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ORIGINAL PAPER

The contribution of intersubspecific hybridization to the breeding of super-high-yielding *japonica* rice in northeast China

Jian Sun · Di Liu · Jia-Yu Wang · Dian-Rong Ma · Liang Tang · Hong Gao · Zheng-Jin Xu · Wen-Fu Chen

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Abstract Hybridization between *indica* and *japonica* rice combined with utilization of ideal plant type has led to the development of high-yielding *japonica* rice in northern China. However, the contribution at the genomic level of intersubspecific hybridization to the increased yield of northern Chinese *japonica* rice is uncertain. In this study, we analyzed the genomic pedigree of descendants of hybridization between *indica* and *japonica* rice grown in northeastern China between 1963 and 2008. Simple sequence repeat markers indicated that since 1990 the genetic diversity among northern *japonica* cultivars was enriched. Genome-wide analysis with subspecies-specific indel and intron length polymorphism markers showed *indica*-allele frequencies were significantly increased in cultivars bred after 1990, and were significantly positively correlated with spikelet number per panicle and significantly negatively correlated with panicle number per plant. Among eight genes controlling agronomic traits, *GN1a* and *GS3* were partially fixed in the genome of northern *japonica* cultivars. In contrast, *Waxy* and *qSH1* were eliminated, whereas *DEP1* and *qSW5* were retained. *Indica* germplasm is an important contributor to the increased yield of northern *japonica* rice. Breeding for high yield and grain quality in combination is a complicated process and difficult to achieve when relying on only one or several functional genes, thus the selection expertise of the breeder remains critical.

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J. Sun · D. Liu · J.-Y. Wang · D.-R. Ma · L. Tang · H. Gao · Z.-J. Xu (✉) · W.-F. Chen (✉)
Key Laboratory of Northeast Rice Biology and Breeding, Ministry of Agriculture, Rice Research Institute, Shenyang Agricultural University, Dongling Road 120, Shenyang 110866, China
e-mail: xuzhengjin2011@hotmail.com

W.-F. Chen
e-mail: wfchen5512@yahoo.com.cn

J. Sun · D. Liu · J.-Y. Wang · D.-R. Ma · L. Tang · H. Gao · Z.-J. Xu · W.-F. Chen
Key Laboratory of Northern Japonica Rice Genetics and Breeding, Ministry of Education, Rice Research Institute, Shenyang Agricultural University, Dongling Road 120, Shenyang 110866, China

Introduction

Development of super rice was an important strategy to improve the production capacity of *japonica* rice in northern China compared with that achieved with dwarf and hybrid rice (Chen et al. 2007). After nearly a decade of cultivation, super rice accounts for more than 60 % of the total area under rice cultivation and has contributed an estimated two billion dollars to the Chinese national economy. *Indica* and *japonica* are two subspecies of Asian cultivated rice (*Oryza sativa* L.) and possess different biological characteristics. The strategy of breeding super-high-yielding rice involves the combination of a novel plant type with strong vigor in *indica–japonica* hybridization. The breeding of novel super rice is crucial to realize further increases in yield (Yang et al. 1984; Xu et al. 2004). Under the strategy of breeding super-high-yielding rice, many high-yielding and high-quality *japonica* cultivars have been bred. The successful breeding of high-yielding *japonica* cultivars has broken the domination

of Japanese cultivars, which were grown previously in northeast China.

The concepts of 'ideal plant type' and 'utilization of intersubspecific heterosis' are important in rice breeding (Chen et al. 2007). In recent years, isolation of the *DEP1* and *IPA1* genes (Huang et al. 2009; Wang et al. 2009; Jiao et al. 2010) has contributed to an improved understanding of the genetic and molecular bases of the 'ideal plant type' of high-yielding *japonica* rice. However, the contribution of intersubspecific hybridization to the high yields of northern *japonica* rice cultivars is not thoroughly understood.

In recent years, most novel rice cultivars bred in northern China were derived from hybridization of *indica* and *japonica*. The introduction of *indica* pedigree has expanded the gene pool of *japonica* cultivars and enabled further increases of yield in northern China. However, the identity and proportion of *indica* alleles incorporated in the genome of rice germplasm cultivated in northern China is unknown (Gu 2010). Recent studies of genome-wide variation in *Oryza* have provided a novel approach to address this question. Subspecies-specific indel and intron length polymorphism (SSILP) molecular markers have been developed that permit quantitative analysis of *indica* and *japonica* components of the rice genome (Shen et al. 2004; Wang et al. 2005; Lu et al. 2009; Zhao et al. 2009). In addition, isolation of genes that contribute to agronomically important traits, such as *GN1a*, *GS3*, *DEP1*, and *qSW5* provides an effective means to assess the contribution of *indica* alleles to *japonica* cultivars derived from hybridization between *indica* and *japonica* (Ashikari et al. 2005; Fan et al. 2006; Shomura et al. 2008; Huang et al. 2009).

