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Identification of Subspecies-specific STS Markers and Their Association with Segregation Distortion in Rice (*Oryza sativa* L.)

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Abstract

Two subspecies, *japonica* and *indica*, have been reported in rice, which differ in several ecotypic traits. However, reproductive barriers in hybrid progenies between subspecies have been major obstacles in breeding programs using inter-subspecific hybridization. As the first step to elucidate the reproductive barriers, we developed subspecies-specific (SS) STS markers in this study. A total of 765 STS primers were designed through comparing DNA sequences at every 2~3cM interval between *japonica* and *indica* rices, which are available at Web DBs such as IRGSP, NCBI, TIGR, and GRAMENE, and tested for subspecies-specificity using 15 *indica* and 15 *japonica* varieties of diverse origin. Of them, 67 STS markers were identified as SS STS markers and their subspecies-specificity scores were estimated. The SS markers were dispersed throughout the genome along chromosomes. Of them, 64 SS markers were mapped on an RIL population derived from a Dasanbyeo (*indica*) / TR22183 (*japonica*) cross. Genomic inclination of RILs was evaluated based on the genotyping with different types of markers. Association test between markers and segregation distortion revealed that segregation distortion might not be the cause of generating SS markers. The SS markers will be applicable to estimate the genomic inclination of varieties or lines and to study the differentiation of *indica* and *japonica*, and ultimately to breed true hybrid rice varieties in which desirable characters from both subspecies are recombined.

Key words: rice subspecies marker, reproductive barrier, segregation distortion, allelic association, genomic inclination

Introduction

Cultivated rice (*Oryza sativa* L.) is known to consist of two major subspecies, *indica* and *japonica*. *Japonica* rice is divided into two subgroups as temperate and tropical *japonica*, and *indica* has several subgroups adapted to diverse environments (Glaszmann 1987; Oka 1988; Khush 1997). Each subspecies has its own ecologically adapted and useful traits, but the reproductive barriers between two subspecies including hybrid sterility (Oka 1988; Liu et al. 2004; Song et al. 2005), hybrid breakdown (Li et al. 1997; Kubo and Yoshimura 2005), segregation distortion (Harushima et al. 2001 and 2002), and suppressed recombination (Ikehashi 1982; Neiman and Linksvayer 2006) have been obstacles for cross breeding between subspecies.

Inter-subspecific hybrid sterility might be governed by hybrid-sterility QTLs (Li et al. 1997; Song et al. 2005). A 'Supergene', described as a tightly linked and conserved region, was also suggested as one of the mechanisms for inter-subspecific hybrid sterility (Hanson 1959; Li et al. 1997). The chromosomal structure of supergenes might be caused by inversion (Tanksley et al. 1992; Li et al. 1997), which also has been considered to be associated with the segregation distortion (Li et al. 1997; Xu et al. 1997; Harushima et al. 2001 and 2002). Subspecies-specific (SS) or species-specific genomic regions could be inherited in a conserved manner to each of subspecies and species from which the SS regions were originated in the progenies of inter-subspecific or inter-specific crosses (Tanksley et al. 1992; Rieseberg 2000; Wang et al. 2001). Thus SS regions may provide a clue to elucidate the mechanisms for reproductive barriers including inter-subspecific hybrid sterility and for the differentiation of rice subspecies.

A number of different markers for classification of *indica* and *japonica* have been tried out, including isozyme (Glaszmann 1987; Zhang et al. 1989; Li and Rutger 2000), protein marker

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(Bi et al. 1997), RFLP (Qian et al 1995) RAPD (Kwon et al. 1999; Jiang et al. 2001; Chin et al. 2003), microsatellite (Chen et al. 2002; Ni et al. 2002), AFLP (Mackill et al. 1996; Cho et al. 1999), STS (Edwards et al. 2004; Shen et al. 2004), SNPs (Feltus et al. 2004), and chloroplast DNA (Dally and Second 1990; Sun et al. 2002). But the development of SS markers through systematic survey of the whole chromosomes using those markers has not been undertaken. The Chinese Beijing Genomics Institute and Chinese Academy of Sciences have opened an internet service on comparative genetics information in rice (Zhao et al. 2004) and identified more than five million SNPs through whole chromosome scanning of *indica* (93-11) and *japonica* (Nipponbare). The data will be very useful for preliminary comparison of genomic structure between *indica* and *japonica*, and for screening potential SS markers. However, since there would be a great deal of allelic variation among varieties within subspecies, the SNPs should be tested for subspecies-specificity using a number of varieties from each subspecies to be used as SS markers.

The objectives of the present study were to identify SS STS markers, which were designed to cover the whole chromosomes at an 2~3cM interval based on the sequence information available at RGP for *japonica* and NCBI for *indica*, and to investigate their relationship with segregation distortion and the allelic association between them through linkage mapping using an RIL population derived from *indica* / *japonica* cross.

Materials and Methods

Plant material

Fifteen *japonica* and fifteen *indica* varieties of diverse geographical origin, classified by classical criteria (Oka, 1988), were used for screening SS STS markers (Table 5). Four bulks of DNA were made to pre-screen SS STS markers by bulked segregant analysis (BSA) method (Michelmore et al. 1991). An F_9 RIL population (DT RIL) was raised by single seed descent method from the cross between Dasanbyeo (*indica*) and TR22183 (*japonica*). The DT RIL was used for construction of genetic linkage map of SS STS markers and for exploring the relationship between SS markers, and segregation distortion. Genomic DNA of each RIL and their parents, Dasanbyeo and TR22183, were extracted from young leaf blades according to Causse et al. (1994).

