Theor Appl Genet (2016) 129:563–575
DOI 10.1007/s00122-015-2648-0

ORIGINAL ARTICLE

Stacking *S5-n* and *f5-n* to overcome sterility in *indica–japonica* hybrid rice

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Received: 28 September 2015 / Accepted: 30 November 2015 / Published online: 24 December 2015

Abstract

Key message **Pyramiding of *S5-n* and *f5-n* cumulatively improved seed-setting rate of *indica–japonica* hybrids, which provided an effective approach for utilization of inter-subspecific heterosis in rice breeding.**

Abstract Breeding for *indica–japonica* hybrid rice is an attractive approach to increase rice yield. However, hybrid sterility is a major obstacle in utilization of inter-subspecific heterosis. Wide-compatibility alleles can break the fertility barrier between *indica* and *japonica* subspecies, which have the potential to overcome inter-subspecific hybrid sterility. Here, we improved the compatibility of an elite *indica* restorer line 9311 to a broad spectrum of *japonica* varieties, by introducing two wide-compatibility alleles, *S5-n* and *f5-n*, regulating embryo-sac and pollen fertility, respectively. Through integrated backcross breeding, two near isogenic lines harboring either *S5-n* or *f5-n* and a pyramiding line carrying *S5-n* plus *f5-n* were obtained, with the recurrent parent genome recovery of 99.95, 99.49, and 99.44 %, respectively. The three lines showed normal fertility when crossed to typical *indica* testers. When testcrossed to five typical *japonica* varieties, these lines allowed significant increase of compatibility with constant agronomic performance. The introgressed *S5-n* could significantly improve 14.7–32.9 % embryo-sac fertility in *indica–japonica* hybrids. In addition, with the presence of *f5-n* fragment, *S5-n* would increase the spikelet fertility from 9.5 to 21.8 %. The introgressed *f5-n* fragment greatly improved anther dehiscence, embryo-sac and pollen fertility in *indica–japonica* hybrids, thus leading to improvement of spikelet fertility from 20.4 to 30.9 %. Moreover, the pyramiding line showed 33.6–46.7 % increase of spikelet fertility, suggesting cumulative effect of *S5-n* and *f5-n* fragment in seed-set improvement of inter-subspecific hybrids. Our results provided an effective approach for exploiting heterosis between *indica* and *japonica* subspecies, which had a profound implication in rice breeding.

Introduction

As a main staple food, increase of rice yield is of immense importance to feed the world population. However, the yield growth rate for rice has been declining during the last decade, contrasting to the continuing increase of populations in major rice-consuming countries (Jeon et al. 2011). Therefore, improved strategies for further increase in grain yield are essential. Asian cultivated rice (*Oryza sativa* L.) comprises two main subspecies, *indica* and *japonica*. Utilization of intra-subspecific heterosis had achieved great success and contributed considerably to rice yield increase. Nevertheless, *indica–japonica* hybrids show stronger heterosis than intra-subspecific hybrids (Zhang et al. 1996; Zhao et al. 1999), which may have the potential to produce

Communicated by T. Sasaki.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-015-2648-0) contains supplementary material, which is available to authorized users.

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higher rice yield. Recently, breeders have infiltrated the *japonica* genetic material into the genetic background of typical *indica* varieties for the breeding of indicaclinous restorer lines. These indicaclinous strains showed compatibility to *japonica* cytoplasm male sterility lines, and were designed for successful breeding of some *indica–japonica* hybrid combinations (Lin et al. 2012; Lu et al. 2007), illustrating the feasibility of partial utilization of *indica–japonica* heterosis. However, breeding for typical *indica–japonica* hybrids is mainly hindered by inter-subspecific hybrid sterility.

A special group of rice germplasm, known as wide-compatibility varieties (WCVs), is able to break the reproductive barrier between *indica* and *japonica* subspecies. The WCVs bearing neutral allele (*S-n*) at hybrid sterility locus are able to produce fertile hybrids when crossed to both *indica* varieties carrying *S-i* and *japonica* varieties with *S-j*. Both WCV/*indica* (*S-n*/*S-i*) and WCV/*japonica* (*S-n*/*S-j*) hybrids have normal fertility, whereas *indica*/*japonica* (*S-i*/*S-j*) hybrid is semi-sterile (Ikehashi and Araki 1984, 1986). Thus, the neutral allele *S-n* can be exploited to improve the seed-setting rate of *indica–japonica* hybrids (Ouyang et al. 2010).

Many factors caused *indica–japonica* hybrid sterility, such as female gametes abortion (Chen et al. 2008; Yang et al. 2012), male gametes abortion (Long et al. 2008), anther indehiscence (Maekawa et al. 1997; Zhang et al. 2004), as well as reduced affinity between the uniting gametes (Liu et al. 2004) and non-synchronization of male and female gamete development in the same spikelet (Liu et al. 1997). In addition, adverse environmental conditions would also reduce the fertility of inter-subspecific hybrids (Li et al. 1997). Genetic analyses have identified a series of loci controlling hybrid embryo-sac and pollen fertility in different *indica–japonica* crosses (Ouyang et al. 2009), and four *indica–japonica* hybrid sterility loci, *S5*, *Sa, DPL1* and *DPL2* have been further cloned (Chen et al. 2008; Long et al. 2008; Mizuta et al. 2010; Yang et al. 2012).

The *S5* and *f5* loci were identified as two major loci regulating hybrid female and male sterility, respectively. The major locus *S5* conferring embryo-sac fertility was regulated by a killer–protector system of three tightly linked genes (*ORFs3–5*). The neutral allele *S5-n* would be compatible with typical *indica* haplotype *ORF3+ORF4−ORF5+* and typical *japonica* haplotype *ORF3−ORF4+ORF5−*, such as *ORF3−ORF4−ORF5n* from *aus* variety Dular and *ORF3+ORF4+ORF5n* from *japonica* variety 02428 (Chen et al. 2008; Yang et al. 2012). Apart from *S5-n*, the neutral allele *f5-n* rescuing pollen fertility had been fine mapped in the same *aus* variety Dular (Wang et al. 2006). However, pyramiding of neutral alleles regulating both hybrid female and male fertility was still lacking in breeding for inter-subspecific hybrid rice, which was of specific interest for breeders.

Molecular marker-assisted backcross breeding was the most effective way for integration of superior alleles into an elite variety. This strategy was widely applied in rice breeding program involving foreground selection and background selection (Chen et al. 2000, 2001; Khanna et al. 2015; Zhou et al. 2003). Moreover, this strategy has been well applied for pyramiding alleles at hybrid embryo-sac sterility loci (Chen et al. 2011). Therefore, it provided opportunity for exploiting the maximum heterosis of *indica–japonica* hybrids, by the transfer of multiple neutral alleles with minimum donor segment into elite typical *indica* or *japonica* varieties. However, traditional marker-assisted breeding had almost relied on RFLP, AFLP, and SSR (Chen et al. 2000, 2001, 2011; Zhou et al. 2003), which were time-consuming and inconvenient. Today, the development in rice genome sequence made molecular markers available for any region of the genome (Zhao et al. 2015). Recently, two SNP arrays of RICE6K and RiceSNP50 with high-density markers covering rice 12 chromosomes have been developed (Chen et al. 2014; Yu et al. 2014), which would speed up the progress of molecular breeding for rice genetic improvement.

As an elite male parent of *indica–indica* hybrids, 9311 was the first sequenced variety in *indica* subspecies. However, it was highly incompatible with *japonica* varieties. The present study attempted to establish an effective molecular breeding system in rice and produce several wide-compatibility lines for subsequent breeding practices, including (1) incorporation of the two neutral alleles, *S5-n* and *f5-n*, regulating hybrid female and male fertility, respectively, into 9311 via integrated backcross breeding strategy including phenotypic selection in early backcross generation, positive and negative selection of target region, and whole genome background selection, and (2) compatibility evaluation of neutral alleles in different *indica–japonica* heterozygous genetic background and conditions. This study would facilitate the utilization of strong heterosis between *indica* and *japonica* subspecies, which had a profound implication in rice breeding.

