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Mining rice new germplasm containing S_5^n gene by functional molecular marker and sequencing

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Inter-subspecific hybrids between *indica* and *japonica* varieties yield strong biological heterosis, but it is difficult to utilize the hybrids directly in commercial production due to sterility of F_1. A special gene S_5^n may overcome the hybrids sterility, which is caused by the interaction between S_5 loci. Recently, S_5^n had been cloned, and it was revealed containing a large DNA deletion sequence that made the gene nonfunctional, compared to S_5^i or S_5^j. We designed a pair of primers flanking the deletion sequence of S_5^n, and then applied to distinguish the varieties with S_5^n or non-S_5^n, convincing result suggested that the primers could be served as functional molecular marker to efficiently identify the new germplasm with S_5^n. Using the functional marker, we surveyed 197 varieties from China National Micro-core Rice Collection, and found ten of which represented S_5^n including following varieties: Haobuka, Sanbangqishiluo, Mubanggu, Xiaohonggu, Mowanggu'neiza, Laozaogu, Fanhaopi, Feie'nuo2, Baoxie-7B, Teqingxuanhui. Among them, two varieties Sanbangqishiluo and Laozaogu was previously reported to contain S_5^n gene. Further sequence analysis on the DNA sequence covering both sides of deletion in S_5^n of the 10 varieties confirmed that the detected sequences in above varieties was identical with those of varieties containing S_5^n, such as 02428 and Linglun. These results suggested that the gene in S_5 locus of the ten varieties was also nonfunctional and it proved the presence of S_5^n gene.

rice, S_5^n gene, wide-compatibility variety, micro-core collection, functional molecular marker

The Asian cultivated rice (*Oryza sativa* L.) is divided into two major subspecies, *indica* and *japonica*. Subspecific hybrids between *indica* and *japonica* varieties exhibit strong biological heterosis, but practically it is hard to directly apply the hybrids for high production because of F_1 sterility[1]. For several decades, many efforts had been made to pursue the solution for overcoming F_1 sterility[2]. The well-known opinion by Ikehashi and Araki [3] considered that F_1 sterility was mainly caused by embryo sac abortion, which was determined by genotype of S_5 locus in chromosome 6, and *indica* was $S_5^iS_5^i$ in genotype and *japonica* $S_5^jS_5^j$. The hybrids between *indica* and *japonica* varieties with $S_5^iS_5^j$ were sterile due to gene interaction between S_5^i and S_5^j. The hybrids from *indica* or *japonica* parent variety with S_5^n (so-called "wide-compatibility gene") would get high or normal fertility, irrespective of whether the hybrids have $S_5^nS_5^i$ or $S_5^nS_5^j$ genotype. Since then, remarkable progress was made for S_5^n gene fine-mapping, mining new germplasm with S_5^n by test-cross and its utilization for development of rice quality varieties[2,5–12].Chen et al.[13] cloned the S_5 gene and revealed that the *indica* (S_5^i) and *japonica* (S_5^j) alleles differed by two nucleotides, which caused two amino acid replacement in corresponding protein and resulted in the sterility of hybrids. S_5^n had a large DNA sequence, a total of 136 bp, deletion in the N terminus of predicted S_5 protein, causing subcellular mislocalization of the protein, and thus was presumably nonfunctional. Therefore, whether the variety with $S_5^nS_5^n$ crossed with *indica* containing $S_5^iS_5^i$ or *japonica* containing $S_5^jS_5^j$, the hybrid was fertile. In this paper, a pair of primes (functional molecular marker) was initially

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designed based on the DNA sequence of both sides in deletion of S_5^n and the corresponding sequence in Nipponbare, and then the functional molecular marker was used to identify the varieties with candidate S_5^n in China National Micro-core Rice Collection. Finally, sequence analysis was conducted about the DNA sequence spanning the deletion sequence of S_5^n in the varieties with candidate S_5^n. Our study demonstrated this method provided a novel approach to identify new germplasm with S_5^n efficiently and accurately and thus could be further applied for the breeding of inter-subspecific hybrids.