Primer designing and screening

The IRGSP (International Rice Genome Sequencing Project) has completed genetic and physical mapping and sequencing of each chromosome (http://rgp.dna.affrc.go.jp/cgi-bin/statusdb/irgsp-status.cgi). To compare the genetic distance in the Nipponbare/Kasalath map with physical contig map of BAC/PAC clones originated from Nipponbare, BAC/PAC clones positioned at every 2~3cM interval in each chromosome were chosen. The sequence of the BAC/PAC clones were aligned to the *indica* (93-11) sequence DB by using Blast Service at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/Genome/PlantBlast.shtml? 10) to pick up *indica* sequences which were perfectly matched except a few InDel polymorphisms to *japonica* (Nipponbare) sequences. Selected indica WGS clones were reversely aligned to *japonica* sequence DB at TIGR (http://tigrblast.tigr.org/) to confirm the single locus at the same chromosomal position where the *japonica* clone was originated. Primers were designed using a online-service software Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to detect the InDel polymorphism between *indica* and *japonica* sequences. The amplicon size for each primer set was determined so that the amplicon contained at least 5% InDel difference of its whole size, 100~400bp. To construct a framework linkage map using DT RILs, a total of 155 SSR markers were chosen in reference to Temnykh et al (2000).

PCR amplification and detection for SSR markers were performed as described in Temnykh et al. (2000) with some modifications. Each 25㎕ reaction mixture contained 50ng DNA, 5 pmol of each primer, 2㎕ PCR buffer [100mM Tris (pH 8.3), 500mM KCl, 15mM $MgCl_2$, 2㎍ gelatin], 250㎛ of each dNTPs and 0.5 unit *Taq* polymerase. The MJ Research PCR system was used for DNA amplification. The thermocycler profile was: 5min at 94℃, 35 cycles of 1min at 94℃, 1min at 55 ℃ or 61 ℃ or 67 ℃, 2min at 72 ℃, and 5min at 72 ℃ for final extension. Amplified PCR products were resolved by electrophoresis on 3% agarose gels. The markers starting with S designate the STS markers.

For preliminary screening of SS markers, bulked-segregant analysis (BSA) method (Michelmore et al., 1991) was adopted. Four DNA bulks including 5 varieties each from four varietal groups (temperate *japonica*, tropical *japonica*, Korean *indica* and *indica*) were made and screened with a total of 765 STS markers (Table 1). Then 30 varieties were screened with pre-selected primers through BSA method, and subsequently SS markers were identified. Selected markers which showed consistent results in three replications were confirmed as SS STS markers.

Subspecies-specificity score of each marker and subspecies-prototype degree of each variety

DNAs from 30 varieties were genotyped with all of the SS STS markers and scored 'a' (*japonica* allele) or 'b' (*indica* allele) for each marker locus. We counted total number of 'a' from *japonica* varieties and 'b' from *indica* varieties. Since some markers showed variation in generating SS alleles among varieties within and inter-subspecies, the concept of subspecies-specificity (SS) concept was employed as follows;

Subspecies-specificity (SS) score of each marker = (Total number of expected alleles in each subspecies) / (Total number of varieties tested; 30) × 100 (%)

For example, if a marker has a SS score 100%, it means that the SS marker generated SS alleles to all of 30 varieties without exception. A marker with SS score equal to or higher than 93.3 (up to 2 exceptions out of 30 varieties) was regarded as an SS marker. In addition, the subspecies-prototype (SP) degree for each variety was calculated in order to describe the relative genomic inclination of each variety toward either subspecies as follows;

If a variety has a SP degree close to 1 or -1, the variety is estimated to have the genomic inclination close to the prototype variety of *japonica* or *indica*, respectively. Genetic similarities among varieties were obtained by Jaccard coefficient using the computer software, NTSYS-pc 2.0.

Linkage mapping

The genetic linkage map was constructed using the "group"

Table 1. Number of STS markers designed in this study and subspecies-specific (SS) STS markers identified in each chromosome

Chromosome	1	2	3	4	5	6	7	8	9	10	11	12	Total	Average
Total STS markers (T)	66	53	66	61	82	60	62	81	64	51	70	49	765	63.8
BSA-selected markers	29	23	32	29	42	21	35	26	37	23	23	18	338	28.2
SS STS markers (S)	5	7	11	1	2	1	6	4	10	7	3	3	67	5.6
S/T (%)	7.6	13.2	16.7	13.1	2.4	1.7	9.7	4.9	15.6	13.7	4.3	6.1	8.8	8.8

command with LOD > 3.0 on a software JoinMap® 3.0 (Piet, 1993). The order of markers in linkage groups was determined using the COMPARE, TRY and RIPPLE commands. One hundred sixty-six DT RILs and a total of 155 SSR and 64 STS markers were used for mapping. Map units (cM) were derived using the Kosambi function (Kosambi 1944).

Results

Identification of SS STS markers

A total of 765 STS primer sets on twelve chromosomes, designed through the comparison of sequences between Nipponbare (*japonica*) and 93-11 (*indica*) using rice genomics DBs such as RGP, NCBI, and TIGR, were screened with four DNA bulks (temperate *japonica*, tropical *japonica*, Korean *indica* and *indica*) as described by (Michelmore et al., 1991), and, of them, 338 primers which showed specificity for two groups, *japonica* and *indica*, were preliminarily chosen for the next step. Then, the 338 primers were screened with 30 varieties. Finally, 67 primer sets were identified and confirmed as SS STS markers by replicated trials at least three times (Table 1). Figure 1 shows an example of an SS STS marker, S7011, which was pre-selected by BSA (A) and then confirmed by screening 30 varieties (B). All of the 15 japonica varieties produced a 229bp amplicon, while all of the 15 indica varieties a 205bp amplicon. Sometimes a *japonica* variety might have an *indica* allele, and vice versa. For example, Norin mochi 1 (*japonica*) possessed *indica* allele when amplified by S01054, which might be regarded as an 'exception'. If there were more than two exceptions for an STS marker, it was discarded. That is, threshold of SS score to be selected as SS markers was 93.3%. The 67 SS STS markers were distributed all over the chromosomes though the ratio of SS markers out of entire markers tested varied along chromosomes. There was only one SS markers detected on chromosome 6, while 11 SS markers were identified on chromosome 3. The average number of SS markers was 5.6 per chromosome, and the average ratio of SS markers out of entire markers tested was 8.8% across chromosomes (Table 1). The information of 67 SS STS markers is summarized in Table 2. The BAC/PAC clones from which STS markers were originated and the marker location within BAC/PAC clones were denoted in a sequence order of

$$\text{Subspecies-prototype (SP) degree of each variety} = \frac{\text{Total number of } \textit{japonica} \text{ SS alleles in each variety} - \text{Total number of } \textit{indica} \text{ SS alleles in each variety}}{\text{Total number of SS markers tested}}$$

base pairs. The SS markers which showed perfect SS scores were S01022, S02026, S02140, S03020, S03041, S04128, S06001, S07011, S09026B, S10003A, S11004A, S11006A, and S12011B.