Materials and methods

Plant materials

Two rice varieties, 9311 and Dular, were used as the parents in this breeding program. 9311 was used as the recipient, which was an elite *indica*-type restorer line developed by Jiangsu Academy of Agriculture Science in China. Dular was used as the donor, which was a landrace *aus* variety

from India conferring a wide spectrum and high level of wide-compatibility (Wang et al. 1998).

Evaluation in wide-compatibility requires testcross experiment with a broad set of tester lines. Here, five typical *japonica* varieties, Laoguangtou83, AnnongwangengB-2, Balilla, Taichung65 and Nipponbare from four countries and three typical *indica* varieties, Nanjing11, IR64 and Guangluai4 from two countries were used as the testers. Nanjing11 and Balilla have been widely used in wide-compatibility breeding programs in China (Gu et al. 1991). IR64 and Taichung65 have been used as the testers in IRRI (International Rice Research Institute) (Kumar and Virmani 1992). Ikehashi and Araki (1984) used Nipponbare as a *japonica* tester in Japan. In addition, based on genome sequencing database of rice core germplasm (http://ricevarmap.ncpgr.cn/), Laoguangtou83 and AnnongwangengB-2 were regarded as typical *japonica* varieties, whereas Guangluai4 belonged to typical *indica* subspecies. Therefore, these three varieties were selected as the testers in this study.

DNA markers and high-throughput arrays

Eight polymorphism insertion/deletion (InDel) markers between 9311 and Dular were developed based on a comprehensive database of rice genomic variations (http://ricevarmap.ncpgr.cn/) (Table S1). Two *S5* co-segregating markers, S5ORF3 and S5ORF5, were used to detect the presence of *S5-n* allele (Yang et al. 2012). Besides, two markers, S5F70 and S5R240, flanking both sides of *S5* locus at 70 and 240 kb, were used to negatively select the linkage segments around *S5* region. The selection for *f5-n* was conducted using two flanking markers, f5F46 and f5R43, which were located 46 and 43 kb away from the upper and lower border of *f5* locus (Li et al. 2006; Wang et al. 2006) (Fig. 1a). Besides, two markers of C1F79 and C1R79 were developed to clean up the genetic background. In addition, two high-throughput arrays, RICE6K and RiceSNP50, containing 5102 and 51,478 InDel/SNP markers, respectively, were used to analyze the genetic background of intermediate materials and the improved lines (Chen et al. 2014; Yu et al. 2014).

DNA extraction and genotyping

Genomic DNA was extracted from fresh leaves of the 10-day-old seedlings following the protocol described by Murray and Thompson (1980). Polymerase chain reaction (PCR) was performed in a MyCycler™ thermal cycler (Bio-Rad, USA), with 20 μl reaction mixture containing 2 μl of genomic DNA (10 ng/μl), 2 μl of 10 × buffer, 0.2 μl of each primer (10 μM), 1.4 μl of $MgCl_2$ (25 mM), 2 μl of dNTP (2 mM), 0.2 μl of Taq polymerase (5 U/μl),

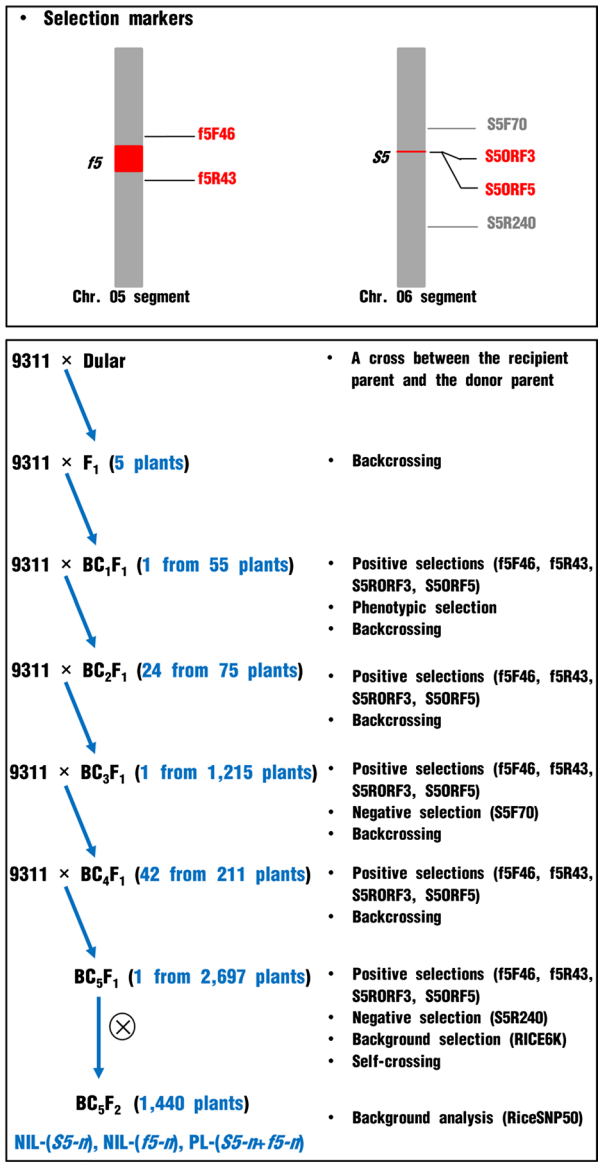


Fig. 1 Breeding scheme for the development of NILs and PL. **a** The *red words* indicate the markers for positive selection, and the *gray words* indicate the markers for negative selection. **b** The procedure for the development of NIL-(*S5-n*), NIL-(*f5-n*) and PL-(*S5-n* + *f5-n*)

and 12 μl ddH_2O. The PCR amplification condition was with one cycle of denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 40 s, with a final extension at 72 °C for 5 min. The amplified product of marker S5ORF5 showed 136 bp difference between 9311 and Dular, which was examined by 1 % agarose gel electrophoresis. The PCR products from other markers showing small InDel variations between the two parents were examined in 4 % polyacrylamide gel with a higher resolution (Table S1). DNA of selected rice plants was analyzed by RICE6K for genetic background selection. The genetic background of three improved lines,

which contained homozygous *S5-n*, *f5-n*, and *S5-n* plus *f5-n* by introgression, respectively, was detected using mixing DNA of 20 individuals from each line. For genome-wide genotyping, DNA amplification, fragmentation, chip hybridization, single-base extension, staining and scanning were conducted by Life Science and Technology Center, China National Seed Co. LTD (Wuhan, China), according to Infinium HD Assay Ultra Protocol (Chen et al. 2014; Yu et al. 2014).

The crossing and selection scheme

The overall breeding scheme followed a recurrent backcross procedure including five generations of backcross and one generation of selfing, combined with whole genome selection strategy, as shown in Fig. 1b. A cross was made between the recipient 9311 and the donor Dular. In backcrossing generations from BC_1F_1 to BC_4F_1, selected individuals heterozygous at both *S5* and *f5* loci were backcrossed to 9311. Stringent phenotypic selection was conducted in BC_1F_1, including four agronomic traits of heading date, plant height, tiller number per plant and glume color. Negative selections on *S5* were carried out in BC_3F_1 and BC_5F_1. In BC_5F_1, the recombinants double-heterozygous at both *S5* and *f5* loci were subjected to background selection using whole genome array RICE6K. Subsequently, the BC_5F_2 population was developed from an individual in BC_5F_1 with the highest proportion of genome recovery of recurrent parent. Meanwhile, the dragged chromosomal segment on the genetic background was selected negatively in BC_5F_2, wherein two near isogenic lines (NILs) harboring either *S5-n* or *f5-n* and a pyramiding line (PL) carrying *S5-n* plus *f5-n* were obtained. Finally, these NILs and PL were further confirmed by whole genome array RiceSNP50 for higher resolution (Chen et al. 2014; Yu et al. 2014).