1 Materials and methods

1.1 Materials

A total 199 rice varieties from China National Micro-core Rice Collection were selected in this study, which kindly provided by the National Crop Germplasm Conservation Center, Chinese Academy of Agriculture. Due to lack of germination two varieties were eliminated, so 197 varieties were used in the study, including 114 *indica* and 83 *japonica* varieties (Table 1). Varieties 02428 (*japonica*) and Linglun (*indica*) and those without S_5^n

Table 1 Varieties of China National Micro-core Rice Collection used in this study

Type	Varieties	Origin of varieties (Province or City)
Indica	Liushizao, Qiuqianbai, Leihuozhan, Zaoxian240	Anhui
	Jinxibai, Jiefangxian, Sanbaili, Taishannuo, Aihechi, Aimi, Hongmisandanbai, Zhenshan97B, Xian'gaiB, Jiangnongzao1B, Nantehao	Jiangxi
	Minbeiwanxian, Lucaihao, Wukezhan, Yizhixiang, Yanshuichi, Hongwan1, Jinyou1	Fujian
	Shuyazhan, Simiao, Esiniu, Qimei, Nanxiongzaoyouzhan, Baikehualuo, Heidu4, Chike'nuo, Qingsiai16B, Guanglu'ai4, Aijiaonante, Guichao2, Ergang'ai, Baoxuan21, Huangsiguizhan	Guangdong
	Hengxianliangchunbengu, Aizaizhan(selection), Hongjinghan'gu, Momi Hong'ainuo, Qiyuexian, Liusha1, Guanglu'ai15-1, Zaoshuxiangheimi	Guangxi
	Dongtingwanxian, Liuyezhan, Xuan'enchangtanqingzhan	Hubei
	Aijiaozao, Wanlixian, JinnanteB, ZhuzhenB, Zhaoyang1B, L301B, Jinnante43B, Baoxie123B, 80B, Baoxie-7B, Gu154, Gui630, IR 661-1(early), PeiC122, Teqingxuanhui, Xianghui91269, Xiang'aizao10, Xiangwanxian1, Xiangwanxian3, Xiangzaoxian7	Hunan
	Xiangdao, Hanmadao	Henan
	88B, Nanjing11, Yangdao2, Zhenxian232	Jiangsu
	Erjiunan1	Zhejiang
	Mamagu, Meihua'nuo, Zhongnong4, Honggu, Sankecun, Chengdu'ai3, Aimakang, Shufeng101, Luke3, Aituogu151, Chengnongshuijing	Sichuan
	Wenxiangnuo, Dawannuo, Qingke, Laozaogu, Qitougu, Zinuo, Haolai, Haoxiang, Haohuangla, Nan'gaogu, Jinzhinuo, Fanhaopi, Biwusheng(selection), Ximaxian, Wuzuihonggu, Dianrui409B	Yunnan
	Zhankenuo, Maweizhan, Cungu'nuo, Feie'nuo2, Yangkenuo, Xiaobaimi	Guizhou
	Jiabala	Xizang
	Taizhongzailai1/Taizhong65, Taizhongxianxuan220	Taiwan
	Menjiading2, Baoerfu	Hainan
Japonica	Funingzipijingzi, Longhuamaohulu, Gaoyangdiandaodahongmang, Shuiyuan300li, Yelicanghua	Hebei
	Zhengdao5	Henan
	Zhonglou1, Jindao1	Shanxi
	Weiguo, Dandongludao, LimingB, 76-1, Liaojing287	Liaoning
	Xingguo	Jilin
	Baimaodao, Heijing2	Heilongjiang
	Muxiqiu, Laohuzhong, Youmangzaojing, Jing7623	Shanghai
	Huangkehannianri, Baigedao, Cunsanli, Ninghui21, JWR221, Guihuahuang	Jiangsu
	Tieganwu, Xiushui115	Zhejiang
	Feidongtangdao, JinghuB, Dangyu5	Anhui
	Jinbaoyin	Fujian
	Sanlicun, Xishi15	Guangdong
	Guangkexiangnuo	Guangxi
	Bawangbian1	Hubei
	Xugunuo, Muguanuo, Hongqi5, AnnongwanjingB, Zaoshu'nonghu6B, Huhui628, Chenwan3, Jing87-304	Hunan
	Xibaizhan, Nantian'gangjiugu, Shanjiugu, Gzhenshan97B	Sichuan
	Haomake(K), Haobuka, Sanbangqishiluo, Qitoubaigu, Mubanggu, Zimi, Xianggu, Xiaohonggu, Wuzidui, Haobayong1, Gongju73, Lengshuigu2, Lengshuinuo, Lamujia, Yuyannuo, Huangpinuo, Mowanggu'neiza, Jixuenuo, Banjiemang, babaili, Beizinuo	Yunnan
	Zegu, Magunuo, Xiangnuo, Hongkezhenuo(2), Youzhan, Guantuibaihe1, Haolvguangzhan	Guizhou
	Maguzi	Shanxi
	Putaohuang	Tianjin
	Taidongludao328, Taizhong65/TaizhongHR539	Taiwan
	Menjiagao1	Hainan
	Zhonghua8, Sibeitichao6	Beijing

