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Variation of the *OsGI* intron and its phenotypic associations in *Oryza rufipogon* Griff. and *Oryza sativa* L.

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ABSTRACT. We analyzed intron 9 of the *OsGI* gene in *Oryza rufipogon* and *Oryza sativa* in order to investigate evolutionary relationships in rice and the relationship between intron variation and phenotype. *OsGI-9* was cloned in 38 *O. rufipogon* populations and in 139 *O. sativa* cultivars and the phylogeny was reconstructed. Seed cold tolerance and dormancy were quantified in *O. sativa*. Three *OsGI-9* band types occurred in *O. rufipogon*: S-type (1.2 kb), F-type (0.9 kb), and FS-type (1.2 and 0.9 kb), whereas only the S-type and F-type occurred in *O. sativa*. The S-type contains two 255-bp repeats, the F-type contains one 255-bp repeat, and the FS-type contains both. All individuals could be divided into 5 groups in the organism's phylogenetic network: S-type

O. rufipogon, F-type *O. rufipogon*, FS-type *O. rufipogon*, S-type *O. sativa*, and F-type *O. sativa*. F-type *O. sativa* are most closely related to F-type *O. rufipogon* and S-type *O. sativa* are most closely related to S-type *O. rufipogon*. Statistical analysis indicated that *OsGI-9* type is significantly correlated with phenotype; most S-type *O. sativa* have strong seed dormancy and cold tolerance, and most F-type *O. sativa* have no seed dormancy and poor cold tolerance.

Key words: *Oryza rufipogon; Oryza sativa*; *OsGI*; Phylogenetic network; Cold tolerance; Seed dormancy

INTRODUCTION

Oryza rufipogon is commonly recognized as the progenitor of *Oryza sativa* and is widely distributed in China, Southeast Asia and Southern Asia. Although both species reproduce asexually and sexually, asexual reproduction predominates in *O. rufipogon*. A high rate of cross-pollination, low reproductive isolation between *O. rufipogon* and *O. sativa*, and rich genetic diversity are valuable traits that have contributed to the improvement of *O. sativa* varieties (Lu et al., 2002). Although useful variations in *O. sativa* can be rapidly identified using genomic techniques and have been widely exploited via transgenic technology (Rakshit et al., 2007), approximately 30-40% of genetic variation was lost during rice domestication. Therefore, a valuable source of genetic variation remains within the wild germplasm (Sun et al., 2001), and it is therefore extremely important to characterize the genetic diversity of *O. rufipogon* and its phylogenetic relationship with *O. sativa*.

Phylogenetic analysis plays an important role in the characterization of evolutionary relationships between species and in the reconstruction of evolutionary processes (Lessa, 1992). Therefore, construction of reliable phylogenetic trees, using appropriate molecular markers and making full use of the phylogenetic information contained therein, is vital (Slade et al., 1994). Previous studies have used morphological features, data from cell research, and molecular markers such as isozymes or RFLP, RAPD, AFLP, SSR, SINEs, and MITE for phylogenetic analyses of A-type genome species (Ren et al., 2003). However, the species' classifications are currently unclear due to the lack of obvious morphological differences and because evolutionary relationships described in different studies are not consistent (Ren et al., 2003). Investigations into the huge potential of nuclear genes for phylogenetic analysis have increased in recent years (Slade et al., 1994; Sang, 2002). However, phylogenetic reconstruction at lower taxonomic levels has been limited due to a lack of sufficient variation within molecular sequences (Doyle et al., 1996). Intron sequences have been widely applied for the reconstruction of phylogeny at lower classification levels as they are rapidly evolving, easily cloned and nearly neutral (Dalebout et al., 2008). Nuclear genes, including introns, in diploid organisms can be homozygous or heterozygous, as a result of insertions or deletions (InDel) or base sequence variation (Creer et al., 2007). Increasing numbers of studies have indicated that hybrid introns, termed intra-individual allele heterozygotes (IIAHs), are common in nature. IIAHs provide a rich source of phylogenetic information, and can reveal potential hybridization or introgression in the analysis of phylogenetic relationships between related species or genetic structures within a species.