In the present study, we used neutral simple sequence repeat (SSR) markers to analyze genetic diversity and relationships among *japonica* rice cultivars that were cultivated in northeast China in the period 1963–2008. By using subspecies-specific indel and SSILP markers, we determined the proportion of *indica* alleles in the *japonica* cultivars and analyzed the correlation between *indica* alleles and yield-related agronomic traits. By genotyping, the gene loci that contribute to yield and grain quality-related traits, we obtained molecular evidence for the contribution of hybridization between *indica* and *japonica* to rice breeding in northern China, and thus provide a theoretical basis for *japonica* rice breeding in the future.

Materials and methods

Plant materials

A total of 78 *japonica* rice (*Oryza sativa* L. subsp. *japonica*) cultivars that were cultivated widely in northeast China between 1963 and 2008 were selected for this study (Table S1). Of these cultivars, 25 were obtained from Heilongjiang province, 16 from Jilin province, 29 from Liaoning province, and 8 from Japan. In addition, 13 *indica* rice (*O. sativa* subsp. *indica*) cultivars were selected as control samples. All of the cultivars were grown at the Shenyang Agricultural University, Liaoning Province, China. The yield and grain quality-related traits, i.e. average panicle length, number of panicles per plant, number of spikelets per panicle, percentage seed set and 1,000 grain weight were measured in 2010 and 2011 for each cultivar.

DNA isolation and SSR, indel and SSILP genotyping

Fresh leaf tissue of each accession was frozen in liquid nitrogen and total genomic DNA was extracted using the Rapid DNA Extraction Kit (Tiangen, China). Sixty-five SSR markers (McCouch et al. 2002; Huang and Zhang 2003) were randomly selected to analyze the population structure and genetic diversity. Subspecies-specific indel and SSILP markers in typical *indica* and *japonica* rice cultivars were applied to analyze the proportion of *indica* alleles in the genome of each *japonica* cultivar. The clone names and physical distances for 34 indel and 55 SSILP markers were obtained from the International Rice Genome Sequencing Project (IRGSP) marker-based physical maps (http://www.rgp.dna.affrc.go.jp/E/IRGSP/download.html). The two marker types were integrated into one genetic map using Mapchart software based on the physical distances (Fig. 1). All of the indel and SSILP markers were used to genotype the 91 cultivars. The PCR reactions were performed in accordance with previously described procedures (Shen et al. 2004; Wang et al. 2005; Zhao et al. 2009). The primers are listed in Table S2.

Detection of the genotypes for yield and quality-related genes

We identified the genotypes of eight functional genes related to yield and grain quality for all 91 cultivars. The genes were those that control the spikelet number per panicle (*GN1a*), grain weight (*GS3*), grain width (*qSW5*), grain weight (*GW2*), plant architecture (*IPA1*), dense and erect panicle form (*DEP1*), high amylose content (*Waxy*), and shattering (*qSH1*). The indel molecular markers and separation of the PCR products by agarose gel electrophoresis were used to detect the genotype of genes that were changed by a fragment deletion between the wild type and mutant type. We used cleavage amplification polymorphism (CAPS) or derived cleavage amplification polymorphism (dCAPS) molecular markers to detect the genotype of functional genes that were changed by a single