gene, including *indica* rice (Nanjing11, IR36, Guanglu'ai4) and *japonica* rice (Qiuguang, Shennong 15 and Shennong 265) were used as control.

1.2 Primer design

Two primers S5-t1 (test primer) and S5-t2 (sequence primer) were designed to study presence or absence of S_5^n gene (spanning the deletion sequence of EU889293 in 02428) which was published by Chen et al.[13]. Both markers contained same forward primer (S5-t1f and S5-t2f) 5′-CGTCTTGCTTCTTCATTCCC-3′ located at 143 bp upstream of start codon ATG of S_5^n. Reverse primer of S5-t1 marker (S5-t1r) 5′-GTAGGTAAACACAGGCAGAG-3′ started at 355 bp downstream of start codon of S_5 in Nipponbare and 219 bp of S_5^n in 02428, respectively (Figure 1). The PCR product by test primers was predicted to be 381-bp length in rice varieties with S_5^n and 517 bp without S_5^n, respectively. Reverse primers of S5-t2 (S5-t2r) 5′-AGAGTTCACAGCGTGATCTG-3′ was located at 688 bp downstream of ATG of S_5 in Nipponbare (552 bp in 02428). PCR product of 695bp covering the deletion sequence of S_5^n was obtained by sequence primers. Primers were synthesized by Shanghai Sangon Biological Engineering Technology and Services CO. Ltd.

1.3 PCR analysis

DNA was extracted from young leaves harvested from rice varieties and PCR was conducted according to Panaud et al.[14] with a little modification. The reaction reagent of 20 μL included 0.15 μmol/L primer, 200 μmol/L dNTP, 1×PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl pH 8.3, 1.5 mmol/L $MgCl_2$, 0.01% glutin), 10—30 ng DNA template and 1 U *Taq*. PCR amplification were performed with PTC-100 PCR machine with the following profile: 5 min at 94℃ to denature, followed by 33 cycles of 1 min at 94℃, 1 min at 58℃, and 1 min at 72℃, and a final extension period of 72℃ for 5 min to complete the reaction. PCR product was checked on 1.0% agarose gel and recorded and repeated thrice. The PCR product was sequenced by Invitrogen (Shanghai), while alignment and similarity analysis were carried out by using MegAlign of DNAstar, clustalx 1.83 and GeneDoc.

2 Results

2.1 Feasibility of S_5^n gene functional molecular marker to detect the varieties with S_5^n gene

Using primers S5t1f and S5t1r markers, PCR amplification was performed on varieties with S_5^n gene, 02428 and Linglun, and varieties without S_5^n gene, including *indica* rice (Nanjing11, IR36, Guanglu'ai4) and *japonica* rice (Qiuguang, Shennong15 and Shennong265), and then the PCR amplification products were checked by using agarose gel. A remarkable 381-bp length DNA band was found in 02428 and Linglun, while 517 bp in the varieties without S_5^n gene, such as Nanjing11 etc. The PCR products were identical with the predicted results and results were verified by triplicate experiments. The results suggested that S5-t1 marker was accurate to distinguish varieties for presence or absence of S_5^n gene. The primers could be considered as gene functional molecular marker of S_5^n.

