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Genetic diversity associated with heading date in some rice (*Oryza sativa* L.) genotypes using microsatellite markers

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Abstract: Heaading date (HD) in rice (*Oryza sativa* L.) is a key agronomic trait with complex nature. Among rice cultivars, due to genotypic diversity a wide range of variation in heading date has been observed. Genetic categorization of heading date have facilitated by the DNA markers and simple sequence repeat (SSR) markers have been recommended as markers of choice. In this study, 14 rice genotypes from different regions were used; two genotypes from each region and differ in their heading date (late flowering time and early flowering time) using 13 SSR primer pairs. The overall results showed that some markers could be discriminated earlier heading than the late heading for each country, and these markers could be associated with heading date. The microsatellite assay generated cultivar-specific alleles in most of the screened genotypes (like RM 585, which has 3 alleles); these may be used as DNA fingerprints for cultivar identification and associated with heading date. Polymorphic information content (PIC) values were varied from 0.47 to 0.78, the highest value belongs to RM2239 (PIC 0.78), while RM223 showed the lowest PIC value (PIC= 0.47). Further analysis will be required to prove this marker (RM 585) such DNA sequence and QTL. Moreover, SSR marker, RM420, was found to be related to generating four alleles, which could be distinguishable for early and late genotypes and would help to reduce the amount of water required for rice growth especially in areas with limited water availability.

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1. Introduction

There is growing pressure to significantly increase rice productivity to meet the ever increasing food demands of human population (Khush, 2005). Rice (*Oryza sativa* L.) is staple food of a major part of human population and also one of the world's most widely cultivated crop (Maclean et al., 2002). As a major cereal crop, it is one of the most differentiated crops with adaptability to a wide range of terrestrial, ecological and climatic regions. Heading date, (HD) or flowering time is one of the key factors determining regional and seasonal adaptation and has been a major target of selection in rice breeding programs (Endo-Higashi and Izawa, 2011; Fujino et al., 2013; Xue et al., 2008).

In rice HD is a complex trait which is governed by multiple genes and regulated by environmental factors, such as day length, temperature, and soil conditions. Rice is a short-day plant, with the distribution of the ancestral species placed in the tropics (Yano et al., 2001). The domesticated rice growing area was stretched to the Northern latitudes by selecting accessions with appropriate heading dates (Izawa, 2007). Depending on conventional breeding for early flowering is slow in reaching progress due to poor understanding of genetic control of early flowering date. Molecular markers helps in identification of marker(s) associated with heading date trait and indirect selection using marker assisted selection (MAS). The DNA-based molecular markers are universal, repeatable, stable and highly consistent (Song et al., 2003). Experiments with different rice genotypes have reported the existence of significant variation in heading date and revealed that *Se5, Hd1, Hd3a, Hd6, and Ehd1* genes are involved in the flowering time of rice, (Kojima et al., 2002; Doi et al., 2004).

Many molecular markers and genetic studies have been applied elucidate mechanism of heading date in rice (Izawa et al., 2003). Among several classes of available DNA markers, simple sequence repeat (SSR) are considered the most proper markers. Due to their

codominant inheritance, multi-allelic nature, reproducibility, highly informative and abundant in plant genomes (Powell et al., 1996). In rice and other plant species large number of SSR markers were developed and mapped (McCouchet al., 2002), these markers are widely used to track loci and genome regions and exploring genetic variability in a rice genotypes for detecting new gene(s) and for further improvement of the germplasm (Brondaniet al., 2006; Jayamani et al., 2007). Moreover, SSR markers in rice have been successfully applied for many purposes like genome mapping; assessment of the genetic diversity and relatedness (Saini et al., 2004).

Recently, SSR markers have facilitated the genetic analysis of heading date, and numerous quantitative trait loci (QTLs) have been identified using mapping populations (Nonoueet al., 2008; Matsubara et al., 2008; Maas et al., 2010). This study aimed to elucidate the diversity of heading date associated marker(s) using SSR markers in rice genotypes from different regions. Such markers would be of great importance in marker-assisted selection for early flowering time, which on the other hand would help to reduce the water requirement rice crop especially in drought prone areas like Egypt.

2. Materials and Methods

2.1 Rice genotypes

Fourteen rice genotypes from different regions (as shown in Table 1), two genotypes from each region differ and highly diverged in their heading date (one late flowering time and another is early flowering time) were used in this study kindly provided by the Institute of Genetic Resources, Kyushu University, and heading date data as well; except for Sakha 101 and 102 from Egypt.

Table 1. Fourteen rice genotypes from different regions and with different heading dates.