Linkage mapping and segregation distortion (SD) of SS STS markers

The chromosomal location of each SS marker was determined on the Nipponbare/Kasalath linkage map of RGP (Harushima et al. 1997) based on the chromosomal location of BAC/PAC clones from which the STS markers were derived (Table 2). Some SS markers were clustered on chromosome 3, 4, 7, 8, 9, 10, and 11.

A framework map was constructed using 155 SSR markers on DT RILs and then 64 SS STS markers developed in this study were mapped together. Three markers on chromosome 7, S07048, S07050A, and S07050C, didn't show polymorphism between both the parents, Dasanbyeo and TR22183, and thus were excluded. Total map distance was 1,317.08 cM (Figure 2(A)). Chromosome 1, 2, 3, 5, 6, 7, and 12 divided into two or three groups probably due to the lack of harboring markers filling the gap between segments. Most of the SS STS markers were mapped in the same order as in the Nipponbare/Kasalath map. However, the map orders of S01157B and S01160 on chromosome 1, S03136 and S03145 on chromosome 3, S04058 and S04060 on chromosome 4, S09073/S09075 and S09093 on chromosome 9, S10001/S10003/S10013 and S10019 on chromosome 10 were reversed (Figure 2(A)).

Of 64 SS markers mapped, 23 markers revealed significant SD, all of which favored indica alleles except only one marker S03020 on chromosome 3 (Figure 2(B)). The SS markers on chromosome 2, 6, 7, 8, and 10 didn't exhibit SD though some of non-SS markers belonged to those chromosomes showed SD. For non-SS markers, 79 markers out of 155 markers showed SD, of which 66 markers favored *indica* alleles and 13 markers favored *japonica* alleles.

Associated transmission of SS markers in DT RILs

It was notable that SS markers were detected in an associated pattern even among independent chromosomes. To test the association between SS markers in DT RILs, expected number of

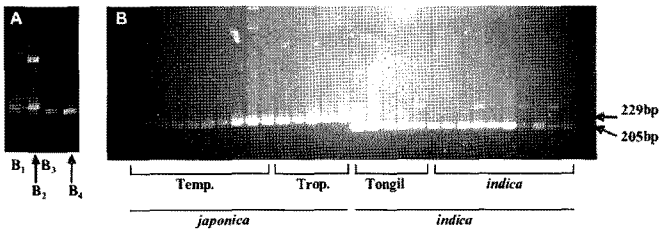

Fig. 1. Screening of subspecies-specific (SS) STS markers. (A) A sample figure of screening SS STS primers using four subspecies bulk DNA (B1: temperate *japonica*; B2: tropical *japonica*; B3: Korean *indica* (Tongil); B4: *indica*). (B) Difference in size amplified DNA by an SS STS marker, S7011 (1~15: *japonica* varieties, 16~30: *indica* varieties).