Compatibility tests

The obtained NILs and PL were testcrossed to three *indica* testers and five *japonica* testers, respectively. The progeny of testcrosses were sowed on May 15th and May 25th, 2014, respectively, at the experimental farm of Huazhong Agricultural University, Wuhan, China. Here, the two sowing dates were described as *S1* and *S2*, respectively. The 25-day-old seedlings were transplanted. Each of the plots consisted of three rows with 10 plants per row at a planting density of 16.7 cm between plants in a row, and 20.0 cm apart between rows. Field management followed the normal agricultural practices.

Trait measurements

Three plants per testcross F_1 were used to investigate the anther dehiscence, the embryo-sac fertility, and the pollen fertility. For anther dehiscence, approximately 150 anthers from flowering panicles of three plants per testcross F_1 were mixed and examined under the dissection microscope. Following the method of Zhang et al. (2004), each anther was given a score of 1 (indehiscent) to 5 (completely dehiscent) based on the degrees of anther dehiscence. The anther dehiscence was evaluated by anther dehiscence index (ADI), defined as

$$\mathrm{ADI} = \frac{\sum(\text{Score} \times \text{number of anthers with the correspinding score})}{\text{total number of anthers.}}$$

The anther with the ADI less than 3 was considered as abnormal.

For embryo-sac and pollen fertility, approximately 200 florets from the middle and upper parts of three normal panicles per plant were collected 1–2 days before flowering, and were fixed immediately in FAA (formaldehyde: acetic acid: 50 % ethanol = 5: 6: 89) for at least 24 h. The florets were then washed with 50 % ethanol and stored in 70 % ethanol at 4 °C (Zeng et al. 2007). Embryo-sac fertility was investigated using the whole-stain clearing method (Song et al. 2005). The egg apparatus was not clear enough using this method. However, because of the low frequency of embryo-sac abortion due to egg apparatus (Zeng et al. 2007), the error was in an acceptable range. Approximately, 150 ovaries per plant were collected for examination in embryo-sac fertility. Meanwhile, pollen grains of six florets per plant were mixed and stained with 1 % I_2–KI solution. Three independent sets were scored, with at least 100 pollen grains from different microscope fields per set. The fully stained grains were estimated as fertile, and the partially stained or unstained ones were defined as abortion by visual inspection. Overall pollen fertility was estimated from the mean proportion of the stained pollen.

For spikelet fertility, five plants in the middle of the central row in each plot were investigated. At maturity, all normal panicles per plant were harvested for examination in the percentage of filled grains.

Evaluation of agronomic performance of the improved lines

Agronomic performance of the obtained NILs and PL was evaluated and compared to the recurrent parent 9311 at Wuhan in the summer of 2014 and at Hainan in the spring of 2015. For the test under each condition, all materials were planted in a randomized complete block design with two replications. The field planting and management followed the procedure mentioned above. The heading date of each line was recorded. For this study, days to heading was referred to as days from the sowing date to the heading date, when the percentage of heading culms reached to the 50 % of total number of fertile tillers in all plants from

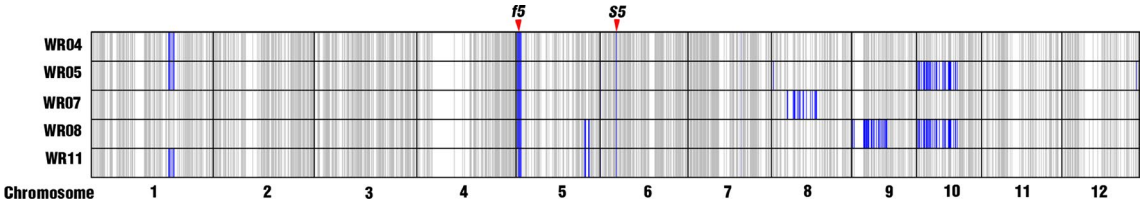


Fig. 2 Whole genome background selection based on five individuals in BC_5F_1 by RICE6K array. Twelve chromosomes of rice are labeled from *1* to *12*. The reference genome is Nipponbare (rice TIGR6.1). The *red triangles* indicate the positions of two target loci, *f5* on chromosome 5 and *S5* on chromosome 6, respectively. The *blue lines* indicate the SNP loci with heterozygous genotypes where genomic fragments of the donor parent Dular were introgressed, and the *gray lines* indicate the SNP loci with the same genotypes as the recipient parent 9311. The plant *WR04* had the maximum genome recovery of the recurrent parent with only three fragments from Dular, including two target loci, *S5* and *f5*, and an additional fragment on chromosome 1

the entire plot. At maturity, five plants in the middle of the central row in each plot were taken and measured for agronomic traits, including plant height, tillers per plant, panicle length, number of spikelets per panicle, spikelet fertility, weight of 1000 grains, and yield per plant.

Data analysis

Statistical analysis was conducted using SPSS statistics 17.0 for Windows (IBM, Armonk, NY, USA). Tests for ADI, pollen, embryo-sac, and spikelet fertility of the test-cross F_1s were performed using one-way ANOVA with the error from plants, and the Fisher's Least Significant Difference (LSD) test was used for multiple mean comparisons. The two-tailed *t* test was used for comparing agronomic traits of each improved line with the control 9311.

Results

Positive and negative selections for development of NILs and PL

Two important hybrid sterility loci, *S5* and *f5*, were confirmed to control embryo-sac and pollen fertility in *indica–japonica* hybrids, respectively (Chen et al. 2008; Wang et al. 2006; Yang et al. 2012). Here, two neutral alleles of *S5-n* and *f5-n* identified in Dular were transferred into an elite *indica* restorer line 9311.

The positive and negative selections were conducted following the described breeding scheme (Fig. 1a, b). In BC_1F_1, 21 plants were identified as containing *S5-n* and *f5-n*, and one individual with the agronomic traits identical to 9311 in heading date, plant height, tiller number per plant and glume color was selected by the conventional phenotypic selection. This plant was subsequently backcrossed to 9311. In BC_2F_1 of 75 plants, 24 individuals containing *S5-n* and *f5-n* were selected to backcross to 9311 by mixing pollen, and a large BC_3F_1 population of 1215 plants was constructed. In BC_3F_1, 223 plants containing *S5-n* and *f5-n* were positively selected, followed by negative selection at *S5*. One plant with homozygous 9311 (recurrent parent) genetic background at marker S5F70 was obtained and subsequently backcrossed to 9311. In BC_4F_1 of 211 plants, 42 individuals containing *S5-n* and *f5-n* were selected to backcross to 9311 by mixing pollen, and a large BC_5F_1 population of 2697 plants was obtained. Similarly, five plants carrying *S5-n* and *f5-n* were selected in BC_5F_1, with homozygous 9311 genetic background at marker S5R240. Therefore, the introgressed segment carrying *S5-n* from the donor parent was narrowed to <326.6 kb in the improved lines. Because of the lack of information in *f5* candidate gene, only positive selection was carried out at *f5* locus (Fig. 1a, b).

Whole genome background selection

The five selected plants were assayed for their genetic backgrounds using the whole genome array RICE6K (Fig. 2). This array contained a total of 1554 SNP markers showing polymorphisms between 9311 and Dular, with the average density of 1 SNP marker per 240 kb. The plant WR04 was found to have the maximum genome recovery of the recurrent parent (99.39 %) with only one additional fragment from Dular. This plant was self-pollinated to produce BC_5F_2. In BC_5F_2 of 1440 plants, with the elimination of the additional fragment using two negative markers C1F79 and C1R79, 20 individuals homozygous for *S5-n*, 24 individuals homozygous for *f5-n*, and 21 individuals homozygous for *S5-n* plus *f5-n* were, respectively, selected as the improved versions of 9311, designated as NIL-(*S5-n*), NIL-(*f5-n*), and PL-(*S5-n* + *f5-n*), respectively (Fig. 1b).