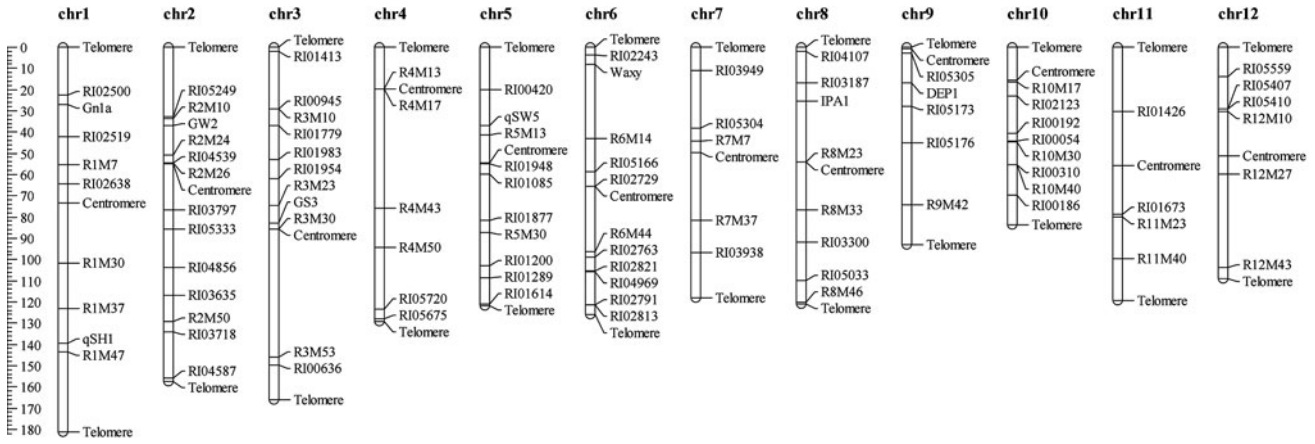

Fig. 1 Linkage map of subspecies-specific indel and SSILP markers as well as functional genes used in the study

Table 1 Molecular markers for yield and quality-related genes used in the study

Marker name	Primer (5′–3′)	Restriction enzyme	Product size (bp)	Marker type
Gn1a-M1F	CTCTTGCTTCATTATCAATC		129/113	Indel
Gn1a-M1R	AAACTACACAAGAATCTGC			
Gn1a-M2F	TGAGGATGCCGTGGAAGACG		172/161	Indel
Gn1a-M2R	TTCGTGTTCGCGCAGGACGT			
qSW5-F	CGTCTTGCAACCAACGCCGATGTTAT		759/1,971	Indel
qSW5-R	GAGCGTGTGTAGGGAAGGAGCTGCATGA			
DEP1-F	AAAGACCAAGGTGCCTCA		466/1,091	Indel
DEP1-R	TGGTTCAACCTCGTCTCATA			
Waxy-F	GCTTCACTTCTCTGCTTGTG	*ACC*1	403+57/460	CAPS
Waxy-R	ATGATTTAACGAGAGTTGAA			
qSH1-F	CAATACATGCATGGATGCGT	*Bsp*HI	379+339/718	CAPS
qSH1-R	TCCATATGTGTGTAGGACCC			
GS3-F	TATTTATTGGCTTGATTTCCTGTG	*Pst*I	294+218/512	CAPS
GS3-R	GCTGGTTTTTTACTTTCATTTGCC			
IPA1-F	CCTGCAGAGCAAGCTCAAGCTCAC	*Sdu*I	295+294/589	CAPS
IPA1-R	CCAGAAGAGCATCGCAGGTTCA			
GW2-F	AATAAAGATGTCCATTCTGTTA	*Hpa*I	23+151/174	dCAPS
GW2-R	GCTCTTCCTGTAACACATATTATG			

nucleotide polymorphism (SNP) between the wild type and mutant type. The primers are listed in Table 1.

Data analysis

The SSR data were used to evaluate genetic structure among the northern Chinese *japonica* cultivars. POPGENE 1.31 software (Yeh 1997) was used to estimate genetic distance, mean number of alleles per locus (N_a), effective number of alleles per locus (N_e), and Shannon diversity index (I). A phylogenetic tree was constructed using the neighbor-joining method based on Nei's (1972) genetic distances using PowerMarker 3.25 software (Liu and Muse 2005) and MEGA 4.0 software (Tamura et al. 2007). STRUCTURE 2.1 software (Pritchard et al. 2000) was used to examine the population structure. Using a burn-in length of 1,000 steps followed by a run length of 1,000 Monte Carlo Markov Chain replicates, the number of subgroups from $k = 1$ to $k = 10$ were tested with a model that assumed admixture and correlated allele frequencies for the simulation.

To investigate genomic pedigree, each PCR band showing polymorphism among the analyzed cultivars was scored as either AA (Nipponbare-type), BB (9311-type), CC (other *indica*-type) or AB (heterozygote) for all indel and SSILP loci. The *indica* allelic frequency (F_i) and heterozygous locus frequency (H_i) of each cultivar were calculated. A correlation analysis was performed to

examine the relationship between *indica*-allele frequency and yield and grain quality-related traits with SPSS 17.0 software (SPSS, Inc., Chicago, USA).