2.2 Identification of varieties with S_5^n gene by using the functional marker

Using primers S5-t1f and S5-t1r, PCR amplification was done with the DNA of 197 varieties. Electrophoresis analysis of PCR products showed that the length of DNA bands from *japonica* varieties Haobuka, Sanbangqishiluo, Mubanggu, Xiaohonggu, Mowanggu'neiza and *indica* varieties Laozaogu, Fanhaopi, Feie'nuo2, Baoxie-7B, Teqingxuanhui were identical with those in the varieties with S_5^n gene, such as 02428 and Linglun (Figure 2). It suggested that might be these varieties having S_5^n gene. However, the other varieties represented only 517-bp length bands similar to those without S_5^n gene, such as Nanjing11 and Qiuguang etc.

2.3 Sequence analysis of S_5 locus in 10 varieties with candidate S_5^n gene

To further determine whether the deletion sequence of S_5

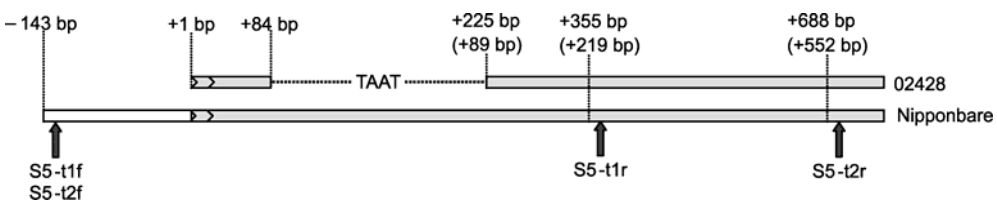

Figure 1 Location of test and sequence primers at S_5^n gene.

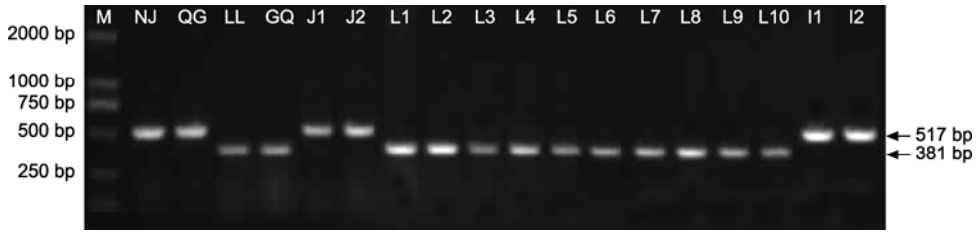

Figure 2 Electrophoretogram of the PCR amplification products using the test primer. M, DL2000 Marker; NJ, Nanjing11; QG, Qiuguang; LL, Linglun; GQ, 02428; J1, Funingzipijingzi (*japonica*); J2, Cunsanli (*japonica*); L1, Haobuka; L2, Sanbangqishiluo; L3, Mubanggu; L4, Xiaohonggu; L5, Laozaogu; L6, Mowanggu'neiza; L7, Fanhaopi; L8, Feie'nuo2; L9, Baoxie-7B; L10, Teqingxuanhui; I1, Liushizao (*indica*); I2, Qiuqianbai (*indica*). Electrophoretogram only showed the DNA band of four varieties without S_5^n gene from the Rice Micro-core Collection (including Funingzipijingzi (*japonica*), Cunsanli (*japonica*), Liushizao (*indica*), Qiuqianbai (*indica*); other varieties were not shown); The DNA band in IR36, Guanglu'ai 4, Shennong15 and Shennong265 were not shown in the electrophoretogram, which were the same as those in the other varieties without S_5^n gene.

locus in 10 varieties were identical with those of 02428 and Linglun, sequence primer S5-t2 was used to amplify S_5 gene sequences of 10 varieties, as well as 02428 and Linglun. Subsequent sequence analysis of PCR products demonstrated that the deletion DNA fragments and DNA sequence spanning the deletion in S_5 locus of 02428 and Linglun were identical with those sequence reported by Chen et al.[13]. It was noteworthy that the similar DNA deletion and sequence spanning the deletion were also found in the 10 varieties, which were newly detected with candidate S_5^n gene, besides the replacement of G at 388bp in 02428 by A in Xiaohonggu, Laozaogu, Mowanggu'neiza and Fanhaopi. The results suggested that the varieties, such as Haobuka, Sanbangqishiluo, Mubanggu, Xiaohonggu, Mowanggu'neiza, Laozaogu, Fanhaopi, Feie'nuo2, Baoxie-7B and Teqingxuanhui contained S_5^n gene indeed.