The genetic backgrounds of three improved lines were further confirmed by the RiceSNP50 array with higher resolution (Fig. S1). A total of 12,524 polymorphism SNP markers were available between 9311 and Dular, with the average density of 1 SNP marker per 30 kb. Introgression

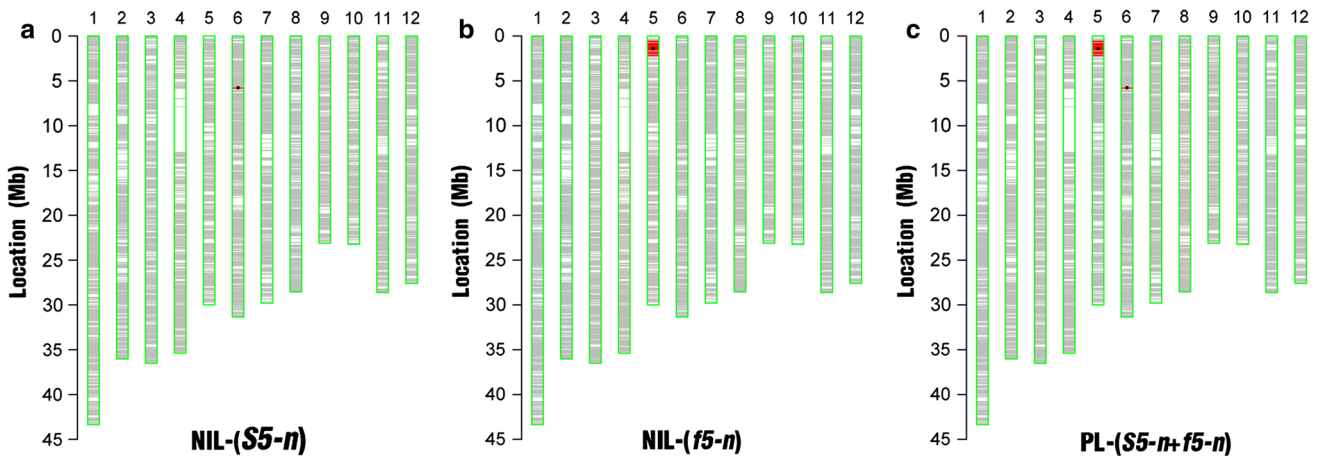


Fig. 3 Genetic background analysis of the three improved lines detected by RiceSNP50 array. **a** NIL-(*S5-n*), **b** NIL-(*f5-n*), and **c** PL-(*S5-n* + *f5-n*). The *black dots* indicate the positions of two target loci, *f5* on chromosome 5 and *S5* on chromosome 6, respectively. The *red lines* indicate the SNP loci with homozygous genotypes where genomic fragments of the donor parent Dular were introgressed, and the *gray lines* indicate the SNP loci with the same genotypes as the recipient parent 9311

fragment compassing *f5-n* was narrowed within 1809.9 kb via random recombination during the backcross process based on whole genome genotyping result from RiceSNP50. Thus, in all, the recurrent parent genome recoveries of NIL-(*S5-n*), NIL-(*f5-n*) and PL-(*S5-n* + *f5-n*) were 99.95, 99.49, and 99.44 %, respectively, without any additional segments (Fig. 3). These results indicated that whole genome background selection based on high-throughput array greatly increased the efficiency and precision of backcrossing.

Compatibility of improved lines to *japonica* testers

To confirm the potential usefulness of *S5-n* and *f5-n* in *indica–japonica* hybrid rice, NILs and PL were testcrossed to five *japonica* testers, respectively, to comprehensively evaluate the wide-compatibility using ADI, embryo-sac fertility, pollen fertility, and spikelet fertility in two conditions (*S1* and *S2*).

Effects of f5-n introgression in indica–japonica hybrids

All testcross F_1s derived from NIL-(*f5-n*) had normal anther dehiscence, significantly higher embryo-sac, pollen, and spikelet fertility than F_1s derived from 9311. Increase of embryo-sac, pollen, and spikelet fertility of NIL-(*f5-n*) ranged from 9.3 to 33.2 %, 22.0 to 51.5 %, and 18.8 to 37.0 %, respectively, compared with the control 9311 (Table 1).

The *indica–japonica* testcross F_1s with single-allele *f5-n* showed white anthers (Fig. 4b) and normal anther dehiscence in the natural field, compared to the ones without *f5-n* showing yellow anther (Fig. 4a) due to the insufficient release of pollen resulting from abnormal anther dehiscence. We further investigated the theca-opening degree of anther and evaluated the ADI of testcross F_1s under stereo microcopy. When testcrossed to five *japonica* testers, the average ADI of testcross F_1s carrying single-allele *f5-n* (4.2 in *S1*, 4.4 in *S2*) showed significant difference from those without neutral alleles (2.6 in *S1*, 2.7 in *S2*). No significant difference was observed between two conditions (Fig. 5a). These results indicated that the anther of *indica–japonica* hybrids dehisced normally to pour sufficient pollen on the stigma when *f5-n* was transferred, while the anther dehisced abnormally with the absence of *f5-n*. One exception was that 9311 also showed normal anther dehiscence when testcrossed with Taichung 65 (Table 1).

The average pollen fertility of the *indica–japonica* testcross F_1s carrying single-allele *f5-n* (82.9 % in *S1*, 84.6 % in *S2*) was much higher than ones without neutral alleles (46.9 % in *S1*, 54.5 % in *S2*) (Fig. 5b). Interestingly, the testcross F_1s with *f5-n* exhibited no significant difference in pollen fertility between the two conditions, whereas those without *f5-n* showed significant difference. Overall, *f5-n* significantly improved the pollen fertility of *indica–japonica* testcrossing F_1s (Fig. 4c, d). When testcrossed to AnongwangengB-2, Balilla, and Taichung65, NIL-(*f5-n*) produced normal level of pollen fertility (>85 %) under one of the two conditions (Table 1).

Notably, the NIL-(*f5-n*) also improved embryo-sac fertility significantly, which was even better than our expectation. The average embryo-sac fertility of *indica–japonica* testcross F_1s with fragment of *f5-n* (59.9 % in *S1*, 73.4 % in *S2*) was significantly higher than those lacking neutral alleles (50.3 % in *S1*, 56.2 % in *S2*) (Fig. 5c). Normal embryo-sacs contained two obvious polar nuclei locating

Table 1 Anther dehiscence index, embryo-sac, pollen, and spikelet fertility of the testcross F_1s with the eight testers