Haplotypes are examined in the majority of current phylogenetic analyses; however, usually only a single IIAH haplotype is examined, and the phylogenetic information contained in hybrid introns has been so far neglected. In addition, increasing numbers of studies have indicated that intron diversity is related to many human diseases and phenotypic variations in animals and plants (Reszka et al., 2006). High outcrossing rates and intron heterozygosity are ubiquitous in *O. rufipogon* (Lu et al., 2002). Therefore, phylogenetic information contained in IIAHs should not be ignored during the reconstruction of *O. rufipogon* phylogeny using introns and for analyses of intron function.

Gigantea (*GI*) is a circadian clock-controlled gene that regulates photoperiodic flowering in *Arabidopsis* (Fowler et al., 1999), and also plays a role in phytochrome B signaling, the circadian clock, carbohydrate metabolism, fruit setting, and cold stress response (Brock et al., 2007). *OsGI*, a single-copy gene, was identified as the rice ortholog of *Arabidopsis GI* and is an important regulator of flowering. In this study, the *OsGI* intron in 38 Chinese *O. rufipogon* samples and in 139 *O. sativa* cultivars from around the world were used for phylogenetic reconstruction, and the correlation between *OsGI* intron variation and *O. sativa* phenotypes were analyzed in order to determine the impact of *OsGI* intron variation on rice evolution.

MATERIAL AND METHODS

Material collection

Thirty-eight *O. rufipogon* populations from China were selected for this study. Twelve populations were obtained from the Hainan Island Province and 26 populations were obtained from 6 inland Provinces: Guangdong (N = 5), Guangxi (N = 17), Yunnan (N = 1), Hunan (N = 1), Fujian (N = 1), Jiangxi (N = 1) (Figure 1). The leaves of individual plants were collected from the populations in Hainan Island, Guangdong, Jiangxi, and Yunnan Provinces. To avoid replication of the same clones, samples were collected at intervals greater than 12 m (Xie et al., 2001). *O. rufipogon* seeds were collected from the populations in Guangxi, Hunan, and Fujian Provinces. Detailed sampling information for all of the *O. rufipogon* samples is provided in Table 1. The *O. rufipogon* samples from Hainan Island, Guangdong, Yunnan, and Jiangxi Provinces were collected by members of our laboratory, and the other samples were provided by the Crop Science Research Institute and the China National Rice Research Institute at the Chinese Academy of Agricultural Sciences and the Guangdong Academy of Agricultural Sciences. The leaves of individual plants from each population were clipped for DNA extraction. Seed samples were germinated on Petri dishes using moist filter paper and the seedlings were harvested for DNA extraction.

The seeds of 139 *O. sativa* cultivars were collected from around the world; 65 cultivars were from various provinces in China and 74 cultivars were from other countries (Table 1). All of the seeds were germinated and the seedlings were harvested for DNA extraction.

Gene amplification and sequencing strategy

Nuclear alleles within *O. rufipogon* individuals may be heterozygous due to the high cross-pollination rate; therefore, it was necessary to account for allele variation. In order to find individual allelic differences and avoid errors during polymerase chain reaction (PCR)

amplification, genomic DNA was extracted from each individual using a high-fidelity polymerase (AccuPrime *Taq* DNA polymerase, Invitrogen) for PCR amplification, and at least 7 clones from each individual were sequenced. Mutations between haplotypes of a single individual located at informative sites were considered to be allelic variation, while mutations located at singleton variable sites were considered to be errors from PCR amplification.

