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Rice

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Identification and Genetic Analysis of Collinearity Loci for Interspecific Hybrid Sterility in Genus *Oryza*

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Abstract

Background Hybrid sterility is a common phenomenon in hybrids between the Asian cultivated rice (*Oryza sativa* L.) and its relatives with AA genome, which limits the utilization of interspecific heterosis and favorable gene introgression. Numerous loci for hybrid sterility have been identified between *O. sativa* and its relatives. However, it remains elusive whether hybrid sterility between different species is controlled by a set of conserved loci, and whether there are variations in the genetic mode of these loci.

Results In this study, six novel hybrid sterility loci for pollen sterility were identified from different cross combinations between *O. sativa* and its three wild relatives. *S59* caused hybrid pollen sterility in hybrids between *O. sativa* and *O. rufipogon*. *S60* and *S61* controlled the hybrid pollen sterility between *O. sativa* and *O. glumaepatula*. *S62*, *S63* and *S64* governed the hybrid pollen sterility between *O. sativa* and *O. barthii*. Genetic and linkage analysis showed that *S59*, *S60*, and *S62* were located in near the same region on the short arm of chromosome 5. *S61* and *S63* were mapped near RM27460 on the short arm of chromosome 12. *S64* was restricted into the 60.27 kb region between RM4853 and RM3372 on the short arm of chromosome 3. The genetic behavior of six novel hybrid sterility loci follows one-locus allelic interaction model, the male gametes carrying the alleles of *O. sativa* in the heterozygotes were selectively aborted except for *S62*.

Conclusions The findings from this research would provide a better understanding for the genetic nature of interspecific hybrid sterility in rice.

Keywords Rice, Interspecific hybrid sterility, Segregation distortion, Collinearity, Orthologous

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Background

Asian cultivated rice (*Oryza sativa* L.) is the primary food crop for half of the global population. Its genetic diversity has become increasingly narrow during domestication from wild ancestors to cultivated rice and modern improvements. The seven relatives of Asian cultivated rice with the same AA genome and similar sequence arrangement as *O. sativa*, are considered to be a valuable source for genetic improvement (Chang 1976; Ohmido et al. 1995; Xiao et al. 1998; Vaughan et al. 2003). However, the severe reproductive isolation of interspecific hybrids between *O. sativa* and its AA genome relatives hinders the utilization of favorable genes in wild rice.

Reproductive isolation is a natural biological phenomenon restricting gene flow between populations, indicating speciation and maintaining species identity (Sano 1986; Orr et al. 2000). Hybrid sterility is a major form of postzygotic reproductive isolation in rice. Since the first report on the phenomenon of hybrid sterility between *O. sativa* subsp. *indica* and *O. sativa* subsp. *japonica* nearly 100 years ago, approximately 60 hybrid sterility loci have been reported in interspecific and intraspecific of rice so far (Kato et al. 1928; Ouyang et al. 2018; Zhang et al. 2022). Till now, 15 hybrid sterility loci have been cloned and characterized. Among those loci, *S1* (Xie et al. 2017; Koide et al. 2018; Xie et al. 2019), *S5* (Chen et al. 2008; Yang et al. 2012), *S7* (Yu et al. 2016), *S13* (Myint et al. 2024), *S22 A/S22B* (Sakata et al. 2021), *Sa* (Long et al. 2008), *Sc* (Shen et al. 2017), *ESA1* (Hou et al. 2019), *hsa1* (Kubo et al. 2016), *qHMS1* (You et al. 2023), *qHMS7* (Yu et al. 2018), and *pf12/RHS12/Se* (Zhou et al. 2023; Wang et al. 2023a, b) fit the one-locus allelic interaction model. Three hybrid sterility pairs, including *S27/S28* (Yamagata et al. 2010), *DPL1/DPL2* (Mizuta et al. 2010), *DSG1/DSG2* (Nguyen et al. 2017), followed the two-locus epistatic interaction model.

During the genetic mapping study of hybrid sterility loci between *O. sativa* and its AA genome relatives, researchers frequently identified six "hot spots" from different species. A comparison of the mapping results for all these hybrid sterility loci suggested that some loci arising from multiple species should be allelic to each other, since they were located to the same chromosome region and had similar genetic pattern (Li et al. 2020). Further research has identified some of these loci as homologous loci. One such locus is the *qHMS7*, which is responsible for the pollen semi-sterility in the hybrid between *O. sativa* and *O. meridionalis*. This locus contains two tightly linked genes, *ORF2* and *ORF3*. *ORF2* encodes a toxic genetic element that leads to the abortion of pollen in a sporophytic manner. On the other hand, *ORF3* encodes an antidote that protects pollen in a gametophytic way. Researchers analyzed the haplotype of *ORF2* and *ORF3* in different AA genome species. They found that *ORF2* was present in all sequenced accessions, while *ORF3* only had one haplotype identified in parts of *O. rufipogon* and most of *O. sativa*. Still, *ORF3* was not detected in *O. meridionalis*, *O. glaberrima*, *O. barthii*, and *O. longistaminata* (Yu et al. 2018). The mapping interval for *qHMS7* is close to several hybrid sterility loci reported previously, such as *S21* (detected from crosses between *O. sativa* and *O. glaberrima*, *O. sativa* and *O. rufipogon*, respectively) and *S23* (identified from the cross between *O. sativa* and *O. glumaepatula*) (Doi et al. 1999; Miyazaki et al. 2007; Sobrizal et al. 2000a, b; Li et al. 2018). Compared to *qHMS7*, *ORF2* and *ORF3* of *S23* also have the "Toxin-Antidote" function (Fang et al. 2019). However, there are significant differences between *S23* and *qHMS7* in their genetic effects. *S23* and *qHMS7* exhibit varying degrees of causing hybrid F_1 pollen abortion, and the expression of *S23* is influenced by the environment. Meanwhile, *qHMS7* is not affected by the environment (Yu et al. 2018; Fang et al. 2019). This result suggests that *qHMS7* and *S23* were hybrid sterility orthologous loci between *O. sativa* and its wild relatives *O. meridionalis*, and *O. glumaepatula*. However, the allele from *O. glumaepatula* is expected to be a distinct allele from *O. meridionalis*.

Another example is the *S1* locus, which plays a crucial role in determining the occurrence of hybrid sterility between two cultivated rice species, *O. sativa* and *O. glaberrima* (Koide et al. 2008b; Garavito et al. 2010; Zhou et al. 2010). The male and female gametes carrying the *O. sativa* allele, *S1-s*, are selectively aborted in the heterozygote, while those carrying the *O. glaberrima* allele (*S1-g*) survive. *S1-g* is composed of three adjacent genes *S1 TPR*, *S1 A4* and *S1 A6* (*SSP*), these three genes produce a complex gamete-killer system, but the *O. sativa* allele *S1-s* lacks all these genes (Xie et al. 2017, 2019; Koide et al. 2018). Several studies identified *S10* as another locus that induces both male and female gamete abortion in a cross between *japonica* and *indica*. Interestingly, *S10* was mapped in a similar position as *S1* on chromosome 6 (Sano et al. 1994), and some further studies suggest that *S1* and *S10* were allelic to each other (Heuer and Miézan 2003; Zhu et al. 2005). A major locus *qpsf6* for hybrid sterility was also identified near the SSR marker RM587 on the short arm of chromosome 6. This QTL is responsible for pollen and spikelet fertility in the cross between *O. sativa* and *O. longistaminata*. Comparing their mapping region and genetic effect indicated that this QTL coincides with *S1* (Chen et al. 2009). In addition, three hybrid sterility loci were identified, and showed good collinearity with *S1* in the hybrid combinations obtained from *O. sativa* crossed with *O. rufipogon*, *O. nivara*, and *O. barthii*, respectively (Yang et al. 2016). These results indicated that the hybrid sterility loci *S1* between *O. sativa* and *O. glaberrima*, *O. barthii*, *O. longistaminata*,