Results

Genetic diversity among rice cultivars grown in northeast China

A total of 188 alleles were detected among the 78 *japonica* cultivars with the 65 SSR markers. The number of alleles per locus ranged from 1 to 7 (average 2.89) among the marker loci. The number of alleles per locus and the Shannon diversity index for the cultivars from Liaoning were the highest among the three provinces, whereas the effective number of alleles per locus (N_e) was highest in the cultivars from Heilongjiang (Table 2). Genetic diversity among cultivars from Jilin was significantly lower than among cultivars from Liaoning and Heilongjiang.

The level of genetic diversity among the cultivars bred in different periods also differed (Table 3). The number of alleles per locus and Shannon diversity index of the cultivars bred after 1990 was higher than those bred prior to 1990. These results indicated that because of utilization of *indica* rice as a germplasm resource, increased genetic diversity among the cultivars grown in northeast China was achieved in the last two decades.

Genetic relationships among rice cultivars cultivated in northeast China

The LnP(D) value showed a peak when $k = 2$ and α values fluctuated distinctly, which demonstrated that the *japonica* cultivars could be divided into two groups (Fig. 2). The genetic constitution of the cultivars from Heilongjiang was distinct from that of the cultivars from Liaoning and Jilin, whereas the cultivars from the latter two provinces were genetically similar.

Table 2 Genetic diversity among rice cultivars grown in the three provinces of northeast China

Province	Genetic diversity index		
	Number of alleles per locus	Effective number of alleles per locus	Shannon diversity index
Heilongjiang	2.28 ± 1.18aA	1.50 ± 0.68aA	0.43 ± 0.39aA
Jilin	1.86 ± 0.84bB	1.42 ± 0.68aA	0.34 ± 0.33Bb
Liaoning	2.49 ± 1.24aA	1.48 ± 0.73aA	0.44 ± 0.41aA

Within each column, the uppercase and lowercase letters indicate a significant difference at the 1 and 5 % levels, respectively

Table 3 Genetic diversity among cultivars bred in different periods

Period	Genetic diversity index		
	Number of alleles per locus	Effective number of alleles per locus	Shannon diversity index
2001–2008	2.63 ± 1.17aA	1.55 ± 0.71aA	0.49 ± 0.38aA
1991–2000	2.40 ± 1.13bB	1.52 ± 0.70aA	0.45 ± 0.39bA
Before 1990	1.81 ± 0.83cC	1.38 ± 0.52aB	0.33 ± 0.35aB

Within each column, the uppercase and lowercase letters indicate a significant difference at the 1 and 5 % levels, respectively

The neighbor-joining tree indicated that the cultivars from Liaoning, Jilin and Heilongjiang provinces represented independent groups (Fig. 3a). When the cultivars were classified into three groups based on the time of cultivar registration, cultivars from each province were relatively evenly distributed throughout the dendrogram (Fig. 3b), which reflected irregularity in the genetic constitution of the cultivars bred in different decades. The clustering of cultivars by geographical origin reflected selection on the basis of ecological conditions, whereas the clustering of cultivars by time of origin reflected artificial selection during rice breeding. Thus, the dominant factor that determined population structure among rice cultivars grown in northeast China was ecological adaptability.

Analysis of *indica* pedigree of northeast China cultivated rice

The subspecies-specific indel and SSILP markers used in the present study were distributed among the 12 chromosomes in the rice genome (Fig. 4). We calculated the *indica*-allelic frequency for five groups of cultivars. The rank order of the groups from the maximum to minimum frequency of *indica* alleles was *indica* (0.848) > Liaoning (0.0764) > Jilin (0.0608) > Heilongjiang (0.0366) > Japan (0.0112).

The frequency of *indica* alleles on chromosomes 5 and 6 was higher than that on all other chromosomes for all *japonica* cultivars. The frequency of *indica* alleles on chromosomes 1, 2, 10 and 11 for Liaoning cultivars was significantly higher than that for Jilin and Heilongjiang cultivars, whereas on chromosome 9 Heilongjiang cultivars possessed a significantly higher frequency of *indica* alleles than Liaoning cultivars. The frequency of *indica* alleles for cultivars bred between 2001 and 2008 was 0.061, whereas the frequency was 0.057 for those bred between 1991 and 2000 and 0.04 for those bred before 1990. These results are consistent with the historical timing of *indica–japonica* hybridizations and showed that the proportion of *indica* alleles differed among the three provinces.