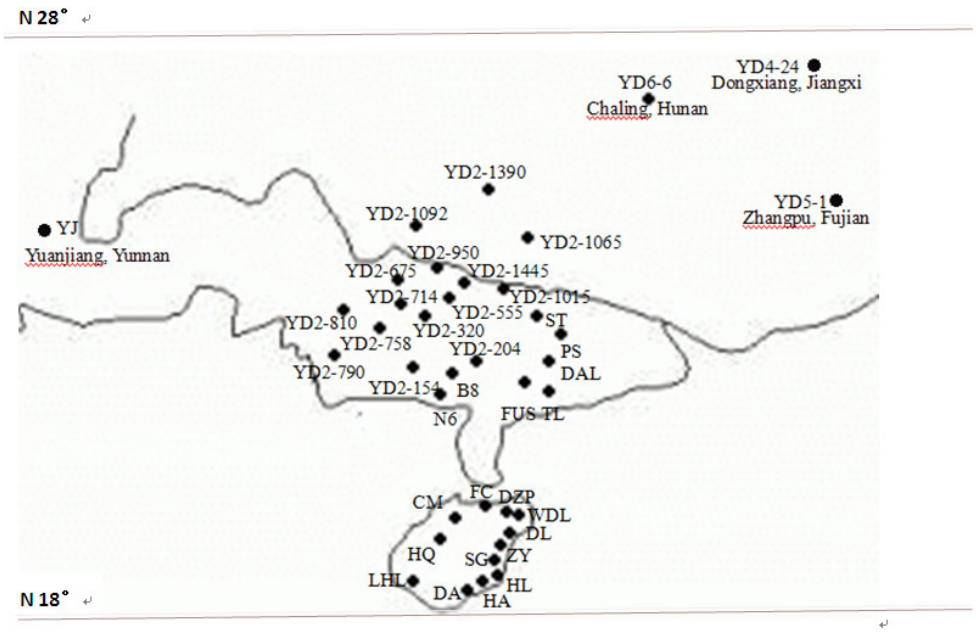

Figure 1. Distribution map of *Oryza rufipogon* populations from China.

DNA extraction, PCR amplification, cloning, and sequence analysis

Based on the sequence of *OsGI* from *O. sativa japonica* Nipponbare (GenBank accession AP003047), specific primers were designed for *OsGI* intron 9 (*OsGI-9*) using the exon-primed, intron-crossing method (forward 5'-ACAATGGCAAGTATAGGCTCC-3' and reverse 5'-ATCTTCATTTCCTGCGTGC-3'; Figure 2). Total DNA was extracted using a modification of the CTAB method (Xie et al., 1999). PCR amplification was performed in a total volume of 50 µL, containing 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each primer, 0.025 U/µL AccuPrime Taq DNA (Invitrogen), and 5 ng/µL template DNA. Amplification was carried out for 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 54°C, and 80 s at 72°C, with a final extension at 72°C for 10 min. The amplification products were separated by electrophoresis on 1.2% agarose gels, stained with ethidium bromide and images were captured using a gel imaging system (GBox, Gene Company). PCR fragments were purified using a DNA gel extraction kit (Axygen Biosciences) and cloned into the pEASY-T3 cloning vector (Transgen Biotech). Independent plasmids were randomly selected and at least 7 positive clones were individually sequenced, aligned and analyzed using Vector NTI advance 10 (Invitrogen).

Table 1. Detailed information on the *Oryza rufipogon* and *O. sativa* samples used in this study.