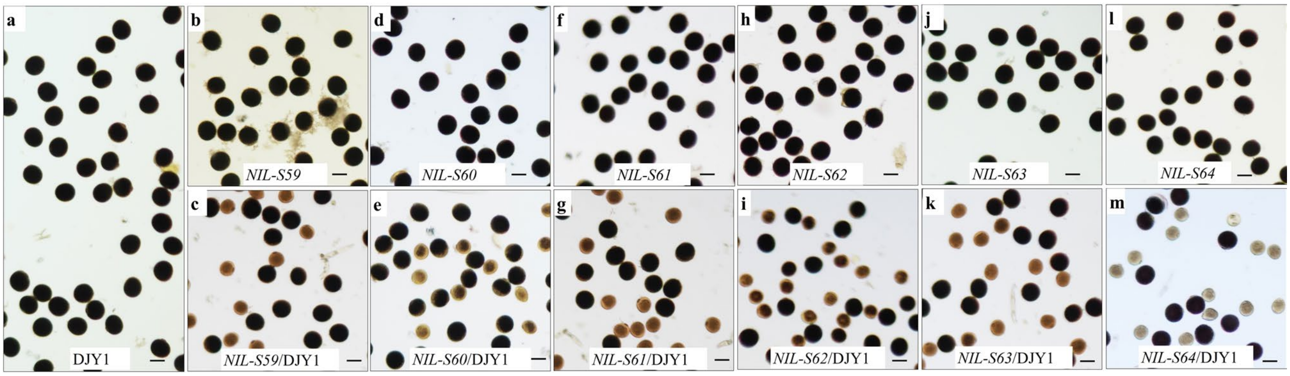

Fig. 1 Pollen grain diagrams of the recurrent parent DJY1, near-isogenic lines (NILs) for each sterility locus, and the hybrid F_1 derived from crosses between the recurrent parent and the NILs for each sterility locus, as visualized by 1% (w/v) I_2-KI staining (**a**–**m**). Scale bar, 100 µm

Table 1 Pollen and spikelet fertility (%) of F_1s and their parents

Parent and F_1	Donor		Generation	Pollen fertility (%)	Spikelet fertility (%)
	Species	Accession			
DJY1	*O. sativa*			96.34 ± 1.73	97.08 ± 2.69
NIL-S59	*O. rufipogon*	Acc.106,138	BC_6F_2	97.43 ± 1.98	93.91 ± 2.92
NIL-S60	*O. glumaepatula*	Acc.105,662	BC_6F_4	95.03 ± 3.6	92.67 ± 3.54
NIL-S61	*O. glumaepatula*	Acc.105,662	BC_6F_3	98.25 ± 0.63	91.62 ± 3.28
NIL-S62	*O. barthii*	Acc.104,061	BC_6F_4	96.01 ± 2.12	92.04 ± 2.89
NIL-S63	*O. barthii*	Acc.100,117	BC_6F_3	97.45 ± 0.90	92.58 ± 2.91
NIL-S64	*O. barthii*	Acc.100,117	BC_6F_3	94.25 ± 2.77	94.86 ± 3.27
NIL-S59/DJY1			BC_7F_1	52.83 ± 2.37	93.53 ± 2.8
NIL-S60/DJY1			BC_7F_1	47.07 ± 3.52	95.06 ± 3.1
NIL-S61/DJY1			BC_7F_1	51.27 ± 1.23	94.12 ± 1.52
NIL-S62/DJY1			BC_7F_1	49.91 ± 0.31	94.57 ± 2.03
NIL-S63/DJY1			BC_7F_1	46.46 ± 2.49	91.77 ± 3.36
NIL-S64/DJY1			BC_7F_1	49.56 ± 0.74	92.51 ± 1.91

O. rufipogon, *O. nivara* should be an orthologous locus. Although some hybrid sterility loci located in the same interval were identified to be homologous loci through further research, while the genetic effects of these orthologous loci are still unclear as they were identified from distinct hybrid crosses in different genetic backgrounds.

In this study, six novel loci for interspecific hybrid male sterility in the hybrids between *O. sativa* and its three wild relatives were confirmed on chromosome 3, 5 and 12, respectively. Among them, *S59*, *S60*, and *S62* were responsible for interspecific hybrid pollen sterility in the crosses between the Asian cultivated rice cultivar DJY1 and *O. rufipogon*, *O. glumaepatula*, and *O. barthii*, respectively. They were located near the RM1024 on chromosome 5 and showed good collinearity. *S61* identified from the cross between DJY1 and *O. glumaepatula*, and *S63* found from the cross between DJY1 and *O. barthii* were both mapped to the same region on chromosome 12. Another locus, *S64*, was located on the short arm of chromosome 3 in the cross between DJY1 and *O. barthii*. This study showed that the hybrid sterility loci identified from different hybrid combinations were mapped in the same or similar chromosome regions, and they may be allelic to each other. In addition, the different degree of segregation distortion and the mode of gamete transmission were observed among the collinearity loci in the same genetic background. These results would be beneficial for further understanding the nature of interspecific hybrid sterility in rice and help us understand the function of collinearity loci for hybrid sterility in *Oryza* genus divergence.

Results

Genetic Analysis of Hybrid Pollen Semi-Sterility

The recurrent parent DJY1 and NILs, harbored the different hybrid sterility loci, had normal pollen and spikelet fertility. In contrast, all six BC_7F_1 hybrids developed from the crosses between NILs and DJY1 exhibited pollen semi-sterility, and the sterile pollen type was stained abortion, while the spikelet fertility of all six BC_7F_1 hybrids was fertile (Fig. 1; Table 1). Pollen fertility in the six BC_7F_2 populations were segregated into fertile and semi-sterile classes, fitting a 1:1 segregation ratio. However, the spikelet fertility of all six BC_7F_2 populations was normal, similar to recurrent parent and NILs (Fig. 2). These results suggest that pollen sterility in the BC_7F_1 hybrids was controlled by a single locus, attributed to single locus male sterility.

Molecular Mapping of Hybrid Sterility Loci

To map the hybrid sterility loci, polymorphic SSR markers from the introgression regions based on the Rice 6k Chips, were utilized to genotype the corresponding BC_7F_2 mapping populations (Table S2). The ANOVA analysis of genotypic and phenotypic data in the mapping populations indicated that there were six loci controlling pollen sterility on chromosome 3, 5, and 12, respectively (Table 2).

Ten polymorphic SSR markers on the short arm of chromosome 5 were used to genotype the 463 individuals in the BC_7F_2 population derived from the cross between NIL-*S59* and DJY1. As a result, the *S59* locus was restricted to a 2.6 cM region flanked by RM17804 and RM1024, at genetic distance of 0.4 and 2.2 cM, respectively (Fig. 3b). The physical distance between the two SSR markers was about 87.8 kb based on the Gramene public database (http://www.gramene.org).