Testers	NILs/PL	Sowing on May 15 (*S1*)				Sowing on May 25 (*S2*)			
♂	♀	ADI	PF (%)	EF (%)	SF (%)	ADI	PF (%)	EF (%)	SF (%)
Laoguangtou	NIL-(*S5-n*)	1.5	39.4 ± 14.0	67.8 ± 5.6	15.6 ± 6.0	1.2	50.3 ± 1.8	67.6 ± 6.8	14.6 ± 3.4
	NIL-(*f5-n*)	3.4	82.1 ± 3.2	56.8 ± 9.6	41.9 ± 4.4	3.2	75.3 ± 0.9	65.2 ± 10.8	47.4 ± 10.1
	PL-(*S5-n* + *f5-n*)	3.9	83.4 ± 4.6	84.1 ± 3.8	61.2 ± 7.8	3.6	74.5 ± 1.8	88.1 ± 0.7	56.4 ± 8.5
	9311(CK)	1.7	34.5 ± 9.0	41.5 ± 3.2	14.5 ± 3.9	1.4	48.7 ± 2.6	44.8 ± 4.3	18.9 ± 6.1
	LSD(0.05)*	–	16.5	11.45	7.72	–	3.56	12.71	10.02
	LSD(0.01)**	–	24.00	16.66	10.63	–	5.18	18.50	13.81
AnnongwangengB-2	NIL-(*S5-n*)	2.4	63.5 ± 0.9	71.4 ± 6.3	32.4 ± 1.8	2.8	58.1 ± 5.7	73.6 ± 6.6	37.8 ± 5.1
	NIL-(*f5-n*)	4.3	85.0 ± 2.4	61.8 ± 5.9	50.8 ± 5.6	4.5	86.0 ± 2.4	66.6 ± 9.9	62.7 ± 4.6
	PL-(*S5-n* + *f5-n*)	4.2	85.6 ± 2.5	75.3 ± 1.7	59.2 ± 6.9	4.6	84.5 ± 1.4	77.3 ± 7.2	75.3 ± 4.0
	9311(CK)	2.1	63.0 ± 6.0	47.8 ± 5.3	23.4 ± 6.3	2.7	59.8 ± 4.1	47.6 ± 11.2	41.7 ± 5.4
	LSD(0.05)*	–	6.55	9.61	7.41	–	7.07	16.75	6.41
	LSD(0.01)**	–	9.53	13.98	10.21	–	10.29	24.37	8.84
Balilla	NIL-(*S5-n*)	2.0	52.1 ± 4.1	86.1 ± 2.0	33.6 ± 12.5	2.4	47.4 ± 4.7	82.1 ± 3.0	20.0 ± 4.6
	NIL-(*f5-n*)	4.1	86.5 ± 0.8	62.5 ± 4.3	52.0 ± 3.6	4.7	87.3 ± 1.3	55.4 ± 4.9	40.6 ± 0.9
	PL-(*S5-n* + *f5-n*)	4.1	88.3 ± 2.6	96.4 ± 1.5	73.9 ± 2.1	4.9	83.1 ± 1.0	97.0 ± 2.6	62.3 ± 3.1
	9311(CK)	2.4	55.1 ± 4.7	53.2 ± 5.0	31.0 ± 9.1	2.5	43.8 ± 6.7	56.3 ± 5.9	20.8 ± 1.6
	LSD(0.05)*	–	6.37	6.69	11.06	–	7.87	8.13	3.91
	LSD(0.01)**	–	9.27	9.73	15.24	–	11.45	11.82	5.39
Taichung65	NIL-(*S5-n*)	4.1	31.7 ± 5.9	76.7 ± 3.8	31.8 ± 9.3	3.8	61.8 ± 2.9	85.8 ± 5.4	19.7 ± 7.7
	NIL-(*f5-n*)	4.7	79.7 ± 2.9	58.4 ± 8.3	62.4 ± 11.4	4.8	93.2 ± 3.6	85.3 ± 6.5	58.4 ± 9.3
	PL-(*S5-n* + *f5-n*)	4.8	80.0 ± 2.0	83.0 ± 2.9	74.5 ± 3.5	4.7	93.4 ± 1.0	93.9 ± 1.5	65.3 ± 3.6
	9311(CK)	4.2	28.5 ± 7.7	59.0 ± 6.4	37.7 ± 6.2	3.7	61.5 ± 1.4	71.1 ± 3.4	21.4 ± 6.4
	LSD(0.05)*	–	9.70	10.82	10.95	–	4.66	8.69	9.48
	LSD(0.01)**	–	14.12	15.74	15.09	–	6.78	12.65	13.07
Nipponbare	NIL-(*S5-n*)	2.3	53.4 ± 3.5	75.2 ± 2.0	26.7 ± 5.6	2.5	60.0 ± 3.3	87.6 ± 5.5	40.7 ± 4.4
	NIL-(*f5-n*)	4.3	80.9 ± 6.2	60.2 ± 3.0	46.1 ± 7.5	4.6	81.3 ± 3.6	94.4 ± 0.3	48.8 ± 4.5
	PL-(*S5-n* + *f5-n*)	4.6	79.0 ± 3.1	94.5 ± 3.0	57.9 ± 3.4	4.9	78.9 ± 2.3	94.6 ± 1.0	68.4 ± 2.8
	9311(CK)	2.9	53.2 ± 2.9	49.9 ± 3.6	16.1 ± 3.3	3.0	58.5 ± 4.6	61.2 ± 8.1	30.0 ± 1.6
	LSD(0.05)*	–	7.84	5.53	7.00	–	6.67	9.26	4.78
	LSD(0.01)**	–	11.41	8.05	9.65	–	9.71	13.47	6.58
Nanjing 11	NIL-(*S5-n*)	4.9	93.7 ± 0.9	96.6 ± 3.1	89.5 ± 2.2	4.7	97.8 ± 0.7	98.0 ± 1.7	80.7 ± 1.4
	NIL-(*f5-n*)	4.9	94.5 ± 0.9	94.9 ± 1.3	87.5 ± 5.0	4.8	97.4 ± 0.4	98.0 ± 0.1	77.7 ± 1.3
	PL-(*S5-n* + *f5-n*)	4.9	94.8 ± 3.2	96.7 ± 2.3	89.0 ± 2.0	4.7	96.8 ± 1.1	97.6 ± 0.7	76.5 ± 3.9
	9311(CK)	4.9	95.2 ± 2.1	96.3 ± 1.3	86.0 ± 0.8	4.9	96.8 ± 1.6	98.8 ± 1.2	77.1 ± 2.2
	LSD(0.05)*	–	3.76	4.05	5.10	–	1.97	2.06	4.19
	LSD(0.01)**	–	5.48	5.89	7.03	–	2.87	2.99	5.78
IR64	NIL-(*S5-n*)	4.5	93.6 ± 0.3	98.0 ± 1.0	90.2 ± 1.9	4.5	93.8 ± 2.1	95.1 ± 0.6	88.6 ± 1.1
	NIL-(*f5-n*)	4.6	94.6 ± 1.4	96.6 ± 1.3	90.8 ± 2.1	4.5	93.2 ± 2.5	95.8 ± 4.0	84.8 ± 2.3
	PL-(*S5-n* + *f5-n*)	4.6	93.8 ± 0.4	98.4 ± 1.0	90.3 ± 3.4	4.2	93.5 ± 2.4	98.0 ± 1.9	89.2 ± 1.7
	9311(CK)	4.6	95.4 ± 0.9	97.2 ± 1.6	89.3 ± 4.2	4.4	93.0 ± 1.8	98.5 ± 1.5	84.8 ± 1.2
	LSD(0.05)*	–	1.64	2.36	5.27	–	4.18	4.71	3.46
	LSD(0.01)**	–	2.38	3.43	7.26	–	6.09	6.85	4.77

above the egg apparatus and a group of antipodal cells at the chalazal end, which can be easily distinguished from the abortive ones (Fig. 4e). Four types of abnormal embryo-sacs were observed in *indica–japonica* testcross F_1s, including degeneration of embryo-sac (Fig. 4f, g), abnormal location of polar nuclei (Fig. 4h), more than two polar nuclei (Fig. 4i), and small embryo-sac (Fig. 4j). Apparently, the testcross F_1s with *f5-n* fragment in *S2*

Table 1 continued

Testers	NILs/PL	Sowing on May 15 (*S1*)				Sowing on May 25 (*S2*)			
♂	♀	ADI	PF (%)	EF (%)	SF (%)	ADI	PF (%)	EF (%)	SF (%)
Guangluai 4	NIL-(*S5-n*)	4.8	89.5 ± 2.5	97.5 ± 0.6	–	5.0	88.0 ± 4.2	98.7 ± 0.6	–
	NIL-(*f5-n*)	4.8	87.9 ± 2.3	97.4 ± 3.6	–	5.0	86.8 ± 3.3	98.8 ± 0.7	–
	PL-(*S5-n* + *f5-n*)	4.9	85.3 ± 1.2	99.2 ± 0.3	–	5.0	89.3 ± 3.1	97.5 ± 1.9	–
	9311(CK)	5.0	88.4 ± 5.2	97.3 ± 2.0	–	4.9	86.9 ± 3.7	97.8 ± 0.8	–
	LSD(0.05)*	–	5.97	4.23	–	–	7.27	2.14	–
	LSD(0.01)**	–	8.68	6.16	–	–	10.57	3.12	–

Pollen, embryo-sac, and spikelet fertility are measured as mean ± SD. Asterisk and double asterisk represent least significant difference at 0.05 and 0.01 probability levels, respectively

ADI anther dehiscence index, *PF* pollen fertility, *EF* embryo fertility, *SF* spikelet fertility

showed significantly different embryo-sac fertility from *S1* (Fig. 5c). These result confirmed that fragment with *f5-n* improved embryo-sac fertility in *indica–japonica* hybrids, and the effect of *f5-n* fragment in female fertility depended on environmental conditions. Notably, the embryo-sac fertility of the testcross F_1s with Nipponbare reached to a normal level of 94.4 % in *S2*, with the increase of 33.2 % compared to 9311. One exception was that NIL-(*f5-n*) showed little effect in embryo-sac fertility when testcrossed to Balilla (in *S2*) and Taichung65 (in *S1*) (Table 1).