3 Discussion

3.1 Rice germplasm with the S_5^n gene can be identified effectively using the gene functional marker and DNA sequencing

In recent years, with the successful cloning of functional genes in rice[15], broad attention has been drawn to usage of gene sequence information in exploring new germplasm. The special sequence and unique function of target genes play a key role in exploring new germplasm with known gene information. Chen et al.[13] reported that S_5^n gene deleted a large DNA fragment, compared with S_5^i gene in typical *indica* rice and S_5^j in typical *japonica* rice. Due to deletion in S_5 locus, S_5^n gene could not be transcribed and translated into the proper protein (Aspartic protease), thus inducing embryo sac fertility in hybrids with the S_5^n/S_5^i or S_5^n/S_5^i genotype. The sequence deletion of the S_5^n gene provides an opportunity to find new rice germplasms with the S_5^n gene directly and effectively. In this study, by comparing S_5^n gene sequence of 02428 with S_5^j of Nipponbare, we designed a pair of specific DNA primers flanking the deletion region of the S_5^n gene. Applying the primers, varieties with the S_5^n gene (02428 and Linglun) and without S_5^n gene (Nanjing11 and IR36) can be distinguished by PCR amplification. These primers can be successfully used as the S_5^n functional molecular markers, determining the existence of the S_5^n gene in screened samples. The functional markers were applied to examine 197 varieties obtained from China National Micro-core Rice Collection, and ten varieties namely Haobuka, Xiaohonggu, Mubanggu, Sanbangqishiluo, Laozaogu, Mowanggu'neiza, Fanhaopi, Feie'nuo2, Baoxie-7B, and Teqingxuanhui were found to be containing S_5^n gene. The DNA sequence of these ten varieties spanning S_5^n gene was analyzed and it was found that the deleted sequence and its flanking sides were identical to 02428 and Linglun, indicating that the S_5 locus of the ten varieties has no function and the S_5^n gene exists. It is worth mentioning that among the ten varieties, two varieties (Sanbangqishiluo and Laozaogu) had already been reported containing S_5^n gene [2,16]. Therefore, we demonstrated here possibility of mining new rice germplasm with the S_5^n gene effectively by using S_5^n functional molecular markers.

3.2 Yunnan Province may be the main location of Chinese rice varieties with the S_5^n gene

Many rice varieties (lines) with the S_5^n gene have been developed by various crossing methods throughout the world (Table 2). In China, a large number of rice varieties containing S_5^n gene were derived from the local va-

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Nanjing11  ATCAACCCAT TTCCTTTCCT ACGTTTGACT GCCTGCCTGC CCCTGAGCAA GCAAGAAAGA AAGAAAGAAG
02428      ATCAACCCAT TTCCTTTCCT ACGTTTGACT GCCTGCCTGC CCCTGAGCAA GCAAGAAAGA AAGAAAGAAG
Linglun    ATCAACCCAT TTCCTTTCCT ACGTTTGACT GCCTGCCTGC CCCTGAGCAA GCAAGAAAGA AAGAAAGAAG
L1         ATCAACCCAT TTCCTTTCCT ACGTTTGACT GCCTGCCTGC CCCTGAGCAA GCAAGAAAGA AAGAAAGAAG
L2         ATCAACCCAT TTCCTTTCCT ACGTTTGACT GCCTGCCTGC CCCTGAGCAA GCAAGAAAGA AAGAAAGAAG
L3         ATCAACCCAT TTCCTTTCCT ACGTTTGACT GCCTGCCTGC CCCTGAGCAA GCAAGAAAGA AAGAAAGAAG
L4         ATCAACCCAT TTCCTTTCCT ACGTTTGACT GCCTGCCTGC CCCTGAGCAA GCAAGAAAGA AAGAAAGAAG
L5         ATCAACCCAT TTCCTTTCCT ACGTTTGACT GCCTGCCTGC CCCTGAGCAA GCAAGAAAGA AAGAAAGAAG
L6         ATCAACCCAT TTCCTTTCCT ACGTTTGACT GCCTGCCTGC CCCTGAGCAA GCAAGAAAGA AAGAAAGAAG
L7         ATCAACCCAT TTCCTTTCCT ACGTTTGACT GCCTGCCTGC CCCTGAGCAA GCAAGAAAGA AAGAAAGAAG
L8         ATCAACCCAT TTCCTTTCCT ACGTTTGACT GCCTGCCTGC CCCTGAGCAA GCAAGAAAGA AAGAAAGAAG
L9         ATCAACCCAT TTCCTTTCCT ACGTTTGACT GCCTGCCTGC CCCTGAGCAA GCAAGAAAGA AAGAAAGAAG
L10        ATCAACCCAT TTCCTTTCCT ACGTTTGACT GCCTGCCTGC CCCTGAGCAA GCAAGAAAGA AAGAAAGAAG
Nipponbare ATCAACCCAT TTCCTTTCCT ACGTTTGACT GCCTGCCTGC CCCTGAGCAA GCAAGAAAGA AAGAAAGAAG