Table 2. Subspecies-specific (SS) STS markers identified in this study

Chrom.	Marker Name[a]	CM[b]	Aligned Clone[c]	F/R	Primer Sequence[d]	Product Size *Jap./Ind.*(*bp*)[e]	Aligned Region(*bp*)[f]	SS score (%)[g]
	S01022	22.6~24.0	AP002484	F/R	catggatgatgcttccctct / ttgacagtggctccacaaag	300/312	47091~46764	100.0
	S01054	54.3	AP002070	F/R	gcgaagcctgctttttgat / cggagatttttccctaaaacaa	237/366	17334~17664	96.7
1	S01140	140.2	AP003255	F/R	gctaggcagactctagctcatca / tggaacaagtagaagcagaagtca	175/188	73193~73418	93.3
	S01157B	157.6	AP003791	F/R	ccctcaatcatcgcaactgt / cagatgcagaaaagcgcata	236/223	89205~89564	93.3
	S01160	160.4	AP004667	F/R	ttgcgatttatttgccagtg / ccaggcatccaatgcttatt	183/171	119228~119467	96.7
	S02026	26.9	AP004184	F/R	tggtccatcatattgccaac / tcctctcagatccgattttca	167/180	70844~71190	100.0
	S02052	52.2	AP005743	F/R	gcagtcggttctcaattggt / gattttccagcccattctca	192/201	42263~42613	93.3
	S02054	54.6	AP004151	F/R	tttgaagcacgagggatctt / ataaaggaccgatgcaaacg	167/157	98176~98594	93.3
2	S02057B	57.9	AP004086	F/R	agcctcttctccctcctcac / tgcaaacaccataacaaccaa	230/241	16170~16398	93.3
	S02081B	81.7	AP004876	F/R	agcggcatattttgcatagc / tgttttgcaggacgcagtag	225/201	113342~113641	96.7
	S02085	85.9	AP006069	F/R	gcgagagtgtacccctttga / tgtgtaccttgcaccctgaa	164/153	111261~111800	96.7
	S02140	140.9	AP004140	F/R	tgggaggaggatattgtgga / tgacaggttgatgtgatggaa	205/220	29024~29308	100.0
	S03010B	10.9	AC118132	F/R	gtgcggatttggttctgttt / gagggagaggccagattctt	215/203	139770~140069	93.3
	S03020	20.3	AC079633	F/R	tttctaggtacattttaagcaagca / catgaatttgaagctgcgagta	183/168	120908~121207	100.0
	S03027	27.9	AC105928	F/R	tgaacattttggtcgtctgg / Ttgacgaagtcaccatagacg	249/232	166195~166490	96.7
	S03041	41.9	AC135209	F/R	gctgacattgtccgaggttt / ccgacgtccaacctaagc	192/201	18840~19190	100.0
3	S03046	46.6	AC137634	F/R	tcacagtttacaggcggaatc / gcaccatgtatagaccattcca	257/248	75608~76026	93.3
	S03048	48.5	AC136767	F/R	gggatgggagaagggaataa / gccagctaggatgttgaagg	179/159	164474~164833	96.7
	S03096	96.6	AC120505	F/R	cacttgcaagctaagcacca / ccttcctgcttgacgagaaaa	169/184	23364~23708	96.7
	S03099	99.6	AL731878	F/R	ctcccaggatgctcactcag / ataatccaagggcacagcac	222/233	43627~44035	96.7
	S03120	120.4	AC092779	F/R	tgtgcgctcgtgattatttt / aaggggagcagataatgcag	243/254	52189~52476	96.7
	S03136	136.0	AC118133	F/R	gcattaaggcacacaaagca / tgtttgtaatccgcatggaa	200/219	32167~32452	96.7
	S03145	145.6	AC084748	F/R	tcacctacaggaagcagcag / gccgtcgttgaagaagtagc	239/251	23667~24014	93.3
	S04058	58.6	AL662947	F/R	gatccatgcagttgattgtga / tcgtcttatctaaaaagaaaatttga	245/226	89597~89896	96.7
	S04060	60.2	AL606618	F/R	tatggttttatccgccaacc / gctacaactaaaacaagaaacgtga	221/203	17349~17588	96.7
	S04077A	77.9	AL606604	F/R	atgtggatggtgggtcctat / agggttcatcgctaatgctg	266/247	100193~100492	93.3
4	S04077B	77.9	AL731638	F/R	tcccaggtgaactacggact / cagcattttcagtggaagca	174/201	91006~91338	93.3
	S04087A	87.1	AL606682	F/R	atgtttggcaatccgctaag / aaagatggttgagcggaaga	255/247	116475~116894	96.7
	S04097B	97.7	AL662957	F/R	tccacagtctgtccgtgaaa / ctccttgtgctgcagaattg	190/200	41474~41883	93.3
	S04128	128.5	AL606641	F/R	tcacgggaaagctttggtat / aacttatgcagccaccatcc	163/181	30192~30593	100.0
	S04129B	129.6	AL606686	F/R	aatcgattcattcgcacaaa / ctttcatgctctgccattga	182/204	152067~152350	93.3
	S05064	64.1	AC104267	F/R	aaagcaagtcacaaacaaaataaa / tgcctcgattttcataagca	245/233	124568~124913	93.3
5	S05080A	80.7	AC109595	F/R	tggccaactttgggaattta / aagagtcgtgcaaatgaaaaga	229/254	24140~24534	96.7
6	S06001	1.7	AP000616	F/R	agctcaatatcaggcaagcag / aaatgacacagttgacctttttgaa	231/248	91692~92034	100.0
	S07011	11.0	AP004263	F/R	ctggatccaaggcatcattc / cttcgctctcaccatcaaca	229/205	90002~90361	100.0
	S07048	48.0~49.4	AP005824	F/R	catggcaccttgagagttga / acacatggagctggcttctc	157/172	16231~16574	96.7
7	S07050C	50.0	AP004349	F/R	tacacgaacgaacgacaagg / cgctgatttgggtaggtctc	209/193	11782~12141	93.3
	S07050A	50.0	AP005200	F/R	ctccacttatggcagcgaat / caagtgaagtgggagcaggt	198/210	99943~100103	96.7
	S07101	101.8	AP003832	F/R	tccaagcttgctttctttcc / tcggtacacacctcctgtga	202/216	42293~42696	93.3
	S07103	103.4	AP005182	F/R	agcatggatccttcatccaa / actccgatttttgcacttcg	211/224	70531~70937	93.3
	S08066	66.5	AP003947	F/R	ttgttccgttgtgtgcaact / gatgcagcgacgtgaaatc	217/238	115103~115441	96.7
8	S08090	90.5	AP004693	F/R	gcgtgtggaagaggagaaag / cagtgagaatctcgcagtcg	212/230	128592~128933	96.7
	S08106	106.1	AP005509	F/R	ttacggattgtcacggtttt / ggaatttgtcactggtttcca	220/194	84595~85014	96.7
	S08107	107.7	AP003888	F/R	ttggtaatgcccatgctaga / cacgattcggtcatttcaga	228/238	8473~10209	93.3
	S09000A	0.0	AP006058	F/R	ccaattcacggtttaacaagg / gccatgaagcttcgttagga	207/234	87489~87831	96.7
	S09026B	26.7	AP005780	F/R	gggaggcagagggaactact / ttatcaggccaggtcctttg	207/182	133227~133586	100.0
	S09040B	40.7	AP005637	F/R	taatatcgcatggcaagacg / actttgcagaggcgacaaac	226/214	103967~104266	96.7
	S09058	58.3	AP005551	F/R	cgtgagaagtccagtccaca / attgatcgattgggggattt	222/233	56675~57073	93.3
9	S09062B	62.4	AP005559	F/R	acgcataccgaatgtgacag / gttggcactcccgattaaaa	250/236	8513~8932	93.3
	S09065	65.1	AP005555	F/R	tgtgttcgacgtttgaccat / gggccagggtacattgaata	237/248	94853~95201	96.7
	S09073	73.6	AC099403	F/R	accaccctgaaccacaacat / tcactgggttctgtgtccaa	233/250	104693~105095	96.7
	S09075A	75.0	AC108753	F/R	gactaacgaacggggcctat / ggcagcccacactatttagg	174/154	64397~64696	96.7
	S09075B	75.0	AC108753	F/R	cctcactcacctggagaagg / cgtccacactaacggacaca	175/160	8282~8641	93.3
	S09093A	93.5	AP006162	F/R	caccgctctcactgtcattc / tccctcagccataaaaccag	259/232	17947~18365	96.7
	S10001	1.9	AC078891	F/R	atcgtggtcgggattatgag / gcatcatggcttttgtgttg	208/229	15102~15440	96.7
	S10003A	3.0	AC025098	F/R	ataagacggacggtcaaacg / atctcttgtgggctttgtgg	234/246	107818~108225	100.0
	S10013A	13.3	AC083944	F/R	agtcgggtcatttcttagcc / ctacgtcctccgtttcacaa	183/170	70557~70976	93.3
10	S10019	19.0	AC123594	F/R	atgcatctacatggcatttg / gatgctgagatgcgattgaa	152/163	131901~132186	93.3
	S10026C	26.1~30.2	AC021893	F/R	tacgtgtccttgtgcctgaa / tttcacccccactgtaaagg	247/227	103229~103588	93.3
	S10071	71.4~67.5	AC113947	F/R	tatggctcaaccctggaaac / cgtgctagtttgttcactgga	167/158	9385~9744	96.7
	S10072	72.5	AC020666	F/R	tgagtgttgcgttgtcttcc / tggtaaggccttgaagatgg	178/202	124298~124633	96.7