In order to map the hybrid sterility locus *S60* which was identified from the cross between *O. sativa* and *O. glumaepatula*, twelve polymorphic SSR markers from the introgressed region on chromosome 5 were used to genotype 488 plants in the BC_7F_2 population from the cross between NIL-*S60* and DJY1. Then, *S60* was located within a 1.4 cM region flanked by RM1024 and RM17812, with genetic distances of 0.5 and 0.9 cM, respectively (Fig. 3c). Another hybrid sterility locus *S61* identified from the cross between *O. sativa* and *O. glumaepatula* was mapped into a 0.5 cM region flanked by RM8109 and RM27478 on the short arm of chromosome 12, at genetic distances of 0.2 and 0.3 cM, respectively (Fig. 4d).

In this study, three hybrid sterility loci identified from the cross between *O. sativa* and *O. barthii*, were resigned as *S62*, *S63*, and *S64*, respectively. A total of 498 individuals from the BC_7F_2 generation crossed by NIL-*S62* and DJY1 were used to detect the pollen fertility and genotype. Then, the *S62* locus was located to a 2.5 cM region flanked by RM1024 and RM17834 on chromosome 5, at genetic distances of 0.9 and 1.6 cM, respectively (Fig. 3d). And *S63* was anchored to a 4.4 cM region flanked by RM27460 and RM27478 on chromosome 12 (Fig. 4e). Nine polymorphic SSR markers were used to genotype 522 individuals in the BC_7F_2 population that derived from NIL-*S64* and DJY1. *S64* was preliminarily confined to a 1.15 cM region between RM3894 and RM22 on the short arm of chromosome 3, at a genetic distance of 1.08 and 0.07 cM, respectively (Fig. 5d). Next, *S64* was mapped into a 60.29 kb region between RM4853 and RM3372 by map-based cloning (Figure S2).

To sum up, we identified six novel interspecific hybrid male sterility loci in the hybrids between *O. sativa* and its three wild relatives on chromosome 3, 5 and 12, respectively. Among them, *S59*, *S60*, and *S62* were responsible for pollen sterility in the crosses between the Asian cultivated rice cultivar DJY1 and *O. rufipogon*, *O. glumaepatula*, and *O. barthii*, respectively. Compared with the previous report, *S59*, *S60*, and *S62* had good collinearity with *S24*, *Sb*, and *f5-Du* for intersubspecific hybrid sterility in Asian cultivated rice (Kubo et al. 2008; Li et al. 2006; Wang et al. 2006). *S61* identified from the cross between DJY1 and *O. glumaepatula*, and *S63* found from the cross between DJY1 and *O. barthii* were both mapped to the same region on chromosome 12. They were located near several previously identified QTL for hybrid pollen sterility from multiple crosses between *indica* and *japonica* varieties (*S25*, *qs12*, *pf12*, *RHS12*, and *Se*), and between *O. sativa* and *O. nivara* (*S36*), as well as between *O. sativa* and *O. glaberrima* (*S39*(t) (Win et al. 2009; Xu et al. 2014; Kubo et al. 2017; Zhou et al. 2023; Wang et al. 2023a, b). Another locus, *S64*, was located on the short arm of chromosome 3 in the cross between DJY1 and *O. barthii*. The mapping region of *S64* was similar to the region of *S19* identified in cross between *O. sativa* and *O. glaberrima* (Taguchi et al. 1999; McCouch et al. 2002; Zhang et al. 2011).

Segregation Distortion of the Six Interspecific Hybrid Sterility Loci

Hybrid sterility often results in segregation distortion due to the abortion of gametes from one of the parents. In this study, the degree of segregation distortion and the mode of gamete transmission for the six interspecific hybrid sterility loci were analyzed by detecting the segregation of three genotypes in BC_7F_2 populations and the transmission ratio of hybrid sterility loci for male and female gamete in the reciprocal cross populations using the tightly linked SSR markers.

Linkage analysis showed that the *S59*, *S60* and *S62* for interspecific hybrid sterility were tightly linked with RM1024 on the short arm of chromosome 5. The interaction between *S59-sativa* and *S59-rufipogon* led to the partial abortion of male gametes carrying the allele of *S59-sativa* in the heterozygotes, and only 1.51% homozygotes of *S59-sativa* existed in the BC_7F_2 population. A similar genetic behavior of gametes abortion was observed in *S60*, with 7.38% homozygotes of *O. sativa* in the BC_7F_2 population. Conversely, for *S62*, the male gametes from *O. barthii* were partially aborted in the heterozygotes, and the male gametes from *O. sativa* were preferentially transmitted to the next generation. Hence, as for *S59* and *S60*, wild species alleles were preferentially transmitted to progeny, whereas the wild species allele at the *S62* locus was selectively aborted in the