No.	Genotypes	Rice genotypes information	
		country	heading date
1	Sakha 102	Egypt	94
2	Sakha 101	Egypt	103
3	Ho 352 (Hokko)	Japan	44
4	Ho 343 (kudai shin 75)	Japan	81
5	Ho 1399 (Baroo66)	Pakistan	52
6	Ho 1400 (Baroo64)	Pakistan	92
7	Ho 393 (Hong mao)	China	45
8	Ho 817 (Hong ma wei)	China	75
9	Ho 1176-1	India	56
10	Ho 1409	India	73
11	Ho 1037	Nepal	40
12	Ho 1030	Nepal	77
13	Ho 1051	Russia	49
14	Ho 1233	Russia	73

2.2. DNA extraction and PCR amplification

Rice seeds of the used 14 genotypes were placed in an oven at 50°C to break seed dormancy. Seeds were soaked in water in a Petri-dish lined with Wattman's filter paper and incubated for 48hrs at 30°C. Pre-germinated seeds were sown in pots and watered daily. Two weeks after sowing young leaf tissues were harvested for DNA isolation. DNA extraction using Cetyltrimethylammonium bromide (CTAB) based on the method of Doyle and Doyle (1990) was used. The procedure involves soaking grained leaves tissue in 600 µl CTAB extraction buffer (100mMTris-Cl, pH 8.0, 25 mM EDTA, pH 8.0, 1.25 M NaCl, 2% CTAB and 3% PVP) for 30-45 minutes at 65°C. Then, mixed with an equal volume of phenol:chloroform (1:1, vol/vol), mixed gently for 2-3 minutes and centrifuged at 14000 rpm for 10 minutes at room temperature. The supernatant is transferred to a fresh sterile micro-centrifuge tube and the DNA was precipitated using an equal volume of ice-cold isopropanol. The DNA is pelleted by centrifugation at 14000 rpm for 10 minutes at room temperature.

After centrifugation, the supernatant is discarded and the DNA pellet is washed twice with 70% ethanol. The pellet is air dried for 1 hr and dissolved in 50 µl of TE buffer (10 mM Tris HCl, pH 8.0 and 1mM EDTA, pH 8.0).The concentration of DNA obtained (ng/µl) was determined on NanoDrop spectrophotometer. The purity levels for all 14 genotypes were accessed by obtaining the absorbance ratio A260/280. Polymerase chain reaction (PCR) amplification was done in a volume of 20µl using 40ng genomic DNA, 0.2 mMdNTP, 1.5 mM $MgCl_2$, 10 pmol of each primer (forward and reverse) and 0.5U *Taq* polymerase. PCR conditions were as follows: 96°C for 1 min, 35 cycles of 96 °C for 30s, 55-57°C (according to primer annealing Tm) for 30 sec, 72°C for 45 sec) and 72°C for 7 min. The reproducibility of the amplification products was checked twice for each primer. After amplification, a 10µl aliquot of the amplified SSR samples was combined with 3 µl of a loading buffer (0.4%(w/v) bromo-phenol blue and analyzed directly on 2% (w/v) agarose gels in 1× TBE buffer (10mM Tris-Borate, 1mM EDTA) containing 0.5 µg per ml of ethidium bromide. A 100bp DNA ladder was used as a size marker to compare the molecular weights of amplified products. After electrophoresis, the gels were documented using Gel Documentation System.

Table 2 Simple sequence repeat (SSR) primers, their sequences and repeat motifs.

Primers	Primer sequence (5'-3') Farward	Primer sequence (5'-3') Reverse	Motifs
RM 510	AACCGGATTAGTTTCTCGCC	TGAGGACGACGAGCAGATTC	(GA)15
RM 585	CAGTCTTGCTCCGTTTGTTG	CTGTGACTGACTTGGTCATAGG	(TC)45
RM223	GAGTGAGCTTGGGCTGAAAC	GAAGGCAAGTCTTGGCACTG	(CT)25
RM315	GAGGTACTTCCTCCGTTTCAC	AGTCAGCTCACTGTGCAGTG	(AT)4(GT)10
RM 21	ACAGTATTCCGTAGGCACGG	GCTCCATGAGGGTGGTAGAG	(GA)18
RM493	TAGCTCCAACAGGATCGACC	GTACGTAAACGCGGAAGGTG	(CTT)9
RM302	TCATGTCATCTACCATCACAC	ATGGAGAAGATGGAATACTTGC	(GT)30(AT)
RM231	CCAGATTATTTCCTGAGGTC	CACTTGCATAGTTCTGCATTG	(CT)16
RM261	GAGCCAAATAAGATCGCTGA	TGCAAGCAGCAGATTTAGTG	(CT)31
RM260	ACTCCACTATGACCCAGAG	GAACAATCCCTTCTACGATCG	C9(CT)8
RM 2239	GACTTGCCAACTCCTTCAATTCG	TCGTCGAGTAGCTTCCCTCTCTACC	(ATAG)7
RM 424	TTTGTGGCTCACCAGTTGAG	TGGCGCATTCATGTCATC	(CAT)9
RM 420	GGACAGAATGTGAAGACAGTCG	ACTAATCCACCAACGCATCC	(AAAT)7

2.3. Microsatellite markers and data analysis

Thirteen SSR primer pairs, their sequences and repeat motifs for these markers can be found in the rice genome Table 2. For each of the defined loci, SSR allelic composition was determined for each genotype. Polymorphism information content (PIC) values which indicating the ability to distinguish between genotypes for each primer was determined using the following formula (Anderson et al., 1993), $PICi = 1-\sum^{n}_{j=1}Pij^{2}$

Where P_{ij} is the frequency of the j^{th} allele for marker i, and summation extends over n alleles.