Table 2. continued

S11004A	4.1	AC136970	F/R	tctctggccttctactcatgg / ttgtgtttctacttggactcttttt	173/157	82162~82401	100.0
S11006A	6.0	AC123525	F/R	atgcgccgtccaacttatac / tggtgcaaaggaatgaacaa	248/261	117709~118068	100.0
S11028	28.6	AC123523	F/R	attcccctggggtagctaga / atgggtgaattgcagagaat	200/220	50917~51205	96.7
S12011B	11.5	AL935154	F/R	tgggggagttctgaaatctg / ttaagttcggtgccccataa	156/178	79392~79730	100.0
S12030	30.0~38.1	AL954157	F/R	tccacatgtaaaccgctgaa / tgagtgatataacaacacacaacca	217/230	67619~67919	96.7
S12109B	109.2	AL732378	F/R	ggactcggataaccgcatta / ggaacgcagcgaaagaat	164/173	61221~61451	93.3

a) STS markers were named as follows : "S"(STS) + "OO" (chromosome) + "OOO" (cM).
b) Estimated distance of each marker in Nipponbare/Kasalath map by RGP in Japan.
c) Clones from which the SS markers were derived.
d) Primer was designed by using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 www.cgi).
e) Estimated product size in bp of *japonica* (former) and *indica* (latter) allele.
f) SS marker position in bp within the corresponding BAC clones.
g) Threshold of SS score for identification of SS marker was 93.3 (%) (See Materials & Methods)

random allelic combinations between all marker-pairs were compared with the observed number of significant allelic association in three groups (Table 3). The rate of significant association between inter-chromosomal marker-pairs was 2.6% in the SS vs SS marker type, which was higher than in other two association types. This means that 2.6% out of all possible allelic pairs between SS markers were transmitted together with each other into certain genotypes of RILs. The same trend was observed for the intra-chromosomal association. This indicates that SS markers were more likely to be transmitted to the progeny not at random, but in an associated manner. SS markers were divided into two groups as D-SS (22 SS markers which showed segregation distortion favoring *indica* allele) and nonD-SS (41 SS markers which showed no segregation distortion) and associated transmission was tested along association type (Table 4). There was no difference in frequency of significant association among association type classified with segregation distortion.

Genomic inclination of 30 varieties and DT RILs

Two subspecies of rice, *indica* and *japonica*, have their own specific traits obtained during differentiation process, some of which breeders have been intended to combine into a genotype to develop a promising variety through inter-subspecific hybridization. However, it has not been so successful due to reproductive barriers between two subspecies and subsequent suppressed recombination. There has been no measure to assess the genonic inclination of breeding lines derived from inter-subspecific crosses, which will be helpful to quantify the recombination of inter-subspecific genomes in a breeding line. We propose a concept "subspecies-prototype (SP) degree" as described in Materials and Methods. The SP degree of 30 varieties used for identifying SS STS markers were estimated as in Table 5. For example, when Gogowiere was tested with 67 SS markers, it generated 66 *japonica*-specific alleles and only one *indica*-spe-

Table 3. Significant intra- and inter-chromosomal association between different types of markers in DT RILs

Association type		Frequency	
		Intra-Chr [c]	Inter-Chr
SS vs SS [a]	Expected (A) [b]	196	1,820
	Observed (B)	116	47
	B/A (%)	59.2	2.6
SS vs non-SS	Expected (A)	810	9,046
	Observed (B)	318	75
	B/A (%)	39.3	0.8
non-SS vs non-SS	Expected (A)	1237	10,544
	Observed (B)	531	73
	B/A (%)	42.9	0.7
Total	Expected (A)	2243	21,410
	Observed (B)	965	195
	B/A (%)	43.0	0.9

a) SS: 64 subspecies-specific markers, non-SS: 154 non-subspecies-specific markers; SS vs SS: association between SS markers, SS vs non-SS: association between SS and non-SS markers, non-SS vs non-SS: association between non-SS and non-SS markers
b) Expected: all possible pairs between markers, Observed: marker pairs showed significant association
c) Intra-Chr: significant association between markers within chromosome, Inter-Chr: significant association between markers across chromosomes

Table 4. Significant intra- and inter-chromosomal association between different types of SS markers in DT RILs