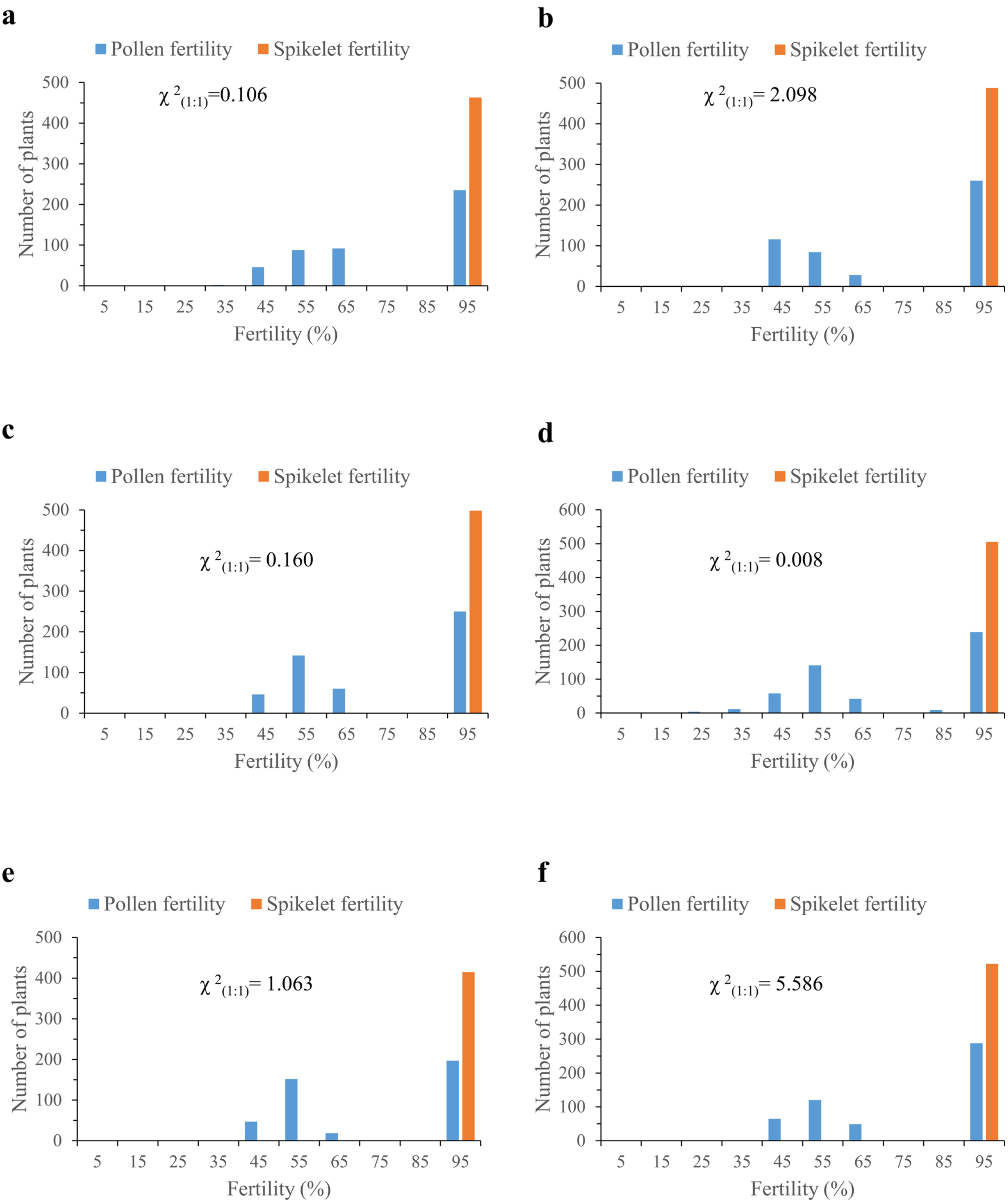

Fig. 2 Frequency distribution of pollen and spikelet fertility in BC$_7$F$_2$ populations. BC$_7$F$_2$ mapping populations derived from NILs × DJY1 for *S59* (**a**), *S60* (**b**), *S61* (**c**), *S62* (**d**), *S63* (**e**) and *S64* (**f**), respectively. The $\chi^2_{(1:1)}$ value reflects the Chi-square test performed on the segregation ratio of pollen fertility in the BC$_7$F$_2$ population, hypothesizing that the ratio of pollen fertile to sterile plants follows the theoretical 1:1 ratio

Table 2 The segregation of marker genotypes in BC_7F_2 populations

Loci	Species	Accession	Generation	Chr	Marker[a]	No. of individuals	Marker segregation			$\chi^2_{1:2:1}$	Aborted gamete
							DD[b]	DW	WW		
S59	*O. rufipogon*	Acc.106,138	BC_7F_2	5	RM1024	463	7 (95.16 ±4.17)[c]	225 (57.24 ±7.13)	231 (98.35 ±3.80)	217.11	*S-DJY1*
S60	*O. glumaepatula*	Acc.105,662	BC_7F_2	5	RM1024	488	36 (95.21 ±11.90)	228 (51.99 ±9.69)	224 (97.49 ±5.86)	146.95	*S-DJY1*
S61	*O. glumaepatula*	Acc.105,662	BC_7F_2	12	RM27460	505	5 (81.41 ±24.43)	249 (52.48 ±8.89)	251 (96.24 ±6.67)	239.76	*S-DJY1*
S62	*O. barthii*	Acc.104,061	BC_7F_2	5	RM1024	498	177 (98.54 ±0. 92)	248 (56.15 ±12.22)	73 (98.41 ±1.44)	43.45	*S-barthii*
S63	*O. barthii*	Acc.100,117	BC_7F_2	12	RM27460	415	21 (94.33 ±2.56)	218 (58.54 ±7.42)	176 (97.48 ±2.59)	116.85	*S-DJY1*
S64	*O. barthii*	Acc.100,117	BC_7F_2	3	RM3372	522	11 (96.67 ±0.81)	235 (51.27 ±6.69)	276 (96.87 ±1.17)	274.24	*S-DJY1*

[a] Marker information is from the Gramene website (http://archive.gramene.org/markers)

, [b] *DD*, *DW* and *WW* indicated DJY1- homozygous, heterozygous and wild rice- homozygous genotypes, respectively

[c] Pollen fertility (%)

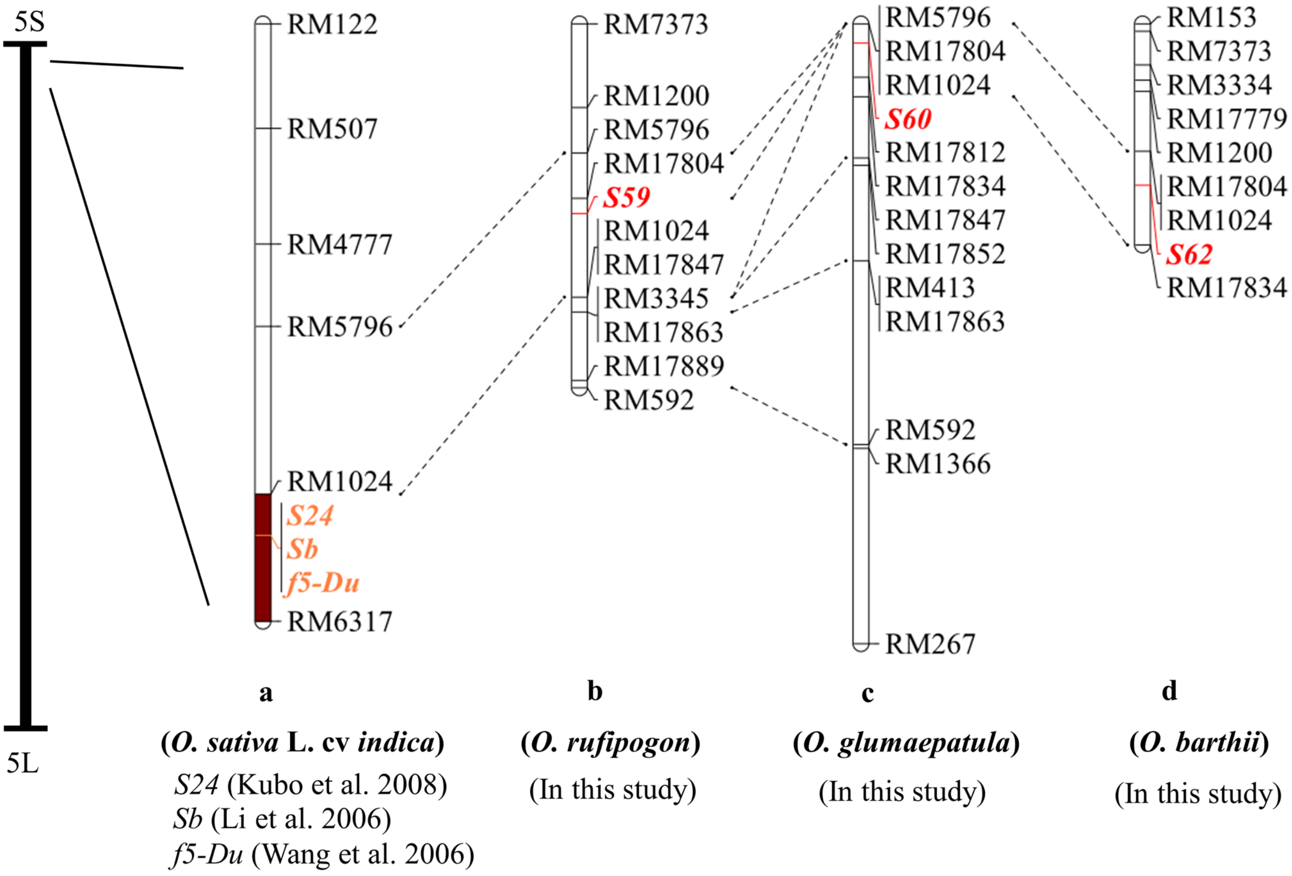

Fig. 3 Genetic location and collinearity analysis for S59, S60, and S62 loci on chromosome 5. **a** The location interval of intraspecific hybrid sterility loci *S24*, *Sb*, and *f5-Du* which were identified from *indica*. **b–d** The genetic location of *S59*, *S60* and *S62*, respectively