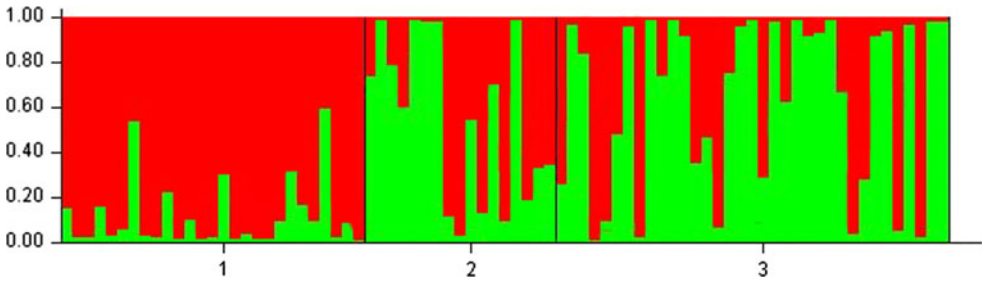

Fig. 2 Population structure obtained by analysis with the STRUCTURE program. Two clusters ($k = 2$, indicated by *green* and *red shading*) were obtained from the simulation using all 78 *japonica* cultivars based on 65 SSR markers. Most Heilongjiang cultivars (*1*) were assigned to the *red group*, and most Liaoning and Jilin cultivars (*2*, *3*) were assigned to the *green group*

Relationship between traits of yield components and frequency of *indica* alleles in *japonica* cultivars

The panicle length, number of panicles per plant, number of spikelets per panicle, percentage seed set, and 1,000-grain weight were recorded for all cultivars in 2010 and 2011. Correlation analysis showed that the frequency of *indica* alleles and spikelet number per panicle were significantly positively correlated ($r = 0.53$, $p < 0.01$), and the allelic frequency was significantly negatively correlated with number of panicles per plant ($r = -0.38$, $p < 0.01$). A non-significant correlation was observed between the frequency of *indica* alleles and percentage seed set ($r = 0.08$), 1,000-grain weight ($r = -0.14$), and yield ($r = 0.15$).

Utilization of elite genes in *japonica* cultivars in northeast China

GN1a can increase the grain number per panicle. Most *japonica* cultivars did not carry this allele (Yan et al. 2009). In the present study, only two *japonica* cultivars carried *GN1a*, which suggested the allele was introduced into *japonica* cultivars in northeast China by selection (Table S3). Although breeders have selected consciously for specific phenotypes, *GN1a* is not completely fixed in the *japonica* cultivars because grain number is a quantitative trait and whether the original *indica* parents carried the elite allele is difficult to trace. The erect panicle gene, *DEP1*, also regulates grain number. We found that 62 % of the cultivars that carried *DEP1* were cultivated in Liaoning, but rarely in the other two provinces (Table 4). *IPA1* changes plant architecture and enhances rice yield (Jiao et al. 2010). Loss of function of *GW2* enhances grain width, weight and yield (Song et al. 2007). We found that the *japonica* cultivars still carried the wild-type alleles of *IPA1* and *GW2* (Table 4), which demonstrated that these two elite alleles have not been utilized in rice breeding in northeast China.

The *qSW5/GW5* locus was detected in most *japonica* cultivars and a few *indica* cultivars that show the wide-grain phenotype (Shomura et al. 2008; Weng et al. 2008). All of the analyzed *japonica* cultivars that were bred by *indica–japonica* hybridization retained the *japonica* allele by selection (Table 4). *GS3* is a quantitative trait locus for grain size that was isolated from the *indica* cultivar Minghui 63. Many *indica* cultivars carry the same allele as Minghui 63, whereas the allele is rare in *japonica* rice (Yan et al. 2009). We found that 96 % of the analyzed *japonica* cultivars carried the Minghui 63 allele (Table 4), which indicated that *GS3* was a major locus and suitable for a *japonica* genetic background, and the locus has become fixed in northern *japonica* cultivars by selection.

In most *japonica* cultivars the mutant *waxy* gene plays an important role in reduction of the amylose content in the rice grain, whereas the mutant gene is not widely present in *indica* cultivars (Olsen et al. 2006). The SNP mutation in the promoter region of *qSH1* causes loss of the *japonica* shattering phenotype, but the mutation is absent in *indica* rice (Konishi et al. 2008). We found that all *japonica* cultivars retained the *japonica*-type allele at the *Waxy* and *qSH1* loci (Table 4), which reflected negative selection against the allele controlling the opposite phenotype in *indica–japonica* hybrid breeding.