The average spikelet fertility of the *indica–japonica* testcross F_1s with single-allele *f5-n* (50.7 % in *S1*, 51.6 % in *S2*) exhibited significantly higher spikelet fertility than those derived from 9311 (24.5 % in *S1*, 26.0 % in *S2*) (Fig. 5d), which indicated that single-allele *f5-n* improved spikelet fertility in *indica–japonica* hybrids (Fig. 4k).

Effects of S5-n introgression in indica–japonica hybrids

NIL-(*S5-n*) improved embryo-sac fertility in *indica–japonica* hybrids, with the effect ranging from 14.7 to 32.9 %. The average embryo-sac fertility of F_1s with single-allele *S5-n* (75.4 % in *S1*, 79.3 % in *S2*) was significantly higher than those lacking neutral alleles (50.3 % in *S1*, 56.2 % in *S2*). In addition, unlike the environmental depended effect of *f5-n* fragment, the effect of *S5-n* in embryo-sac fertility was stable in different environmental conditions (Fig. 5c). However, the average spikelet fertility of the *indica–japonica* testcross F_1s with single-allele *S5-n* (28.0 % in *S1*, 26.6 % in *S2*) was not improved (Figs. 4k, 5d). Only when testcrossed to Nipponbare, NIL-(*S5-n*) exhibited significantly higher spikelet fertility than 9311 under both of the two conditions (Table 1).

Besides, NIL-(*S5-n*) exhibited no effect in anther dehiscence and pollen fertility. The testcross F_1s carrying single-allele *S5-n* showed no significant difference in either the average ADI (2.5 in *S1*, 2.5 in *S2*) or pollen fertility

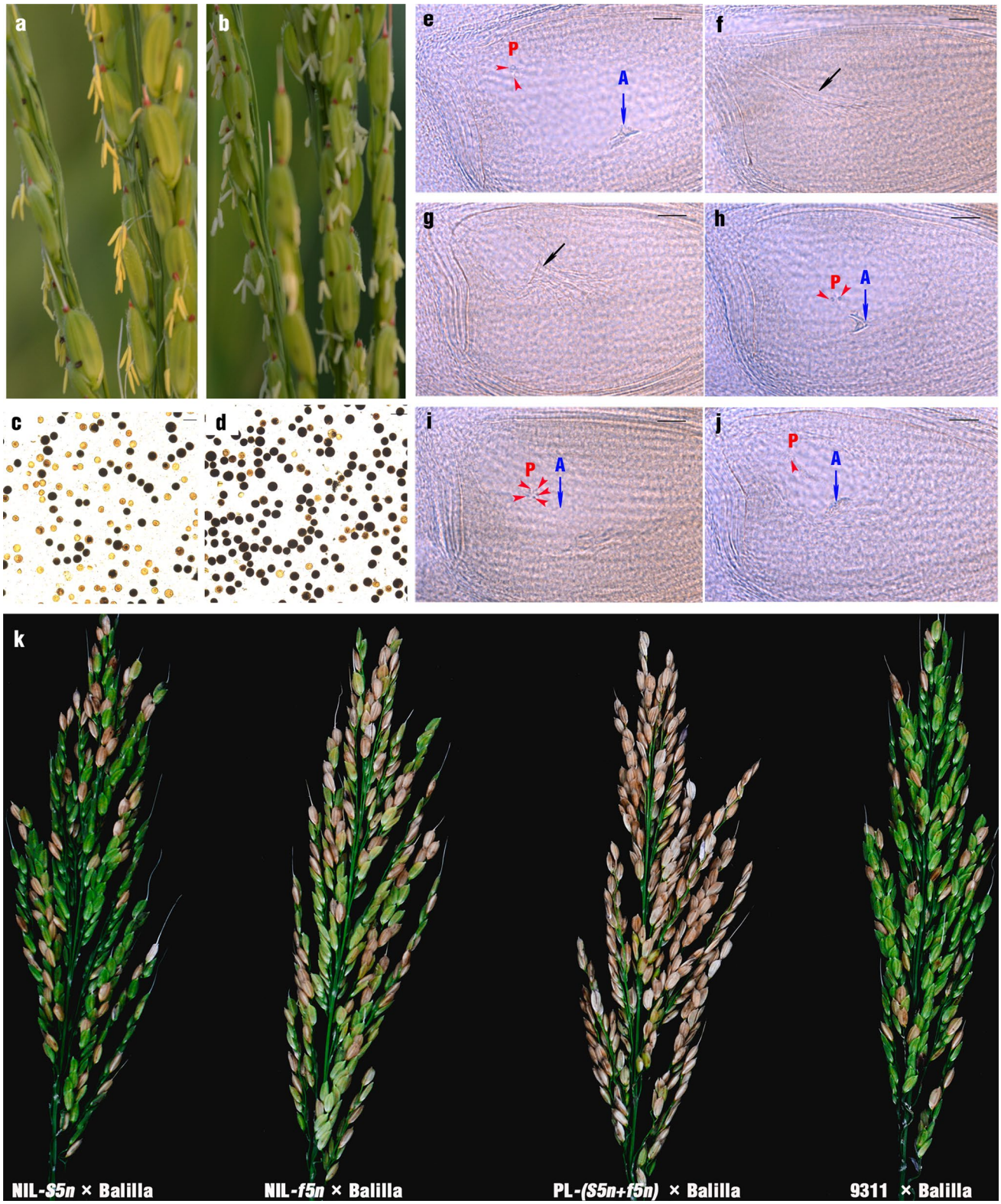
Fig. 4 Evaluation of fertility related traits of the hybrid plants derived from crosses between 9311 and the improved lines to the *japonica* testers. **a** Indehiscent anthers of the bloomed spikelet from the testcross F_1 "9311 × Laoguangtou" in the natural field (in *S1*). **b** Normal anthers of the bloomed spikelet from the testcross F_1 "NIL-(*f5-n*) × Laoguangtou" in the natural field (in *S1*). **c** Pollen grains of the testcross F_1 "9311 × Nipponbare" (in *S1*). **d** Pollen grains of the testcross F_1 "NIL-(*f5-n*) × Nipponbare" (in *S1*). **e–j** Embryo-sac structures of *indica–japonica* hybrids. One *red arrow* indicates one polar nuclear. The *blue arrow* denotes the position of the antipodal cells. The *black arrow* shows the remarkable trace of embryo-sac degeneration. **e** Fertile embryo-sac with normal polar nuclei (*P*) and antipodal cell (*A*), **f**, **g** embryo-sac degeneration, **h** embryo-sac with polar nuclei located in abnormal position, **i** embryo-sac with more than two polar nuclei. **j** Abnormal embryo-sac with smaller size. **k** Spikelet fertility of the improved lines testcrossed to the *japonica* tester Balilla (in *S2*). *Scale bars* 50 μm

(48.0 % in *S1*, 55.5 % in *S2*) when compared with those without neutral alleles (Fig. 5a, b).