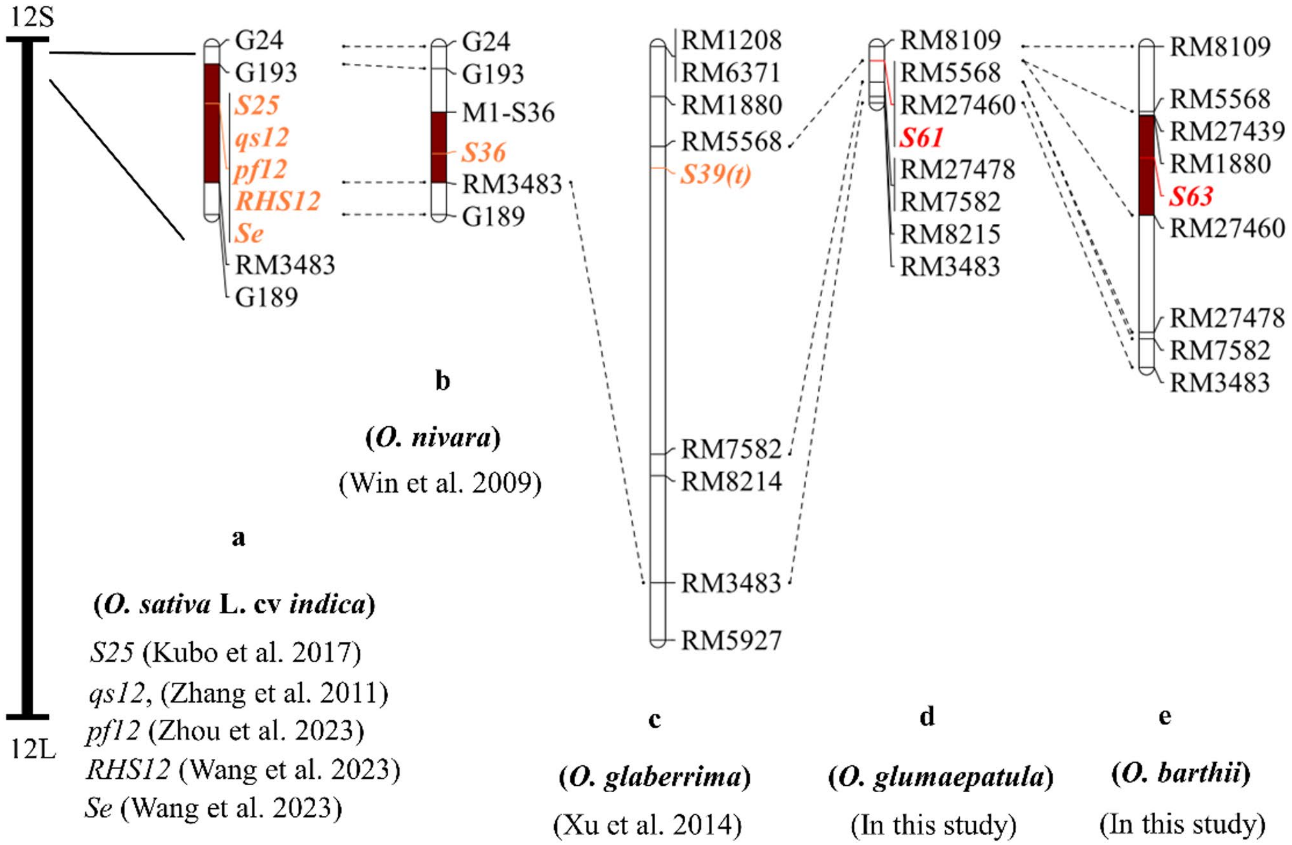

Fig. 4 Genetic location and collinearity analysis for S61, S63 loci on chromosome 12. **a–c** *S25*, *S36*, *S39*(t), *qs12*, *pf12*, *RHS12* and *Se* were mapped into similar region on chromosome 12. **d–e** the genetic location of *S61* and *S63*, respectively

heterozygotes. Reciprocal cross populations were raised to further analyze the genetic effect of the *S59*, *S60* and *S62*, respectively (Table 3). The results showed that for *S59* and *S60*, when F_1 was used as the male parent to cross with DJY1 and NIL, respectively, the ratio of the surviving male gamete *S59-sativa* and *S60-sativa* deviated significantly from the expected ratio of 1:1 in the progeny. However, when F_1 was used as the female parent, the ratio of heterozygous and homozygous plants in the progeny was consistent with the segregation ratio of 1:1 as expected. These findings suggest that male gamete of *S59-sativa* and *S60-sativa* cannot be transmitted normally, while the female gamete of *S59-sativa* and *S60-sativa* can be transmitted normally. Regarding *S62*, when F_1 was used as a female parent crossed with recurrent parent DJY1 and NIL, respectively, the ratio of heterozygous and homozygous plants fitted into 1:1, suggesting that female gametes of both *S62-sativa* and *S62-barthii* can be transmitted equally. Interestingly, when F_1 was used as male parent, it was found that the transmission ratio of the male gamete *S62-sativa* was significantly higher than that of *S62-barthii*. It was suggested the male gamete of *S62-barthii* cannot be transmitted normally (Table 3). Taken together, although *S59*, *S60* and *S62* were located within a similar region, the degree and direction of gamete segregation distortion varied among the different loci.

S61 and *S63* were located on the same interval on the short arm of chromosome 12. Extreme segregation distortion was found in *S61*, only 0.99% homozygotes of *S61-sativa* were found in the BC_7F_2 population. The interaction between *S61-sativa* and *S61-glumaepatula* led to the nearly complete abortion of male gametes carrying the allele of *S61-sativa* in the heterozygotes (Table 2). Compared to *S61*, the gamete elimination effect of *S63* was lower, 5.06% homozygotes of *S63-sativa* were found in the BC_7F_2 population. In addition, the gamete transmission ratio of *S61* and *S63* were investigated by reciprocal crosses. For *S61*, the ratio of heterozygous and homozygous plants deviated significantly from the expected ratio of 1:1 in four reciprocal cross populations, regardless of whether the heterozygote was used as either the male or the female parent. And the transmission rate of *O. sativa*-type gamete was significantly higher when heterozygous plants were used as female parents. The results showed that both male and female gamete of *S61-sativa* cannot be transmitted normally. For *S63* locus, when F_1 was used as the male parent to cross with