Discussion

Enhancement of genetic diversity among northeast Chinese rice cultivars by *indica–japonica* hybridization

From the 1950s to the 1980s, 97.2 % of the *japonica* cultivars grown in northeast China derived from a handful of Japanese cultivar parents, which led to an inevitable annual decline in genetic diversity (Qi et al. 2006). The present research shows that the genetic diversity of *japonica* cultivars grown in northeast China was greatly enhanced in the 1990s, which coincided with the application of

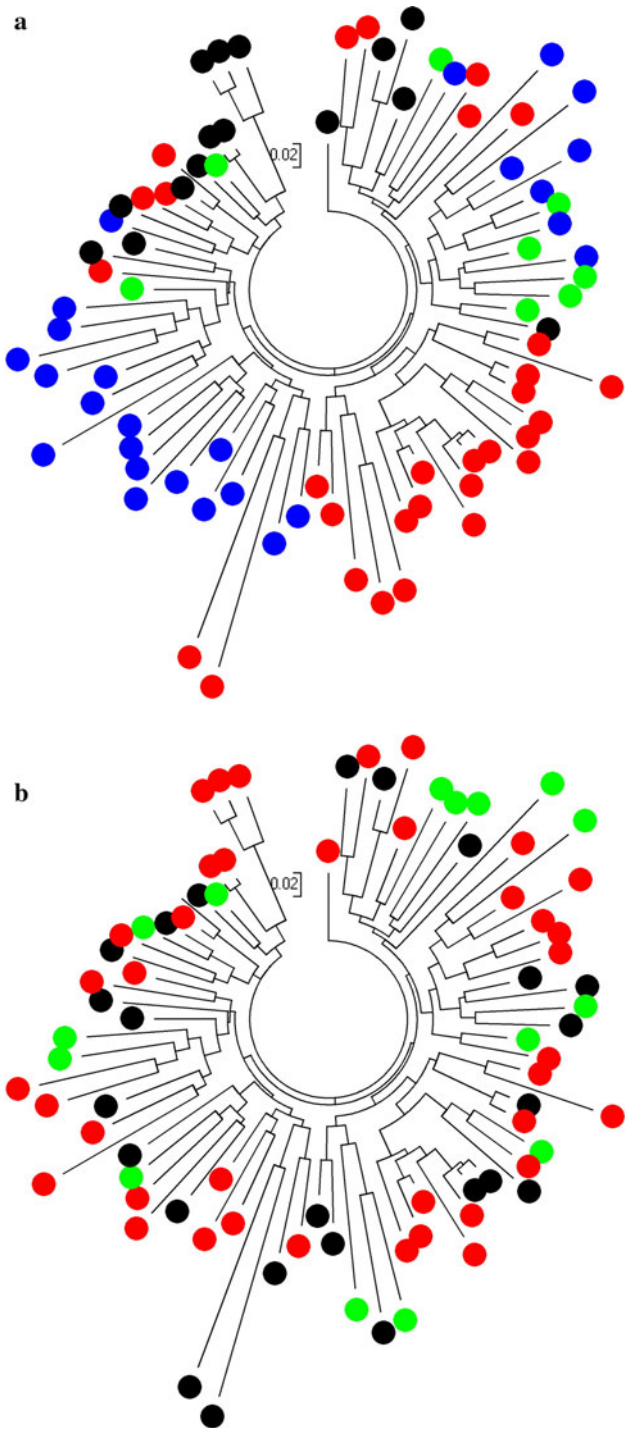

Fig. 3 Neighbor-joining tree of 78 rice cultivars derived from Nei's (1972) genetic distances between the cultivars from **a** Liaoning, Heilongjiang, Jilin and Japan, which are indicated in *red*, *blue*, *black* and *green*, respectively; and **b** different registration periods, where cultivars bred after 2000 are indicated in *red*, those bred from 1991 to 2000 in *black*, and those bred before 1990 in *green*

indica–japonica hybridization in rice breeding in northern China. We found that the frequency of *indica* alleles in cultivars increased significantly over time, especially in the cultivars that were bred after 1990. This result implied that *indica–japonica* hybridization has made an important contribution to the enhancement of genetic diversity of cultivated rice in northeast China.

The present research demonstrated that the introduction of *indica* pedigree caused differentiation in the genetic structure of *japonica* rice cultivated in northeast China. Whether the two rice subspecies have a single or multiple origins is still under debate (Londo et al. 2006; Ge and Sang 2011; Molina et al. 2011), but it is certain that the *indica* and *japonica* genomes are the consequence of long-term ecological adaptation. Severe natural selection pressure caused by the extreme ecological conditions in northern China created a strong genetic bottleneck, which lead to the genomic diversity of the *japonica* cultivar population and the genetic differentiation of the cultivars from the three provinces of northeast China. In future breeding programs, it is advisable to introduce a certain proportion of *indica* genes, on the basis of its different ecological adaptability, to achieve the genomic integration of both rice subspecies and thus maximize the adaptability of cultivars to different ecological environments in northern China.