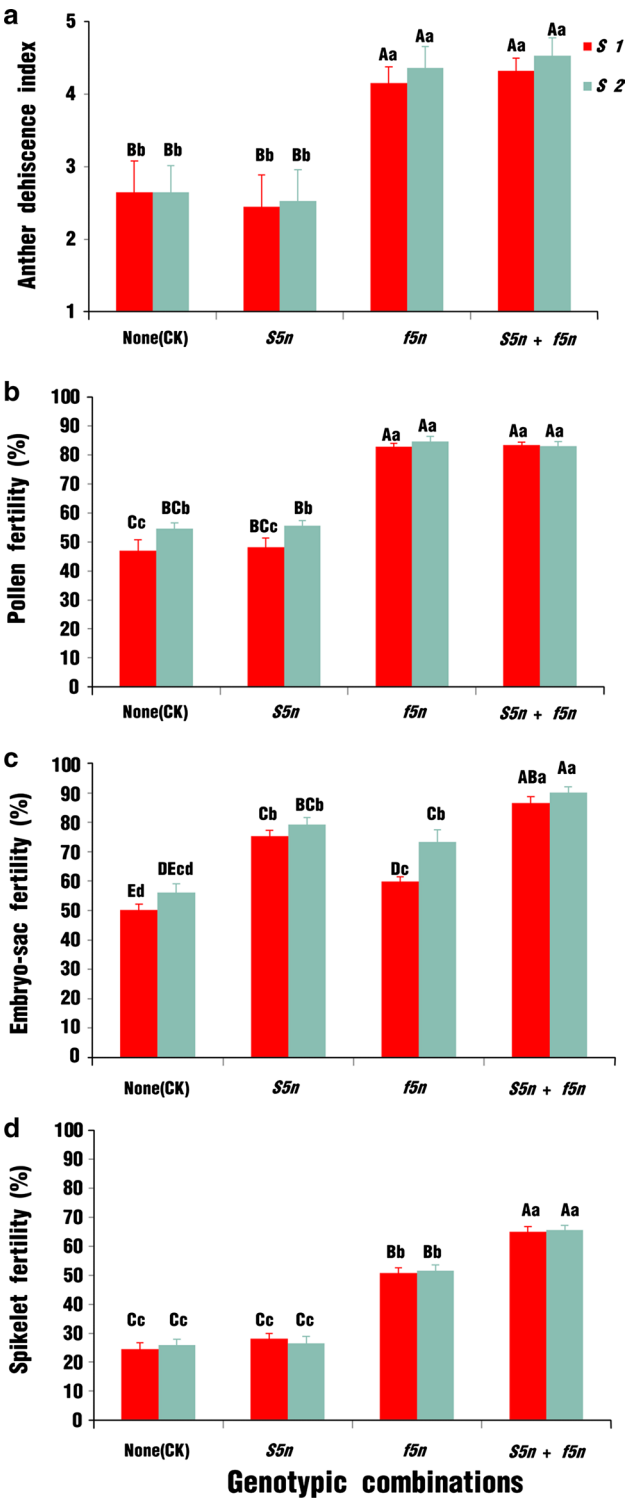
Cumulative effects of f5-n plus S5-n introgression in indica–japonica hybrids

In particular, F_1s of PL-(*S5-n* + *f5-n*) possessed significantly higher embryo-sac and spikelet fertility than both F_1s of NIL-(*S5-n*) and NIL-(*f5-n*). As shown in Fig. 5c, compared with testcross F_1s carrying either single-allele *f5-n* or *S5-n*, the average embryo-sac fertility of the double-neutral allele-pyramiding testcross F_1s (86.7 % in *S1*, 90.2 % in *S2*) was the highest. Therefore, *S5-n* and fragment with *f5-n* improved embryo-sac fertility in *indica–japonica* hybrids with cumulative effect. Such pyramiding effect of *S5-n* and *f5-n* fragment in female fertility did not depend on environmental conditions. Especially, "PL-(*S5-n* + *f5-n*) × Balilla" and "PL-(*S5-n* + *f5-n*) × Nipponbare" had normal level of embryo-sac fertility (>90 %) under both conditions.

Moreover, the average spikelet fertility of the *indica–japonica* testcross F_1s harboring double-allele *S5-n* and *f5-n* (65.0 % in *S1*, 65.5 % in *S2*) was the highest. This indicated that the spikelet effect of *S5-n* depended on the



presence of *f5-n* (Fig. 4k), which was in accordance with the result reported earlier by Wang et al. (2006). When testcrossed to AnongwangengB-2, Balilla and Taichung65, PL-(*S5-n* + *f5-n*) also produced normal level of spikelet fertility (>70 %) under one of the two conditions. These results indicated that PL-(*S5-n* + *f5-n*) possessed higher compatibility to *japonica* than both of NIL-(*S5-n*) and NIL-(*f5-n*) (Table 1).



◀**Fig. 5** The average ADI (**a**), pollen (**b**), embryo-sac (**c**), spikelet fertility (**d**) of the testcross F_1s between the improved lines and the five *japonica* testers under two conditions (*S1*, *S2*). The four genotypic combinations in the *horizontal axis*, none (CK), *S5-n*, *f5-n*, and *S5-n* + *f5-n*, denote the testcross F_1s provided by 9311, NIL-(*S5-n*), NIL-(*f5-n*), and PL-(*S5-n* + *f5-n*), respectively. The value of each genotypic combination is determined by the average ADI and fertility of the testcross F_1s from five *japonica* varieties. The different capital and lowercase letters above the error bars indicate ranking by LSD test at 0.01 and 0.05 probability levels (starting from *A*, *B* is significantly different from *A*, *C* is significantly different from *B*, and so on). *Error bars*, SEM

The F_1s of PL-(*S5-n* + *f5-n*) also showed equal ADI and pollen fertility of F_1s of NIL-(*f5-n*). The testcross F_1s carrying double alleles *S5-n* and *f5-n* are identical to those harboring single-allele *f5-n* in the average ADI (4.3 in *S1*, 4.5 in *S2*) and pollen fertility (83.3 % in *S1*, 82.9 % in *S2*).

Compatibility of improved lines to *indica* testers

When testcrossed to three *indica* testers, the improved lines and 9311 showed normal anther dehiscence (4.2–5.0), embryo-sac fertility (95.1–99.2 %), pollen fertility (85.3–97.8 %) and spikelet fertility (76.5–90.8 %) under two conditions (Table 1). Therefore, these lines harboring *S5-n* and *f5-n* alleles remained compatible to *indica* varieties.

Agronomic performance of the improved lines

The three improved lines, NIL-(*S5-n*), NIL-(*f5-n*), and PL-(*S5-n* + *f5-n*), were further self-pollinated to propagate the BC_5F_3 for the evaluation of agronomic performance. As shown in Table 2, agronomic traits of three improved lines were identical to the recurrent parent 9311 in both environments of Wuhan and Hainan. No significant difference was observed in terms of days to heading, plant height, yield and yield component, suggesting that the two introgression regions containing *S5-n* and *f5-n* did not carry the adverse genetic effect related to agronomic traits. Therefore, these lines allowed high compatibility to either *indica* or *japonica* subspecies with constant agronomic performance.

Discussion

Wide-compatibility alleles at *indica–japonica* hybrid sterility loci would facilitate the utilization of inter-subspecific heterosis in rice. Using an integrated backcross breeding strategy with five generations of backcrossing followed by one generation of selfing, we successfully transferred two neural alleles, *S5-n* and *f5-n*, into an elite *indica* restorer line 9311, to obtain three improved lines. The NIL-(*S5-n*) and NIL-(*f5-n*) contained the fragment carrying *S5-n* and *f5-n* region within 326.6 and 1809.9 kb, respectively, and the PL-(*S5-n* + *f5-n*) pyramided both of the two fragments. The introduction of the two neutral alleles had significantly increased the compatibility of the improved *indica* lines to *japonica* varieties, with the maintenance of agronomic performance of the original 9311.