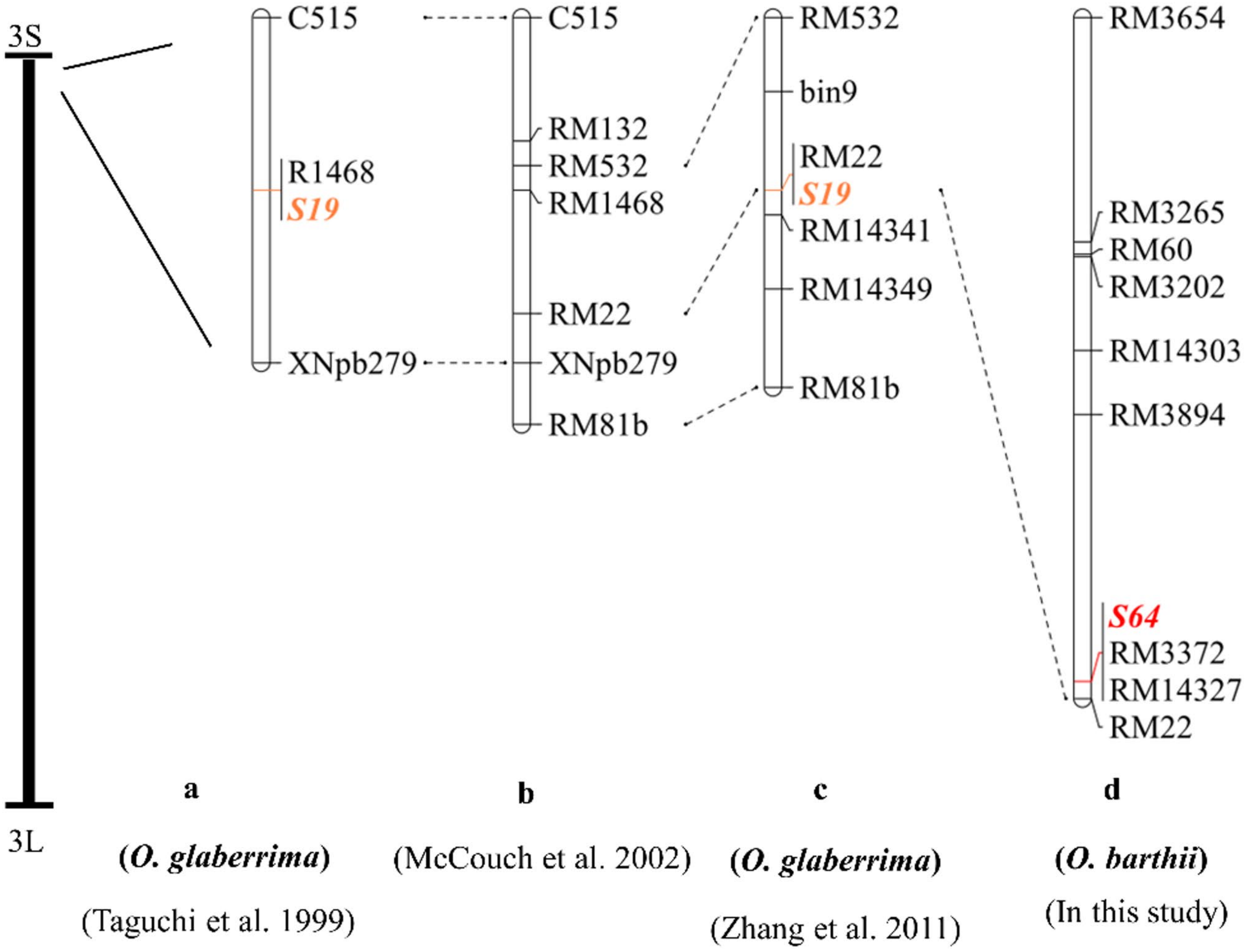

Fig. 5 Genetic location and collinearity analysis for S64 locus on chromosome 3. **a–c** The genetic location of *S19* identified from *O. glaberrima*. **d** *S64* from *O. barthii* was restricted into the 60.27 kb region between RM4853 and RM3372 in this study

Table 3 The transmission ratio of hybrid sterility loci *S59*, *S60* and *S62* on chromosome 5 for male and female gamete in the reciprocal cross populations

Loci	Marker (RM1024, Chr. 5)		Progeny genotype				$\chi^2_{(1:1)}$	Transmission ratio	
	Female genotype	Male genotype	*DD*	*WW*	*DW*	Total		D	W
S59	*DD*	*DW*	21		93	114	45.474	0.18	0.82
	DW	*DD*	68		75	143	0.343	0.48	0.52
	WW	*DW*		108	17	125	65.73	0.14	0.86
	DW	*WW*		126	127	253	0.004	0.50	0.50
S60	*DD*	*DW*	26		159	185	95.616	0.14	0.86
	DW	*DD*	78		107	185	4.546	0.42	0.58
	WW	*DW*		76	7	83	57.361	0.08	0.92
	DW	*WW*		119	106	225	0.751	0.47	0.53
S62	*DD*	*DW*	87		4	91	75.703	0.95	0.05
	DW	*DD*	48		40	88	0.727	0.55	0.45
	WW	*DW*		10	75	85	49.706	0.88	0.12
	DW	*WW*		36	48	84	1.714	0.57	0.43

DD, *DW* and *WW* indicated DJY1- homozygous, heterozygous and wild rice- homozygous genotypes, respectively

Table 4 The transmission ratio of hybrid sterility loci *S61* and *S63* on chromosome 12 for male and female gamete in the reciprocal cross populations

Loci	Marker (RM27460, Chr. 12)		Progeny genotype				$\chi^2_{(1:1)}$	Transmission ratio	
	Female genotype	Male genotype	*DD*	*WW*	*DW*	Total		*D*	*W*
S61	*DD*	*DW*	7		93	100	73.96	0.07	0.93
	DW	*DD*	29		71	100	17.64	0.29	0.71
	WW	*DW*		50	8	58	30.414	0.14	0.86
	DW	*WW*		38	12	50	13.52	0.24	0.76
S63	*DD*	*DW*	21		179	200	124.82	0.11	0.89
	DW	*DD*	34		45	79	1.532	0.43	0.57
	WW	*DW*		80	6	86	63.674	0.07	0.93
	DW	*WW*		130	89	219	7.676	0.41	0.59

DD, *DW* and *WW* indicated DJY1- homozygous, heterozygous and wild rice- homozygous genotypes, respectively

Table 5 The transmission ratio of hybrid sterility locus *S64* on chromosome 3 for male and female gamete in the reciprocal cross populations

Locus	Marker (RM3372, Chr. 3)		Progeny genotype				$\chi^2_{(1:1)}$	Transmission ratio	
	Female genotype	Male genotype	*DD*	*WW*	*DW*	Total		*D*	*W*
S64	*DD*	*DW*	21		146	187	93.563	0.11	0.89
	DW	*DD*	94		102	196	0.327	0.48	0.52
	WW	*DW*		113	16	129	72.938	0.12	0.88
	DW	*WW*		104	87	191	1.513	0.46	0.54

DD, *DW* and *WW* indicated DJY1- homozygous, heterozygous and wild rice- homozygous genotypes, respectively

DJY1 and NIL, respectively, the ratio of the surviving male gamete *S63-sativa* deviated significantly from the expected ratio of 1:1 in backcross populations. However, when F_1 was used as the female parent, the ratio of heterozygous and homozygous plants in the progeny was consistent with the expected ratio of 1:1. These findings suggest that male gamete of *S63-sativa* cannot be transmitted normally, while the female gamete of *S63-sativa* can be transmitted equally (Table 4).

S64 is a pollen sterility locus located on the short arm of chromosome 3. The interaction between *S64-sativa* and *S64-barthii* resulted in the partial abortion of male gametes carrying the allele of *S64-sativa* in the heterozygotes, the homozygous *O. sativa* plants were less than homozygous *O. barthii* plants in the BC_7F_2 population (Table 2). The reciprocal cross test also indicated that the male gamete transmission rate of *O. barthii* was significantly higher when heterozygous plant was used as male parent (Table 5). This result indicated that pollen semi-sterility in hybrid and segregation distortion at *S64* locus were due to the selective abortion of pollen grains carrying the *O. sativa* allele.