Nanjing11  GGATTAAATT TGCTCGCTCC TACGAATCCT GCCCCTGAGT AACAATGACT GACTTTTAAT TTGTTTGCAG
02428      GGATTAAATT TGCT..... ..... ..... ..... .....TAAT .....
Linglun    GGATTAAATT TGCT..... ..... ..... ..... .....TAAT .....
L1         GGATTAAATT TGCT..... ..... ..... ..... .....TAAT .....
L2         GGATTAAATT TGCT..... ..... ..... ..... .....TAAT .....
L3         GGATTAAATT TGCT..... ..... ..... ..... .....TAAT .....
L4         GGATTAAATT TGCT..... ..... ..... ..... .....TAAT .....
L5         GGATTAAATT TGCT..... ..... ..... ..... .....TAAT .....
L6         GGATTAAATT TGCT..... ..... ..... ..... .....TAAT .....
L7         GGATTAAATT TGCT..... ..... ..... ..... .....TAAT .....
L8         GGATTAAATT TGCT..... ..... ..... ..... .....TAAT .....
L9         GGATTAAATT TGCT..... ..... ..... ..... .....TAAT .....
L10        GGATTAAATT TGCT..... ..... ..... ..... .....TAAT .....
Nipponbare GGATTAAATT TGCTCGCTCC TACGAATCCT GCCCCTGAGT AACAATGACT GACTTTTAAT TTGTTTGCAG

Nanjing11  CTAGGGTGGG GATCGAGATG GTGATCTTGG AGCAGCCACA GCTGCTCCTT CTTCTTCTTC TTCTTGTAGC
02428      ..... ..... ..... ..... ..... ..... .....
Linglun    ..... ..... ..... ..... ..... ..... .....
L1         ..... ..... ..... ..... ..... ..... .....
L2         ..... ..... ..... ..... ..... ..... .....
L3         ..... ..... ..... ..... ..... ..... .....
L4         ..... ..... ..... ..... ..... ..... .....
L5         ..... ..... ..... ..... ..... ..... .....
L6         ..... ..... ..... ..... ..... ..... .....
L7         ..... ..... ..... ..... ..... ..... .....
L8         ..... ..... ..... ..... ..... ..... .....
L9         ..... ..... ..... ..... ..... ..... .....
L10        ..... ..... ..... ..... ..... ..... .....
Nipponbare CTAGGGTGGG GATCGAGATG GTGATCTTGG AGCAGCCACA GCTGCTCCTT CTTCTTCTTC TTCTTGTAGC