Table 2 Agronomic performance of the improved lines and the recurrent parent 9311 in Wuhan and Hainan

NILs/PL	Days to heading (day)	Plant height (cm)	Panicle number	Panicle length (cm)	Number of spikelet per panicle	1000-grain weight (g)	Seed-setting rate (%)	Yield per plant (g)
Wuhan (2014)								
NIL-(*S5-n*)	92.0	123.6 ± 2.5	7.2 ± 0.4	24.5 ± 0.9	214.6 ± 11.5	27.3 ± 0.5	85.9 ± 2.5	42.5 ± 4.9
NIL-(*f5-n*)	92.0	123.4 ± 3.7	7.0 ± 0.7	24.2 ± 0.9	220.0 ± 22.0	27.8 ± 0.6	86.8 ± 4.2	42.9 ± 7.4
PL-(*S5-n* + *f5-n*)	92.0	125.0 ± 2.0	6.8 ± 1.3	24.8 ± 0.4	229.1 ± 16.1	27.5 ± 0.6	86.9 ± 3.2	42.4 ± 4.6
9311 (CK)	92.0	124.5 ± 3.5	6.8 ± 1.1	25.0 ± 0.9	219.2 ± 30.3	26.9 ± 0.7	85.0 ± 4.1	42.8 ± 7.0
Hainan (2015)								
NIL-(*S5-n*)	114.0	105.9 ± 1.5	7.2 ± 1.1	22.3 ± 0.5	154.5 ± 13.4	30.7 ± 0.5	94.3 ± 2.1	34.1 ± 5.6
NIL-(*f5-n*)	114.0	106.1 ± 2.4	7.2 ± 1.3	22.7 ± 0.2	157.3 ± 6.0	30.0 ± 0.5	93.6 ± 4.2	34.1 ± 7.0
PL-(*S5-n* + *f5-n*)	114.0	106.0 ± 2.9	6.8 ± 0.8	22.4 ± 0.5	166.4 ± 6.9	30.1 ± 1.2	93.9 ± 1.1	34.0 ± 4.0
9311 (CK)	114.0	107.5 ± 2.7	7.0 ± 0.7	22.7 ± 0.4	162.6 ± 9.9	30.4 ± 0.9	93.8 ± 1.9	34.6 ± 4.2

The value of agronomic traits was measured as mean ± SD

In this breeding program, combinatorial backcross breeding strategy had greatly hastened the dissection of linkage drag and recurrent parent genome recovery, including phenotypic selection in early backcrossing generation, negative selection of the target loci, and whole genome background selection based on high-density array. Phenotypic selection with respect to heading date, plant height, tiller number per plant and glume color was conducted in the BC_1F_1 segregation population in this study, which contained the widest distribution of phenotype based on higher genetic variance. The cost-effective phenotypic selection could be conducted within a short period of time in early generation, which was able to facilitate and accelerate the recovery of recurrent parent genetic background (Khanna et al. 2015; Singh et al. 2013).

In the previous study, the target segments were narrowed to <3.8 and <6.1 cM, respectively, using negative selection with small backcrossing population, because of the deficiency in saturated molecular marker (Chen et al. 2000, 2001; Zhou et al. 2003). However, in this study, using negative selection based on high-density physical map and large backcrossing populations, *S5* region was narrowed within 326.6 kb, thus greatly reducing the risk of the linkage drag. In contrast, *f5* region was introduced within 1809.9 kb by random recombination during the same backcrossing process without negative selection. Thus, these results clearly demonstrated that negative selection could knock out the linkage drag of target region precisely.

This work primarily reported a successful application of whole genome background analysis using two high-density SNP arrays. Compared with the low throughput and uneven genome-wide distribution background selection using traditional DNA markers such as RFLP, AFLP, and SSR (Chen et al. 2000, 2001; Suh et al. 2011; Zhou et al. 2003), high-density markers were available in RICE6K and RiceSNP50 arrays, thus generating 1554 and 12,524 polymorphism SNP markers between 9311 and Dular, respectively, which were higher throughput and more precise in detection of the genetic background. More importantly, the cost of genotyping by SNP array is continually decreasing, which would be acceptable for breeding gradually. Indeed, Khanna et al. (2015) have compared 500 genome-wide SSR markers and 50,051 SNP markers for genetic background analysis of the NILs, which demonstrated that SNP markers provided higher resolution of genetic background, and also showed higher cost effective than SSR markers. Therefore, whole genome SNP array-based background selection might become a feasible strategy with higher precision and efficiency for crop genetic improvement, with the development of high-throughput and resolution genotyping platform in whole genome scale. To sum up, our study represented a successful example of breeding for typical *indica* line with high compatibility to typical *japonica* testers, using integrated approaches. This strategy provided the potential for utilization in a high level of *indica–japonica* heterosis.

Previously, Wang et al. (2005) proved that *f5-n* could increase the pollen fertility and spikelet fertility in *indica–japonica* heterozygous background (Zhenshan97/Balilla). Interestingly, in this study, two concomitant changes occurred with the transferring of the *f5-n* fragment, including an accompanied increasing of ADI and embryo-sac fertility exhibited in the *indica–japonica* hybrids. Noticeably, Zhang et al. (2004) and Zhao et al. (2007) have reported that *Sb* and *S31* loci controlled anther dehiscence and embryo-sac fertility in other *indica–japonica* crosses, respectively, both of which were localized in adjacent region of *f5* locus. Therefore, the observed concomitant increasing in ADI and

embryo-sac fertility of *indica–japonica* hybrids might be due to the pleiotropic effects of *f5* locus. Alternatively, the tight linkage between *f5* locus and the other hybrid incompatibility loci in this introgressed segment was responsible for the effect. These results further confirmed the existence and usefulness of the neutral allele *f5-n* in the wide-compatibility breeding program.

Pyramiding of neutral alleles was essential, since the spikelet fertility of inter-subspecific hybrids was codetermined by numerous hybrid sterility loci influencing female and male units (Ouyang et al. 2009). Indeed, Zhang et al. (2006) reported that the hybrid pollen fertility decreased along with the increase of male sterility loci in *indica–japonica* hybrids. Furthermore, the results in our study showed that the hybrid embryo-sac and spikelet fertility increased by cumulative effect of *S5-n* and *f5-n* fragment in *indica–japonica* hybrids. Thus, PL-(*S5-n* + *f5-n*) exhibited higher compatibility to *japonica* than either NIL-(*S5-n*) or NIL-(*f5-n*), implying that one neutral allele alone was far from sufficient to overcome *indica–japonica* hybrid sterility, whereas pyramiding of more neutral alleles for other hybrid sterility loci would be the most effective and workable strategy in breaking the reproductive barrier.

In this study, with the pyramiding of *S5-n* and *f5-n*, the embryo-sac fertility, anther dehiscence, pollen fertility, and eventually spikelet fertility of the *indica–japonica* hybrid would increase significantly. In particular, the spikelet fertility of three *indica–japonica* crosses with pyramiding of *S5-n* plus *f5-n* had reached to a normal level under one of the two conditions. However, we still notice that the spikelet fertility of the crosses "PL-(*S5-n* + *f5-n*) × Taichung 65" was partial-sterile despite the normal pollen and embryo-sac fertility in *S2* (Table 1). This suggested that other aspects such as reduced affinity between the uniting gametes (Liu et al. 2004) and non-synchronization of male and female gamete development (Liu et al. 1997) may contribute significantly to *indica–japonica* reproductive barriers. Another important concern was that the effect of *S5-n* and *f5-n* varied in different heterozygous genetic background and environmental conditions, due to the existence of a number of hybrid sterility loci other than *S5* and *f5*, as well as loci sensitive to environment (Li et al. 1997). Therefore, breeding strategy in future might focus on pyramiding more neutral alleles and environmental insensitive sterility loci into typical *indica* or *japonica* lines based on different *indica–japonica* crosses, using this combinatorial wide-compatibility breeding strategy, to develop *indica–japonica* hybrid rice possessing high yield and yield stability.

Acknowledgments We are indebted to Dr. Jie Hu from Huazhong Agricultural University for discussion. This research was supported by the Science and Technology Major Projects of Genetically Modified Organisms Breeding of China (High Yield Transgenic Rice Breeding, Grant No. 2011ZX08001-004 and 2014ZX08001-004-001), the National High Technology Research and Development Programs of China (863 Programs, "Breeding of New Varieties of Green Super Rice", Grant No. 2014AA10A604), and the National Natural Science Foundation of China (Grant No. 31371599).

Author contribution statement YO, TM and QZ conceived and designed the experiments; JM, GL, JH, HY, FZ and TM performed the experiments, including artificial crossing, genotyping, and selecting for breeding in the field; JM analyzed the data; YO and JM wrote the paper.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest associated with this study.

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