Discussion

Six Novel Hybrid Sterility Loci Identified in *O. sativa* and its Three Wild Relatives

In this study, six loci responsible for interspecific hybrid sterility were identified from the crosses between *O. sativa* and its three wild relatives, including *S59* identified from the cross between *O. sativa* and *O. rufipogon*, *S60* and *S61* identified from the cross between *O. sativa* and *O. glumaepatula*, *S62*, *S63*, and *S64* identified from the crosses between *O. sativa* and *O. barthii* (Figs. 3, 4 and 5). By now, three loci responsible for hybrid sterility between *O. sativa* and *O. rufipogon* have been reported, including *S6*, *S21*(t) and *qss6-b* (Sano 1990; Miyazaki et al. 2007; Koide et al. 2008a, 2012; Yang et al. 2016). *S6* and *qss6-b* were mapped on chromosome 6, and *S21*(t) was located on the long arm of chromosome 7. Therefore, *S59* should be a new locus controlling pollen sterility in hybrids between *O. sativa* and *O. rufipogon*. Six loci responsible for hybrid pollen sterility between *O. sativa* and *O. glumaepatula* were reported, including *S12*, *S22 A*, *S22B*, *S23*(t), *S27*, *S28*, and *S56*(t) (Sano 1994; Sobrizal. et al. 2000a, b, 2001, 2002; Zhang et al. 2018). The chromosomal location of *S12* was unknown (Sano 1994). *S22 A* and *S22B* were mapped on chromosome 2 (Sakata et al. 2014, 2021), *S23*(t) was mapped on chromosome 7 (Sobrizal et al. 2000b). *S27* and *S28* were located on chromosome 4 and chromosome 8, respectively (Sobrizal et al. 2001; Sobrizal et al. 2002; Yamagata et al. 2010), and *S56*(t) was mapped into the region between RM20797 and RM1093 on chromosome 7 (Zhang et al. 2018). Thus, *S60* located on chromosome 5 and *S61* located on chromosome 12 were novel loci for interspecific hybrid sterility between *O. sativa* and *O. glumaepatula*. The *qss6-c* locus was located on chromosome 6, which was responsible for both pollen and spikelet sterility between *O.*

sativa and *O. barthii*. It had a good co-linear relationship with *S1* (Sano 1990; Yang et al. 2016). Therefore, *S62*, *S63*, and *S64* are novel loci that control male gamete development in hybrids between *O. sativa* and *O. barthii*.

Different Alleles of the Orthologous Loci Showed Different Genetic Effect on Hybrid Sterility

In this study, *S59*, *S60*, and *S62* were located on chromosome 5 and shared the similar chromosome region with *S24*, *Sb*, and *f5-Du*, which were mapped as pollen killer for intersubspecific hybrid sterility in the Asian cultivated rice (Kubo et al. 2008; Li et al. 2006; Wang et al. 2006). Another two loci, *S61* and *S63*, the comparison of map positions showed that *S61* and *S63* on chromosome 12 had good co-linear with *S25*, *qs12*, *pf12*, *RHS12*, and *Se* identified from multiple crosses between *indica* and *japonica* varieties (Kubo et al. 2017; Zhou et al. 2023; Wang et al. 2023a, b), and *S36* identified from the cross between *O. sativa* and *O. nivara* (Win et al. 2009), as well as *S39*(t) identified from the cross between *O. sativa* and *O. glaberrima* (Xu et al. 2014). The mapping region of *S64* coincided with *S19*, which was identified from the cross between *O. sativa* and *O. glaberrima* (Taguchi et al. 1999; McCouch et al. 2002; Zhang et al. 2011). Furthermore, the sterile pollen of these loci heterozygotes showed stained abortion phenotype by I_2-KI staining. Comparing the mapping results and type of sterile pollen in heterozygotes indicated that these loci from different species should be allelic to each other. In previous studies, researchers speculated that hybrid sterility loci consistently detected at the same or similar positions on chromosomes across different species should be orthologous loci, and the hypothesis was confirmed by the molecular characterization of some hybrid sterility loci, such as *S1*, *S5*, *S27*, *Sa*, and *qHMS7* (Long et al. 2008; Yamagata et al. 2010; Win et al. 2009; Yang et al. 2012; Xie et al. 2017; Koide et al. 2018; Yu et al. 2018; Xie et al. 2019; Fang et al. 2019).

Although some orthologous loci for hybrid sterility were identified, it was difficult to investigate the genetic effects for different alleles because those loci were derived from different crosses in distinct genetic backgrounds. In this study, the near-isogenic lines harboring the hybrid sterility loci in the same background were used to compare the genetic effect of orthologous loci. The male gametes carrying *O. sativa* alleles at *S59* and *S60* loci were selectively aborted in the heterozygotes, while the wild species allele at the *S62* locus was selectively aborted in the heterozygotes. In addition, the male gametes carrying the *japonica* allele at *S24*/*Sb*/*f5-Du* were selectively aborted in heterozygous plants (Kubo et al. 2008; Li et al. 2006; Wang et al. 2006). Thus, this hybrid sterility "hot spot" locus was observed in the different hybrid combinations of interspecific and intersubspecific, suggesting that this conserved locus originated before the divergence of AA genome species and had a significant impact on the reproductive isolation with the distinct genetic pattern.

At another hybrid sterility "hot spot" on chromosome 12, the male gametes from *O. glumaepatula* and *O. barthii* in the heterozygotes were preferentially transmitted to progeny. However, even when heterozygous plants of *S61* as maternal parent to cross with DJY1 and NIL-*S61*, the transmission rate of *O. sativa*-type gamete and *O. glumaepatula*-type does not fit the expected ratio 1:1 in the offspring of the hybrid (Table 4). It is suggested that *S61* conferred a different segregation distortion pattern from *S63*. *S25*, *qs12*, *pf12*, *RHS12*, and *Se* were identified from *indica* and *japonica* crosses and showed significant preferential transmission of the *indica* alleles, while some loci identified from the cross between *japonica* and its wild relatives showed significant preferential transmission of the wild relatives alleles (Win et al. 2009; Xu et al. 2014; Kubo et al. 2017; Zhou et al. 2023; Wang et al. 2023a, b). In addition, *S64* was identified for interspecific hybrid sterility between *O. sativa* and *O. barthii*, which was located in the same region as *S19* identified from the cross between *O. sativa* and *O. glaberrima* (Taguchi et al. 1999; McCouch et al. 2002; Zhang et al. 2011). Similar to *S19*, the *O. sativa* allele of *S64* were not completely transmitted to the progeny via male gamete. The presence of co-linearity among various sterility loci suggests that similar loci in different species of the *Oryza* genus may be responsible for controlling reproductive barriers among AA genome species. Identifying and characterizing more hybrid sterility loci from other *Oryza* species will help shed light on the difference in genetic effect of those allelic loci on hybrid sterility and understand the function of allelic hybrid sterile loci in *Oryza* speciation.

Strategies for Overcoming the Interspecific Hybrid Sterility

The abundant genetic diversity of AA genome species in genus *Oryza* played an important role in the Asian cultivated rice improvement. Interspecific hybrid offspring exhibit stronger heterosis compared to intersubspecific hybrids. However, the severe reproductive isolation of interspecific hybrid makes it difficult to utilize interspecific heterosis. Understanding the genetic nature was the basis for utilizing interspecific heterosis or transferring favorable genes to the Asian cultivated rice. By now, great progress has been made in the cloning and molecular characterization of the hybrid sterility loci, for example, *S5* (Chen et al. 2008; Yang et al. 2012), *S7* (Yu et al. 2016), and *ESA1* (Hou et al. 2019) for female sterility, *Sa* (Long et al. 2008), *Sc* (Shen et al. 2017), *qHMS1* (You et al. 2023), *qHMS7* (Yu et al. 2018), *pf12*/*RHS12*/*Se* (Zhou et al. 2023; Wang et al. 2023a, b) for male sterility, *S1* for both male and female sterility (Xie et al. 2017; Koide et al. 2018; Xie et al. 2019). Based on the above information, artificial wide-compatible lines can be made to overcome the hybrid sterility by CRISPR/Cas9 gene editing and RNA interference technologies (Yu et al. 2016; Shen et al. 2017; Xie et al. 2017; Koide et al. 2018; Hou et al. 2019; Xie et al. 2019).