Association type		Frequency	
		Intra-Chr [c]	Inter-Chr
D-SS vs D-SS [a]	Expected (A) [b]	35	407
	Observed (B)	19	5
	B/A (%)	54.3	1.2
D-SS vs nonD-SS	Expected (A)	65	1,699
	Observed (B)	37	18
	B/A (%)	56.9	1.1
nonD-SS vs nonD-SS	Expected (A)	90	1,460
	Observed (B)	60	24
	B/A (%)	66.7	1.6
Total	Expected (A)	190	3,566
	Observed (B)	116	47
	B/A (%)	61.1	1.3

a) D-SS: 23 SS markers showed segregation distortion, nonD-SS: 41 SS markers showed non-segregation distortion ; D-SS vs D-SS: association between D-SS markers, D-SS vs nonD-SS: association between D-SS and nonD-SS markers, nonD-SS vs nonD-SS: association between nonD-SS and nonD-SS markers
b) Expected: all possible pairs between markers, Observed: marker pairs showed significant association
c) Intra-Chr: significant association between markers within chromosome, Inter-Chr: significant association between markers across chromosomes

cific allele for S10013A. Then, the SP degree of Gogowiere was (66-1)/67=0.970, which means that approximately 97% of Gogowiere genome is of *japonica* origin. If SP degree of a variety is 1.0 or -1.0, the variety might be regarded as *japonica*- or *indica*-prototype variety, respectively. According to this concept, Tong88-7, Ilpumbyeo, Dongjinbyeo, Hapcheon1, Nagdongbyeo, Shingeumobyeo, Nipponbare, Hwacheonbyeo, Malagit sinaguing, B581A6, CP-SLO and Azucena will be the *japonica*-prototype varieties, and Dasanbyeo, Milyang23, IR36, IR64, and Tadukan will be the *indica*-prototype varieties. However, SP degree of Basmati370 was 0.109, which inclined toward rather the *japonica* side although it was tested in *indica* group based on its geographical origin.

A total of 166 DT-RILs were genotyped with 155 SSR and 64 SS STS markers, and genetic similarity degree to parents by SSR markers and SP degree of each line by SS markers was evaluated. Figure 3 shows the frequency distribution of genetic similarity degrees using (A) 76 SSR markers which revealed non-SD and (B) 79 SSR markers which revealed significant SD, and SP degrees using (C) 41 SSR markers which revealed non-SD and (D) 23 SSR markers which revealed SD (D). The average genetic similarity degree and SP degree estimated by non-SD markers and non-SD SS markers were -0.034 and -0.013, respectively, which were very similar to each other. When the DT-RILs were evaluated with (B) 79 SD-SSR markers and (D) 23 SD-SS markers, the average genetic similarity degree and SP degree were -0.190 and -0.239, respectively, which were also very similar to each other. However, SP degree graph based on SS markers exhibited wider-range distribution than the graph of genetic similarity degree based on general SSR markers.

Discussion

Identification of SS markers

A total of 765 STS markers, which were designed through the comparison of sequences between Nipponbare (*japonica*) and 93-11 (*indica*) at every 2~3 cM interval throughout genome, were tested for subspecies-subspecificity (SS) in rice, and 67 SS STS markers were identified. To determine the SS markers, two exceptions against 30 varieties, which was comparable to the SS score 93.3%, were allowed for subspecies-specificity. A lot of markers in most chromosomes showed SS scores very close to the threshold suggesting that if only a few varieties were used, the SS score of the unselected markers would be higher and subsequently might have been selected as SS markers. However, since 30 varieties used for screening were from various geographical origins, the SS markers identified in this study are expected to be quite concrete and widely applicable.

Why were the SS markers generated and maintained in a subspecies-specific manner? One possible explanation is that the specific alleles or allelic combinations that govern certain traits associated with subspecies differentiation (Oka, 1988). The second possible explanation is that physical linkage of alleles within chromosome may play an important role of generating SS markers. If differences in chromosome structure caused by inversions exist between two subspecies (Li et al 1997), the supergenes would be conserved in each subspecies without recombination. SS markers located in a clustered form on chromosome 3, 4, 7, 8, 9, 10, and 11 might be caused by this type of physical linkage. Further studies are in progress in relation to the origin of SS markers.

The fact that number of SS markers varied along chromosomes might reflect the relative importance of each chromosome in differentiation of subspecies in rice. That is, chromosomes possessing relatively high number of SS markers such as chromosome 3 and 9 might be more important in the process of subspecies differentiation. However, we assume that there should be more SS markers if we compare the sequences between *indica* and *japonica* varieties in a finer scale.

Table 5. SP (Subspecies-prototype) degree of 30 test varieties used for identification of SS markers

Japonica			*Indica*		
Name	Origin	SP degree	Name	Origin	SP degree
Ilpumbyeo	Korea	1.000	Dasanbyeo	Korea	-1.000
Dongjinbyeo	"	1.000	Milyang23	"	-1.000
Hapcheon 1	"	1.000	Chungcheongbyeo	"	-0.970
Nagdongbyeo	"	1.000	Hankangchalbyeo	"	-0.970
Shingeumobyeo	"	1.000	Nampungbyeo	"	-0.910
Hwacheongbyeo	"	1.000	China1039	China	-0.940
TR22183	China	0.881	IR36	IRRI	-1.000
Tong88-7	"	1.000	IR64	"	-1.000
Norin mochi 1	Japan	0.881	IR21015	"	-0.970
Nipponbare	"	1.000	New Sabramati	India	-0.970
Gogowiere	Indonesia	0.970	ARC10239	"	-0.940
Malagit sinaguing	Philippines	1.000	Basmati370	"	0.104
B581A6	"	1.000	Chinsurah Boro2	Bangladesh	-0.851
Azucena	"	1.000	Tadukan	Philippines	-1.000
CP-SLO	USA	1.000	Tetep	Vietnam	-0.910
Average		0.982	Average		-0.889