Introduction of elite *indica* alleles to improve the morphological and physiological characteristics of *japonica* cultivars

Hybridization between *indica* and *japonica* is an effective breeding strategy to create improved rice germplasm (Chen et al. 2007). The present research demonstrated that the frequency of *indica* alleles showed a highly significant positive correlation with grain number per spike, which helped to further understand the contribution of *indica* alleles to the breeding of *japonica* cultivars in northeast China. The number of vascular bundles in the stem of *indica* rice is higher than that of *japonica* rice, and consequently the number of primary branches in *japonica* rice is lower than that of *indica* rice, which limits the maximum number of grains per spike in *japonica* rice (Xu et al. 2004). Therefore, we inferred that the contribution of *indica* germplasm might increase the number of vascular bundles and thus increase the grain number in *japonica* rice.

We found that a certain proportion of *indica* alleles and the genes that control grain weight, i.e. *GN1a* and *GS3*, had been introduced by breeders into northeast Chinese *japonica* cultivars to improve yield, and the inherent *japonica* elite alleles *qsw5* and *dep1* were retained. In terms of grain quality, consumers in northern China prefer the taste of sticky rice and thus a breeding objective is to eliminate the high-amylose starch gene *Waxy* carried by *indica–japonica* hybrids or recombinants. To accommodate the harvesting