3. Results and Discussion

A wide range of variation in flowering date was obvious among the 14 rice genotypes which were selected in this study from different regions. Egyptian cultivars, Sakha 101 was the most latest in heading date with 103 days, whereas Ho1037 genotype, which was derived from Nepal was earliest genotypes with 40 days as shown in Table (Table 1). SSR markers used in this study amplified polymorphic bands using 14 rice genotypes (Table 3). The lowest amplicon size (121bp) belonged to RM302 and the highest amplicon size (284bp) belonged to RM420. The number of microsatellite alleles of used markers ranged from 2 to 5 alleles of which RM2239 marker produced the highest numbers of alleles (5 alleles) while RM510 and RM 302 produced the lowest numbers of alleles (2 alleles) with motif (GA)15 and (GT)30(AT)8, respectively. Value polymorphism information content (PIC) provides an assessment of discriminating powerful of any marker(s) based on the number of alleles at a locus and comparative rates of these alleles,. PIC values were varied from 0.47 to 0.78, the highest value belong to RM2239 (PIC 0.78) while RM223 showed the lowest PIC value (PIC= 0.47). Some microsatellite assay generated cultivar-specific alleles in most of the screened genotypes (like RM 585, which had 5 alleles); these may be used as DNA fingerprints for cultivar identification.

Analysis of genetic diversity revealed that the SSR marker, RM585, was found to be superior and produced different alleles from each region. It could be added to the useful markers for heading date assist selection. Overall results showed that the some markers could be discriminate the early heading than the late heading for each country, and these markers could be associated with heading date. Moreover, RM420 was found to be related to generate four alleles which could be distinguishable for early and late genotypes. Such markers could be of great importance in marker-assisted selection of early flowering time in rice genotypes, which ultimately would help to produce rice in short duration and reduced quantity of water.

Rice cultivars show a highly divergence of natural variability in heading date and photoperiod sensitivity, yet these differentiations of natural variation is not completely understood and there were rare information about it (Nonoue et al., 2008). Functional and non-functional alleles of *Hd1* were reported to be associated with flowering time, concluding that *Hd1* is a major determinant of flowering time variations in cultivated rice (Takahashi et al., 2009). In addition, the RM510 was attributed to induce flowering under short day conditions (Tamaki et al., 2007; Samuel 2009). Similarly**,** *qhd6* gene was linked with RM510 (Bai et al., 2011). SSRs located in the coding regions in rice (Cho et al., 2000) and other crops (Gupta et al., 2003; Choudhary et al., 2009), are under strong selection pressure with very limited chances of mutations (Varshneyet al., 2005). However, in the present study, we observed slightly higher average PIC values for the trait-linked SSRs (0.78) than those reported earlier (Singh et al., 2004; Joshi and Behera, 2006; Prabakaran et al., 2010), which might be due to high diversity of rice genotypes selected in this study.

Moreover, the SSR markers used in the study were carefully selected on the basis of their high PIC values reported earlier. Higher PIC values for some SSRs similar to our findings were also reported (Jayamani et al., 2007; Ram et al., 2007). Apparently, we have used rice microsatellite (RM) markers linked to QTLs affecting days to heading (trait-linked markers).

Further, when markers linked to known QTLs were tested in a germplasm with large variation for heading dates. Landraces of rice that have been cultivated for many generations in a certain region, being shaped by many factors such as stresses crop management and handling. In some cases, quick changes can take place, especially when the landrace is taken to a different region or when new materials are cultivated in close proximity with the original landrace (Flavio 2013). High levels of differentiation within rice genotypes could be for adaptation to climate conditions of the region, combined with differ with heading date (Zeven, 1998).

A comparison of the genetic construction of flowering time among different crops and rice should consider major points. First, temperature and day length is responsible for large variation in flowering time and heading date. Second, difference in the regulatory mechanisms might be due to difference in cultivation areas and reproduction methods of these species (Rosloski et al., 2010). Molecular characterization using SSR markers is playing an important role to identify gene for days to heading. They have become a popular type of co-dominant molecular marker in genetic analysis and plant breeding application (Choi et al., 2011).