Nanjing11  AGCTGCAGCT GCAACCGGCG CCACAGCAGC CGACGACGAG TTGGAGTGTC CCTCCTCCAT CTTCGGTAAG
02428      ..... ....CCGGCG CCACAGCAGC CGACGACGAG TTGGAGTGTC CCTCCTCCAT CTTCGGTAAG
Linglun    ..... ....CCGGCG CCACAGCAGC CGACGACGAG TTGGAGTGTC CCTCCTCCAT CTTCGGTAAG
L1         ..... ....CCGGCG CCACAGCAGC CGACGACGAG TTGGAGTGTC CCTCCTCCAT CTTCGGTAAG
L2         ..... ....CCGGCG CCACAGCAGC CGACGACGAG TTGGAGTGTC CCTCCTCCAT CTTCGGTAAG
L3         ..... ....CCGGCG CCACAGCAGC CGACGACGAG TTGGAGTGTC CCTCCTCCAT CTTCGGTAAG
L4         ..... ....CCGGCG CCACAGCAGC CGACGACGAG TTGGAGTGTC CCTCCTCCAT CTTCGGTAAG
L5         ..... ....CCGGCG CCACAGCAGC CGACGACGAG TTGGAGTGTC CCTCCTCCAT CTTCGGTAAG
L6         ..... ....CCGGCG CCACAGCAGC CGACGACGAG TTGGAGTGTC CCTCCTCCAT CTTCGGTAAG
L7         ..... ....CCGGCG CCACAGCAGC CGACGACGAG TTGGAGTGTC CCTCCTCCAT CTTCGGTAAG
L8         ..... ....CCGGCG CCACAGCAGC CGACGACGAG TTGGAGTGTC CCTCCTCCAT CTTCGGTAAG
L9         ..... ....CCGGCG CCACAGCAGC CGACGACGAG TTGGAGTGTC CCTCCTCCAT CTTCGGTAAG
L10        ..... ....CCGGCG CCACAGCAGC CGACGACGAG TTGGAGTGTC CCTCCTCCAT CTTCGGTAAG
Nipponbare AGCTGCAGCT GCAACCGGCG CCACAGCAGC CGACGACGAG TTGGAGTGTC CCTCCTCCAT CTTCGGTAAG
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Figure 3 Comparison of deletion region and its both side sequence in S_5 locus among the 10 rice varieties with S_5^n gene and other control varieties (only adjacent sequence was displayed, other parts were not shown). L1, Haobuka; L2, Sanbangqishiluo; L3, Mubanggu; L4, Xiaohonggu; L5, Laozaogu; L6, Mowanggu'neiza; L7, Fanhaopi; L8, Feie'nuo2; L9, Baoxie-7B; L10, Teqingxuanhui. The sequence of Nanjing11 was quoted from the sequence of Chen et al.[13]; the sequence of Nipponbare was extracted from the *Oryza sativa Japonica* Group genomic DNA, chromosome 6, PAC clone P0701E03 (http://ncbi.nlm.nih.gov/sites/entrez).

Table 2 Rice varieties containing the S_5^n gene

Type	Name of varieties
Indica	Peidi[2], Aizizhan[2], Laozaogu[2],a), Taiwanluxian[2], PeiC[2], Peiai 64[5,6], Ce03[6], Guang09[6], Guangqin 1[6], Ce64[6], Hong C311[6] , Linglun[6,7], Zhong413[8], Kuntlan[8], Aus371[8], Zhongdafei[8], Qiaizhan[8], Yu92qiu14[8], Suzaozhan[8], Hainanzhan[8]
Japonica	02428[2,4,8,16], Morobereken[2], Tyahak[2], Shinkei 8544[2], Cpslo17[4,5,16] , Ketan Nanka[2,16,17],Sanbangqishiluo[16],a), PeiC311[5], Lunhui422[5], Lemont[4,5,10,17], Bellmont[17] , Jw12[2], Jw28[2], Jw201[2], Rtn50-1-1[8], Rexmont[8], Labelle[8], Copslo[8], Lebonnet[8], Newbonnet[8], Nortai[8], Newrex[8], Kp502-3[8]
Restore line	SWR78[10], 4183[18] , SWR20[19], Minghui 86[9], H921[20]
Sterile line	Peiai 64S[2,7] , Suqiu A[10]
Others[b)]	PadiBujang Pendex[2], Dular[4,5,21], Aus373[2,5],Reyan 2[2], Zhenxiqiuguang[5], Jian12[5], E-146[5], Pecos[5], Pelde[5], Calotoc[5], S26[6], S18[6], T984[6], MZ/6355-EBR[6], AUS1[6], AUS3[6], AUS5[6], Padi-buha-bulu[6],Gundilgendjah[6],Sade AUS[6], Aiga[5,22,24], a), Haogelao[16], Woaiga[23,24],a), Hua'nuo[23,24],a), Chongtui[24],a), E'aiga[24],a), Dabaigu[24],a), Maobaigu[16,24],a), BP176[17], MCP231-2[17]

a) The local varieties in Yunnan Province; b) no exact data about the concerned type given.

rieties native to Yunnan Province. Examined by the S_5^n functional markers and DNA sequencing, a total of ten rice varieties with the S_5^n gene were identified from the China National Micro-core Rice Collection, including five *indica* and five *japonica* cultivars. Among them, seven varieties were from Yunnan Province, two from Hunan Province, and one from Hubei Province, suggesting that Yunnan's varieties are a rich source of S_5^n gene[25]. Therefore it is recommended that rice varieties belonging to Yunnan Province might be examined extensively, for the S_5^n gene in order to enrich genetic resources for *indica-japonica* hybrid breeding.

