepwater rice were classified as indica, indicating the high diversity of deepwater rice in Bangladesh.

In this study, five varieties with very small grain were included (Supplemental Table 1). Yuan et al. (1992a) has indicated that some Aman varieties, for example, Kallijra from Bangladesh, Chhote Dhan from Nepal, Badsha Bog from India, with very small grain (10 g for 1000 grain weight) and large panicles (>300 grains) should be classified as japonica based on their high hybrid fertility with japonica and negative phenol-reaction. The results here showed that four accessions (CH1, CH18, CH31 and CH36) out of five small grain varieties clustered into japonica group, and other one (CH19) clustered with the indica group.

Most wild rice populations in China are similar to the japonica subspecies whereas wild rice in South Asia is similar to indica subspecies (Sun et al. 2002). What therefore is the origin of the many traditional landraces of Bangladesh that belong to the japonica subspecies? There are several possible explanations (a) Bangladesh japonica varieties may have evolved from japonica like wild rice of South Asia. (b) Japonica varieties in Bangladesh may have evolved from introduced rice germplasm from East Asia where rice was first domesticated (Molina et al. 2011, Vaughn et al., 2008a, 2008b) in a similar way to evolution of japonica varieties after being introduced from China to Southeast Asia (Fuller and Sato 2008). (c) Southeast Asian japonica varieties could have been introduced to South Asia and introgressed with local rice germplasm. It would be instructive to compare japonica varieties of Bangladesh with tropical japonica from Southeast Asia.

In conclusion, in the present study, Bangladesh traditional varieties can be divided into three groups corresponding to japonica, Aus and indica. The varieties within each ecotypes according to cropping system were genetic divergent and complex. A particular group of deepwater rice and varieties with very small grains are special types of Bangladeshi rice, were mainly japonica. This information will facilitate the effective use of the Bangladesh rice germplasm.

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