Table 3. Simple sequence repeat (SSR) no., of bands, Polymorphic, expected allele size, band size range and polymorphic information content (PIC).

Primer name	Bands No.,	Polymorphic (%)	EAS* (bp)	Band size range	PIC**
RM 510	2	100%	122	118-130	0.51
RM 585	3	100%	233	198-229	0.67
RM223	2	100%	165	137-149	0.47
RM315	4	100%	133	133-163	0.76
RM 21	3	100%	157	144-153	0.59
RM493	3	100%	211	195-281	0.60
RM302	2	100%	156	121-134	0.50
RM231	3	100%	182	157-182	0.68
RM261	1	0.00%	138	135	0
RM260	4	100%	111	154-212	0.70
RM 2239	5	100%	123	130-191	0.78
RM 424	4	100%	239	218-280	0.75
RM 420	4	100%	197	197-284	0.75

*EAS = expected allele size **PIC polymorphic information content

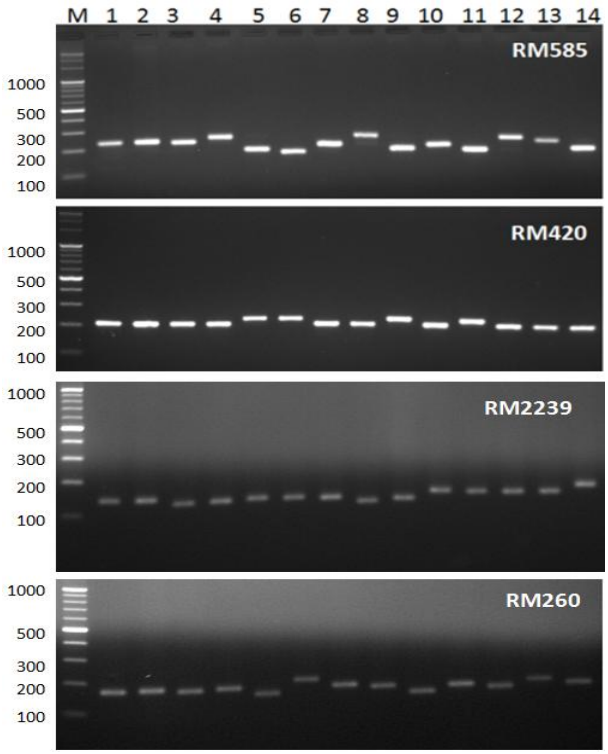

Fig. 1 Amplified products from genomic DNA of 14 rice genotypes using RM585, RM420, RM 2239 and RM 260 primers. The lanes represent, M: 100-bp DNA marker, 1= Sakha 102; 2=Sakha101; 3= Ho 352; 4= Ho 343; 5= Ho 1399; 6= Ho 1400; 7= Ho 393; 8= Ho 817; 9= Ho 1176-1; 10= Ho 1409; 11= Ho 1037; 12= Ho 1030; 13= Ho 1051 and 14= Ho 1233.

SSR markers assisted molecular characterization will also been useful in integrating genetics, physical and sequence based maps of rice and served as an efficient tool for breeders and geneticists to link phenotypic and genotypic variations**.** Thus closely marker linked to the target gene can act as a "tag" which can be used for direct selection for early heading date. Furthermore**,** Some heading date genes are located near SSR markers like, Hd4 was reported to be near and linked to RM7110 and Hd2 was found to be near the markers RM1306 and RM420 on chromosome 7 (Fujino and Sekiguchi 2005; Lin et al. 2003). In our study, RM420 was the best to differentiate genotypes from each region and suggested to be linked to Hd2, indicating that these QTL may be the same loci. Moreover, based on chromosomal location (chr7) detected to be same as the QTL, *Se*7.1 with is near to (RM214), in addition, QTL (chr8) was also considered to be same as *Se*8 (RM25) (Gu and Foley, 2007). It was suggested that *Se*7.1 might also correspond with *Hd4*, reported in the Nipponbare, a Japanese cultivar (Lin et al., 2003). Several other QTLs detected on other chromosomes were also seems to be located on the same

chromosomal region (Gramine; http://www.gramene.org). These loci have been consistently identified from different crosses of various genetic backgrounds, suggesting that these loci may be used for the rice breeding programs of early heading plants. The outcome of this work has been the identification of a SSR marker linked to the heading date gene(s).

Conclusions

The Rice heading date is one of the serious aspects determining regional and seasonal adaptation and has been a main target of selection in rice breeding programs. Different rice genotypes were used to study molecular diversity and MAS for flowering time using 13 SSR primer pairs. The microsatellite assay produced some alleles (markers) which were cultivar-specific in most screened genotypes; those markers could be used as MAS for rice cultivar identification and associated with heading date. Such marker(s) could be used for hybrid rice breeding.

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