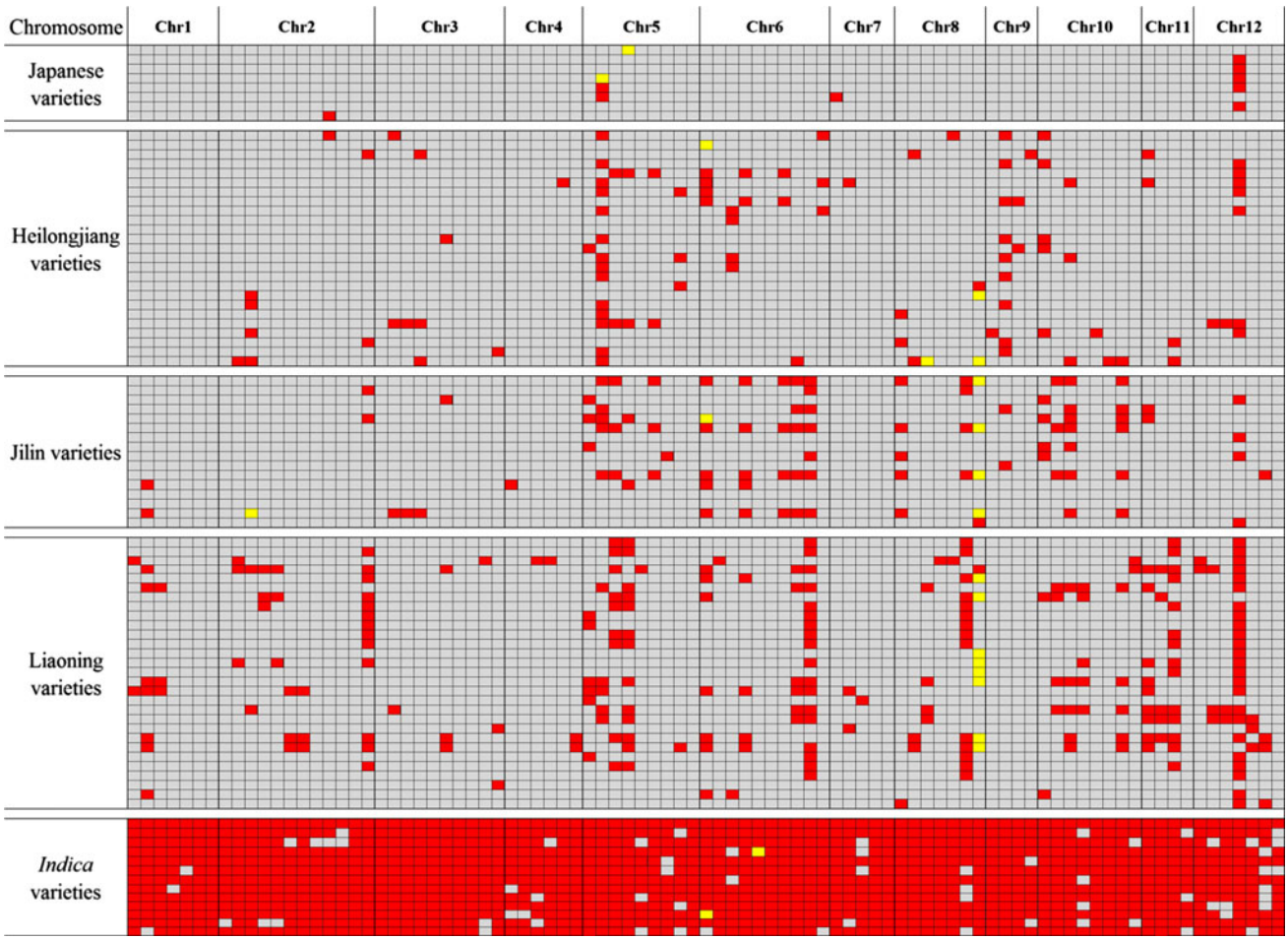

Fig. 4 Genotype pattern for 89 loci that differentiate *indica* and *japonica* rice for the 12 chromosomes in the analyzed cultivars from different regions. A *gray square* indicates a *japonica* allele, a *red square* indicates an *indica* allele, and a *yellow square* indicates a heterozygous site

Table 4 Frequency of mutation type for the yield and quality-related genes

Groups	Grain number *GN1a*	Plant architecture *DEP1*	Plant architecture *IPA1*	Grain weight *GW2*	Grain weight *GS3*	Grain weight *qSW5*	Shattering *qSH1*	Quality *Waxy*
Japan (8)	0.13	0.00	0.00	0.00	0.88	1.00	1.00	1.00
Heilongjiang (25)	0.00	0.04	0.00	0.00	0.96	1.00	1.00	1.00
Jilin (16)	0.00	0.06	0.00	0.00	0.94	1.00	1.00	1.00
Liaoning (29)	0.07	0.65	0.00	0.00	1.00	0.97	1.00	1.00
Indica (13)	0.54	0.00	0.00	0.00	0.85	0.54	0.00	0.31

The number in parentheses indicates the sample size

habits of local residents, the non-shattering allele *qsh1*, which originated in *japonica* rice, was retained, whereas the shattering allele *qSH1* from the *indica* genome was eliminated by selection. In recent years, a number of yield-related genes in rice have been cloned. Some of these genes such as *GS3* and *GN1a*, which are common in *indica* rice, have already been completely or partly fixed in northern *japonica* cultivars. *IPA1* and *GW2* were isolated from mutants and play important roles in increased yield of their cloned parents (Song et al. 2007; Jiao et al. 2010). Although these two genes have not been utilized in the breeding of *japonica* cultivars in northern China, these genes might help to improve grain yield by marker-assisted selection in the future. On one hand, elite *indica* alleles have not been fully utilized in northern *japonica* rice, thus there is great potential to use them for future breeding of *japonica* rice. On the other hand, some inherent morphological and physiological traits of *japonica* rice have been

changed by functional genes, which may not be suitable for a *japonica* genomic background and ecological environments in northeast China. Thus, achieving a balance in the proportion of *indica* alleles in the *japonica* background in accordance with the experience of the breeder and breeding objectives is of utmost importance.

Although the average frequency of *indica* alleles in the whole genome of cultivars grown in Liaoning province was significantly higher than that of cultivars from Jilin and Heilongjiang, the frequency of *indica* alleles on chromosome 9 in Heilongjiang cultivars was significantly higher than that of cultivars grown in Liaoning province. The reason for this might be because most Liaoning cultivars (62 % in the present study) carried the inherent *japonica* gene *dep1* on chromosome 9, whereas this gene was rare in Heilongjiang cultivars, which led to the divergence during the artificial selection of *dep1*. Decay of linkage disequilibrium in rice is less than 200 kb (Huang et al. 2010) and the marker density used in the present study was much lower than this level. High-density SNP haplotype maps are required to test this hypothesis in the future.

Future development of breeding through hybridization between *indica* and *japonica*

Rice breeding is a complicated process with the goals of achieving high yield as well as high grain quality. It is difficult to achieve both objectives when relying on one or several genes, thus the selection expertise of the breeder is critical. The present study confirmed the appropriateness of the breeding strategy for *japonica* rice in northern China using molecular and population genetic techniques. The breeding strategy in future to achieve further increase in yield of *japonica* rice should be to introduce an appropriate proportion of *indica* alleles into the genome, on the basis of adaptability to local environmental conditions, to fix and integrate elite *indica* alleles in the genome directly and rapidly by means of transgenic, genome-wide marker-assisted selection.

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