3.3 Can the S_5^n gene be referred to as the "wide-compatible" gene?

The S_5^n gene, which could overcome the inter-subspecific hybrid sterility, has been widely accepted as a "wide-compatibility gene" since it was first proposed by Ikehashi and Araki[3]. A large number of studies showed that apart from S_5 at least six loci (such as S_7 and S_8) lead to sterile embryo sacs in hybrids due to allelic and epistatic interaction[2], therefore S_5^n gene is unable to overcome the abortion caused by other loci. In addition to the embryo sac sterility, pollen sterility, abnormal fertilization, abnormal embryogenesis, and endosperm development could also contribute to *indica-japonica* hybrid sterility. Low pollen fertility along with embryo sac abortion is regarded as two important causes of inter-subspecific hybrid sterility[26]. In some hybrids, pollen sterility has pronounced effect as compared to embryo sac abortion. Pollen and embryo sac sterility in *indica-japonica* hybrids are controlled by two different genetic systems. It was reported that pollen sterility was controlled by at least six loci, and the degree of pollen sterility was affected by allelic and epistatic interaction at various loci[27], while similar gene interaction controled embryo sac sterility in hybrids[2]. These results showed that the mechanisms of *indica-japonica* hybrid sterility are very complicated. Overcoming sterility in *indica-japonica* hybrid requires a number of genes, besides S_5^n gene. Therefore, naming S_5^n gene alone as a "wide compatible gene" should be considered as deliberate, and due to this reason S_5^n gene was not referred to as "wide compatible gene" in this study. In addition, the test-cross method to detect the S_5^n gene was also deserved to be argued. Traditionally, if the tested variety is crossed with a certain number of typical *indica* and *japonica* varieties, and their F_1 hybrids possess 70% or higher spikelet fertility, this variety is considered to contain the wide compatible gene[2]. It could be inferred that if spikelet fertility of all test cross F_1 are over 70%, then the main genes controlling both embryo sac and pollen fertility should be attributed as widely compatible. Clearly, other "neutral" genes were also required to overcome the F_1 sterility, in addition to the S_5^n gene. Otherwise, the spikelet fertility of test-cross F_1 would be less than 70%. It was speculated that if the method of test-crosses was exclusively used to detect materials with or without the S_5^n gene, two kinds of misjudgments would be encountered. Firstly, the tested material was assumed to be lacking S_5^n gene, because of low spikelet sterility of test-cross F_1, actually it happened due to low embryo sac fertility which was caused by genetic interaction between other locus (such as S_7 or S_8), but not S_5, although it had the S_5^n gene. Secondly, the material was considered as non-S_5^n germplasm for low hybrid's spikelet fertility, which might be the result of high pollen sterility, caused by the interaction of pollen sterility genes from the tested materials and species, even if the tested materials contained the S_5^n gene. Moreover, test-cross method's results might be affected by environmental factors that become one of the important reasons for the inconsistent results obtained by different

researchers with the same material[2,24,28]. Certainly, reliability of the test-cross method should not be denied, but this method may not be valid to determine the genotype under specific circumstances, for example, some materials with S_5^n gene could be lost. However test-cross method was believed to be the best method for detecting S_5^n gene before cloning of this gene. Since the S_5^n gene has been cloned, an effective method should be established to explore new germplasm carrying the S_5^n gene. In this study, a novel method was proposed to explore new germplasm containing S_5^n gene, based on the DNA information of S_5^n gene, and it was proved to be feasible. The procedure of the method mainly included preliminary screening of germplasm with functional molecular markers of S_5^n gene and subsequent sequencing of the special DNA segment.

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