Furthermore, transferring neutral allele of the major hybrid sterility loci to the target parent to raise wide-compatibility lines as bridge parent is an effective strategy to overcome hybrid sterility. For example, scientists have proved that the utilization of wide-compatible genes of *S5* and *f5*, as well as the *japonica* alleles at *Sc* and *pf12* locus could overcome intersubspecific hybrid sterility (Zhou et al. 2023). Pyramiding the *indica* alleles at *Sb*, *Sc*, *Sd* and *Se* loci and the neutral allele at *S5* locus in *japonica* genetic background through MAS are compatible with *indica* rice varieties, the F_1 hybrids showed fertile pollen and spikelet (Guo et al. 2016).

In interspecific of AA genome species in the *Oryza* genus, there have been successful cases of improving the fertility of both pollen and spikelet in interspecific hybrids by using either single locus or multiple hybrid sterility loci pyramiding lines as bridge parents (Deng et al. 2010; Li et al. 2023). Additionally, our study suggests that employing a single allele to raise a bridge parent to overcome hybrid sterility has its limitations due to allelic differentiation. Therefore, bridge parents developed by pyramiding hybrid sterility loci identified from more species in *O. sativa* genetic background would effectively improve the interspecific hybrid sterility between *O. sativa* and more than two species.

Conclusions

In this study, six novel hybrid pollen sterility loci for interspecific hybrids were identified and designed as *S59*, *S60*, *S61*, *S62*, *S63*, and *S64*, respectively. These six loci could be classified into three groups based on collinearity analysis. The first group contained three loci, *S59*, *S60*, and *S62* responsible for interspecific hybrid pollen sterility in the crosses between DJY1 and *O. rufipogon*, *O. glumaepatula*, and *O. barthii*, respectively. All of them were located in the same region on chromosome 5. The second group included two loci, *S61* and *S63*, they were mapped to the same position on chromosome 12 and identified from the crosses between *O. sativa* and *O. glumaepatula*, *O. barthii*, respectively. *S64* from *O. barthii* was located on chromosome 3 and belonged to the third group. Meanwhile, the alleles from different species at the same locus differed in the degree of segregation distortion and gamete transmission mode because of allelic differentiation. This study provides insight into the genetic basis of conserved loci for interspecific hybrid sterility, which played an important role in understanding the hybrid sterility and divergence of genus *Oryza*.

Materials and Methods

Plant Materials

An *O. rufipogon* accession, Acc.106,138; an *O. glumaepatula* accession, Acc.105,662; two accessions of *O. barthii*, Acc. 104,061 and Acc. 100,117, introduced from the International Rice Research Institute (IRRI) as male and donor parents, were crossed with a *japonica* variety of *O. sativa*, Dianjingyou 1 (DJY1). The F_1 as female parent was backcrossed with DJY1 as a male parent to obtain BC_1F_1 progenies. The plants with pollen fertility below 90% were selected to make successively backcross from BC_1F_1 until BC_5F_1/BC_6F_1. Subsequently, the semi-sterile plants identified in the BC_5F_1/BC_6F_1 generations were subjected to multiple rounds of self-fertilization to establish 24 distinct populations for further study and analysis. Mixed pools of pollen fertile and semi-sterile plants for each population were constructed and screened using Rice 6k Chips (Illumina, USA) for introgressed segments associated with pollen sterility (Figure S1, Table S1). After comparing the introgressed segments associated with pollen sterility of these populations with the localization intervals of reported hybrid sterility loci, six pollen semi-sterile families were selected for further research, including one from the cross between *O. sativa* and *O. rufipogon*, two from the cross between *O. sativa* and O. *glumaepatula*, one from the cross between *O. sativa* and Acc. 104,061 of *O. barthii*, and two from the cross between *O. sativa* and Acc. 100,117 of *O. barthii*. The introgressed segments associated with pollen sterility were identified on chromosome 3, 5, and 12, respectively. Since no hybrid sterility loci have been reported in these chromosome regions between *O. sativa* and its three wild relatives (Table S1), the hybrid sterility locus identified from the cross between DJY1 and *O. rufipogon* was designed as *S59*, and another two loci identified from the cross between DJY1 and *O. glumaepatula* were named as *S60* and *S61*, respectively. Three loci controlled the hybrid sterility between DJY1 and *O. barthii* were designed as *S62*, *S63*, and *S64*, respectively.

Polymorphic screening was conducted using SSR markers in the introgression regions based on the results of genome-wide screening by Rice 6k Chips in the six populations mentioned above (Table S2). The individuals harboring the homozygous genome fragments from wild relatives were selected as NILs in BC_6F_3/BC_6F_4 populations by SSR polymorphic markers. NILs were crossed with the recurrent parent DJY1, and then were self-fertilized to produce the BC_7F_2 populations. The individuals of BC_7F_2 populations were used to map the interspecific hybrid sterility loci. To study the gamete elimination and transmission pattern, reciprocal crosses were produced between heterozygous plants in BC_7F_1 and DJY1, and between heterozygous plants in BC_7F_1 and NILs.

All plant materials were grown in paddy field in Breeding Field, Yunnan Academy of Agricultural Sciences (YAAS), Xishuangbanna (100° E, 20°N), Yunnan Province, P. R. China.

Phenotype Evaluation

Five to ten spikelets were collected from the upper and middle branches of the main panicle of each individual 1–2 days before anthesis and fixed in 70% ethanol (Doi et al. 1998). To evaluate the pollen fertility of each plant, 3–6 anthers from each spikelet were mixed and stained with a 1% iodine-potassium iodide (I_2-KI) solution. The observed pollen grains were classified into four categories: typical abortion, spherical abortion, stained abortion, and fertile. At least 300 pollen grains were observed under three independent microscopic fields. The pollen fertility score was determined as the average percentage of fertile pollen grains across the three views. The spikelet fertility was investigated by scoring the fertilized spikelet rate of three panicles on each plant.

DNA Extraction and PCR Protocol

Genomic DNA for simple sequence repeat (SSR) analysis was extracted from the fresh leaves of each plant using a simple DNA extraction method (Edwards et al. 1991). Rice SSR markers were chosen from previously published SSR markers in rice (McCouch et al. 2002). The PCR reaction was performed in a total volume of 10 μl, containing about 40 ng of template DNA, 0.2μM of each primer, and 5 μl 2×*Taq* PCR StarMix Loading Dye-free (Vazyme, Nanjing, China). The reaction mixture was incubated at 94℃ for an initial 5 min, followed by 32 cycles of 94℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 40 s, with a final extension step of 7 min at 72℃. The PCR products were separated on an 8% non-denaturing polyacrylamide gel and detected using silver staining.

Genomic DNA for Rice 6k Chips was extracted from the fresh/frozen leaf samples using the CTAB method (Murray and Thompson 1980). The protocol of Rice 6k Chips as Infinium HD Assay Ultra Protocol Guide (https://support.illumina.com.cn/downloads/infinium_hd_ultra_assay_protocol_guide_(11328087_b).html) was used.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12284-025-00803-6.

Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Supplementary Material 4.

Supplementary Material 5.

Supplementary Material 6

Supplementary Material 7

Author contributions

TDY and ZY conceived and designed the experiments. YY carried out most of the experiments and wrote the manuscript. PQH, LYG, SXC participated in phenotyping and genotyping. LJ, ZJW and DXN participated in developing plant materials. All authors have read and agreed to the published version of the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Institutional review board statement

Not applicable.

Informed consent

Not applicable.

Competing interests

The authors declare no competing interests.

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