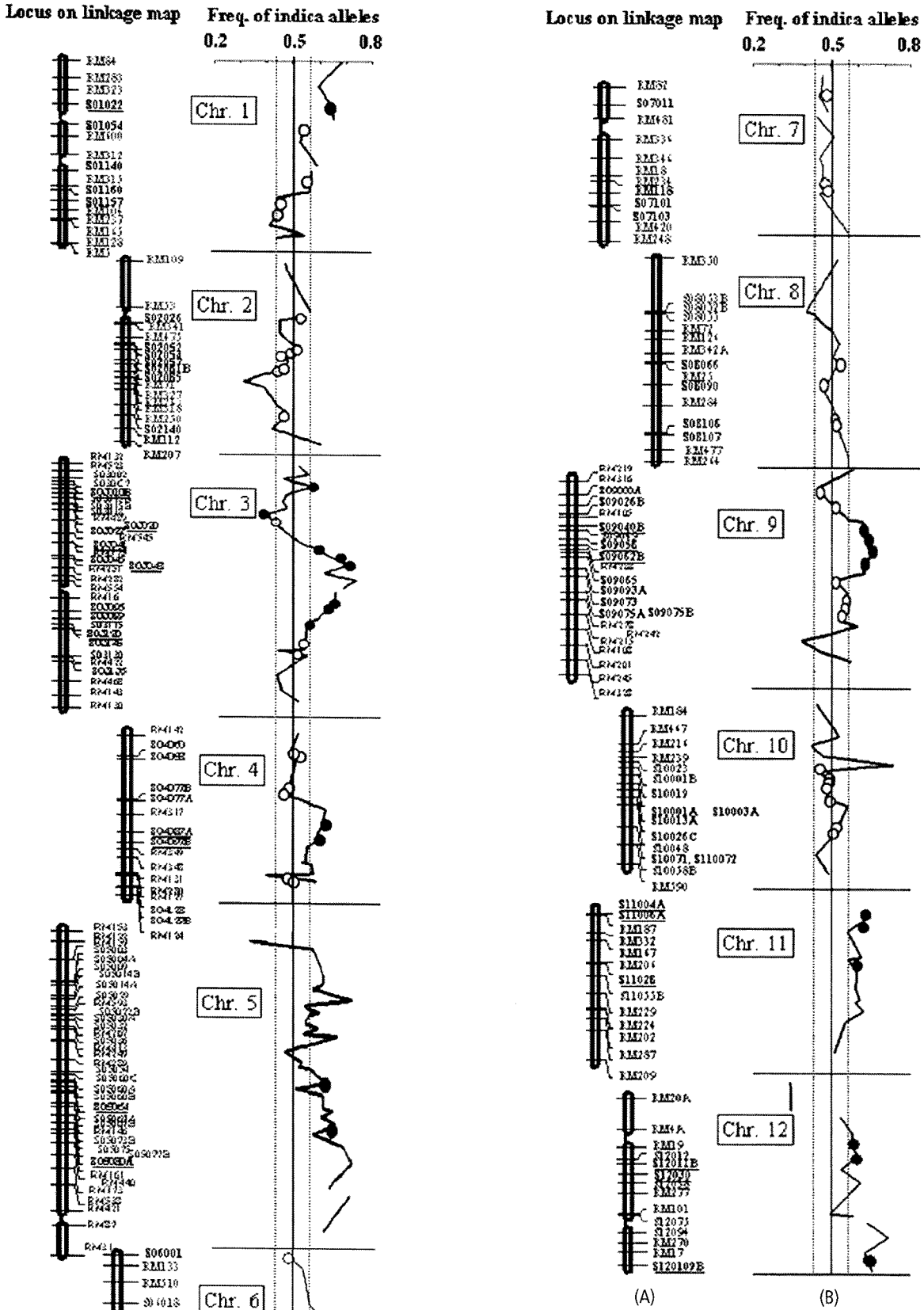

Fig. 2. Linkage map and segregation distortion of subspecies-specific (SS) markers. (A) Linkage map of SS markers in DT RIL. Large and bold STS markers are SS markers. Underlined markers are the SS markers which show significant segregation distortion. Thin solid lines show linkage gaps or regions with a distance >50 cM. (B) Segregation distortion of frame-map markers and SS markers in DT-RIL. Two dotted lines around y axis of 0.5 show both thresholds at 5% significance level for segregation distortion. Frame-map markers which show segregation distortion are positioned outside of both thresholds. Solid black dot: SS markers showing significant segregation distortion. Open dot : SS markers not showing segregation distortion

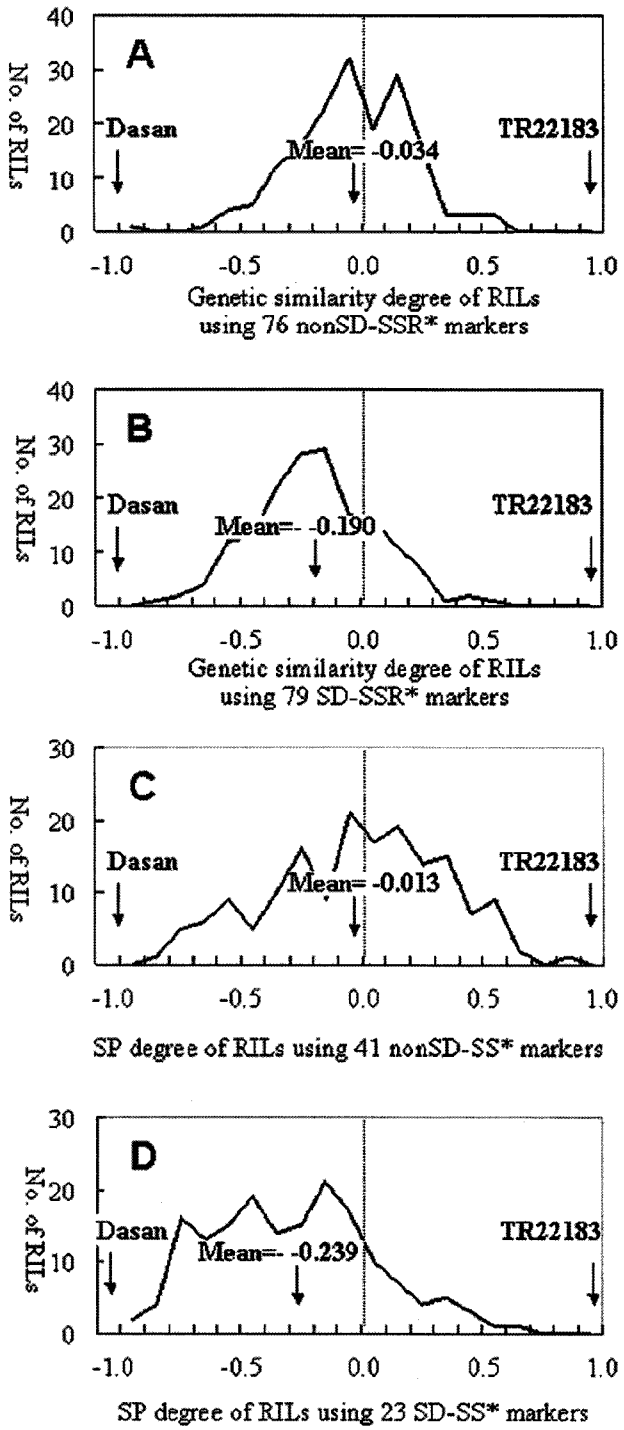

Fig. 3. Frequency distribution of DT-RILs based on (A and B) genetic similarity degree using non-subspecies-specific markers and (C and D) SP degree using subspecies-specific SS markers.

SS markers and reproductive barriers between subspecies

SD is one of the reproductive barriers that frequently occur in inter-subspecific crosses in rice (Harushima et al. 2001 and 2002). Of 64 SS markers mapped in this study, 23 markers showed significant SD, all of which favored *indica* alleles except only one marker S03020 on chromosome 3 (Figure 2(B)). Xu et al (1997) reviewed the possible reason of SD; i) differential transmission, ii) post-zygotic selection prior to genotypic evaluation, iii) male gametophytic selection, and iv) environmental effects. It is not clear what would be the cause of SD in this study. Anyway, since SD did not occur in all of the SS loci and the ratio of SD at SS marker loci was rather lower than at non-SS marker loci, SD might not be the cause of generating SS markers.

Chromosomal regions such as 0~50cM of chromosome 4, 10~30cM of chromosome 5, 0~40cM of chromosome 6, 20~50cM of chromosome 8, 70~110cM of chromosome 11, and 60~110cM of chromosome 12 contained no SS STS markers. Considering that some inter-subspecific hybrid sterility QTLs and S loci have been reported nearby these regions (Oka 1988; Li et al. 1997; Sorbizal et al. 2001; Song et al. 2005), the SS-STS markers seemed not to be directly associated with hybrid sterility between subspecies. However, STS markers were designed at 2.85cM interval on the average that it might not be informative enough to fully deduce the relationship between SS markers and hybrid sterility though hybrid sterility might be caused by large scale of polymorphism between parents, i.e. inversion (Tanksley et al. 1992; Rieseberg et al. 2000; Li et al. 1997). The relationship remains to be further studied in a finer scale.

Application of SS markers and SP degree

SS markers are a series of alleles specifically present in *indica* or *japonica* and a kind of key markers in terms of subspecies genomes in rice, and thus will be applicable to quantitatively assess the genomic inclination of breeding lines or germplasms toward *indica* or *japonica*. Varietal classification and evaluation of genetic relationship among varieties or varietal groups can be effectively conducted using SS markers. There have been a series of studies on the differentiation of subspecies and their relationship in rice (Garris et al. 2005; Londo et al. 2006; Ni et al 2002; Sun et al 2002; Vaughan et al 2003). However, all of them were implemented with a few markers or genes or unselected markers. The SS markers developed in this study are expected to be a good measure for that purpose because the SS markers would be key markers with respect to the subspecies differentiation and they were chosen from scanning whole chromosomes. The SS markers will also be applicable in hybridization breeding to combine desirable characters from both subspecies into an elite line through evaluation of genomic inclination of breeding lines.

The SP degree of the varieties used in this study except Basmati 370 was near to 1.0 (*japonica*) or -1.0 (*indica*) indicating that varietal classification by the concept is well fitted to the traditional classification by morphological traits or geographical origin. Furthermore, using this concept we will be able to quantify the genomic resemblance of varieties to either *japonica* or *indica*. In case of Basmati 370, the SP degree was 0.109 indicating the intermediate genomic constitution or rather close to *japonica*. This agrees to the results of Garris et al. (2005) that Basmati rices were independently clustered closed to the *japonica* group.

When 166 DT-RILs were genotyped with non-SD markers (Fig. 3), genetic similarity degree and SD degree of RILs were near to 0 regardless of subspecies-specificity of each marker, indicating that the non-SD markers were distributed quite evenly over RILs. However, genetic similarity degree and SP degree of

DT-RILs estimated with SD markers were conspicuously skewed toward the *indica* parent suggesting that *indica* alleles at SD marker loci were transmitted to DT-RILs at a higher frequency than *japonica* alleles as most of the SDs occurred favoring *indica* alleles. This preferential transmission of *indica* alleles seems to be the reason that mostly *indica* type progenies are produced more than *japonica* type progenies in hybridization breeding between indica and *japonica* rices (Oka, 1988). In this regard, provided that some desirable traits of each species are linked to the SD markers, breeding endeavor to recombine the traits into an elite line might have been barred. The linkage relationship between SD markers and subspecies-specific traits remains to be studied.

In addition, SP degree graph based on SS markers exhibited wider-range distribution than the graph of genetic similarity degree using general SSR markers (Fig.3). This indicates that SS markers are likely to magnify genomic inclination of RILs towards either *indica* or *japonica* and could be used as key markers to evaluate the genomic constitution of varieties.

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