Code	Origin	Taxa	Samples type	Number of samples
DA	Dong'ao, Hainan Province, China	*O. rufipogon*	Individual plant	128
DZP	Dazhipo, Hainan Province, China	*O. rufipogon*	Individual plant	18
LHL	Ledong, Hainan Province, China	*O. rufipogon*	Individual plant	7
SG	Shangen, Hainan Province, China	*O. rufipogon*	Individual plant	3
HQ	Danzhou, Hainan Province, China	*O. rufipogon*	Individual plant	14
WDL	Donglu, Hainan Province, China	*O. rufipogon*	Individual plant	14
ZY	Zhongyuan, Hainan Province, China	*O. rufipogon*	Individual plant	13
DL	Dalu, Hainan Province, China	*O. rufipogon*	Individual plant	16
HA	Houan, Hainan Province, China	*O. rufipogon*	Individual plant	4
HL	Hele, Hainan Province, China	*O. rufipogon*	Individual plant	3
FC	Fuchen, Hainan Province, China	*O. rufipogon*	Individual plant	4
CM	Chengmai, Hainan Province, China	*O. rufipogon*	Individual plant	7
YJ	Yuanjiang, Yunnan Province, China	*O. rufipogon*	Individual plant	18
PS	Pengshan, Guangdong Province, China	*O. rufipogon*	Individual plant	16
ST	Shatian, Guangdong Province, China	*O. rufipogon*	Individual plant	15
TL	Tanlu, Guangdong Province, China	*O. rufipogon*	Individual plant	17
DAL	Daling, Guangdong Province, China	*O. rufipogon*	Individual plant	13
FUS	Fushi, Guangdong Province, China	*O. rufipogon*	Individual plant	6
YD4-24	Dongxiang, Jiangxi Province, China	*O. rufipogon*	Individual plant	52
N6	Beihai, Guangxi Province, China	*O. rufipogon*	Seeds	10
B8	Bobai, Guangxi Province, China	*O. rufipogon*	Seeds	10
YD2-154	Lingshan, Guangxi Province, China	*O. rufipogon*	Seeds	10
YD2-204	Yulin, Guangxi Province, China	*O. rufipogon*	Seeds	10
YD2-320	Guixian, Guangxi Province, China	*O. rufipogon*	Seeds	10
YD2-555	Guiping, Guangxi Province, China	*O. rufipogon*	Seeds	10
YD2-675	Wuming, Guangxi Province, China	*O. rufipogon*	Seeds	10
YD2-714	Hengxian, Guangxi Province, China	*O. rufipogon*	Seeds	10
YD2-758	Fusui, Guangxi Province, China	*O. rufipogon*	Seeds	10
YD2-790	Zongzuo, Guangxi Province, China	*O. rufipogon*	Seeds	10
YD2-810	Long'an, Guangxi Province, China	*O. rufipogon*	Seeds	10
YD2-950	Shanglin, Guangxi Province, China	*O. rufipogon*	Seeds	10
YD2-1015	Tengxian, Guangxi Province, China	*O. rufipogon*	Seeds	10
YD2-1065	Hexian, Guangxi Province, China	*O. rufipogon*	Seeds	10
YD2-1092	Liucheng, Guangxi Province, China	*O. rufipogon*	Seeds	10
YD2-1390	Xiangshan, Guangxi Province, China	*O. rufipogon*	Seeds	10
YD2-1445	Wuxuan, Guangxi Province, China	*O. rufipogon*	Seeds	10
YD5-1	Zhangpu, Fujian Province, China	*O. rufipogon*	Seeds	10
YD6-66	Chaling, Hunan Province, China	*O. rufipogon*	Seeds	10
Mudanjiang 19	Heilongjiang Province, China	*O. sativa*	Seeds	10
Ji 91-2605	Jilin Province, China	*O. sativa*	Seeds	10
Liao 201	Liaoning Province, China	*O. sativa*	Seeds	10
86XW-17	Ningxia Province, China	*O. sativa*	Seeds	10
9011	Xinjiang Province, China	*O. sativa*	Seeds	10
91-13-11	Shangxi Province, China	*O. sativa*	Seeds	10
Laolongxu	Shangxi Province, China	*O. sativa*	Seeds	10
Xinong 8116	Shangxi Province, China	*O. sativa*	Seeds	10
Qiannong 5782	Guizhou Province, China	*O. sativa*	Seeds	10
Jinxi 870441	Shanxi Province, China	*O. sativa*	Seeds	10
Kenyu 16	Hebei Province, China	*O. sativa*	Seeds	10
Zhonghua 11	Beijing, China	*O. sativa*	Seeds	10
Zhonghua 8	Beijing, China	*O. sativa*	Seeds	10
Zhongzuo 8604	Beijing, China	*O. sativa*	Seeds	10
6017	Beijing, China	*O. sativa*	Seeds	10
Yinfang	Tianjing, China	*O. sativa*	Seeds	10
Putaohuang	Tianjing, China	*O. sativa*	Seeds	10
Lujing 1	Shandong Province, China	*O. sativa*	Seeds	10
Zhengdao 5	Henan Province, China	*O. sativa*	Seeds	10
Xinyang 14	Henan Province, China	*O. sativa*	Seeds	10
Wanjing 1	Anhui Province, China	*O. sativa*	Seeds	10
Anxuan 4	Anhui Province, China	*O. sativa*	Seeds	10
Liushizao	Anhui Province, China	*O. sativa*	Seeds	10
Wuyujing	Jiangsu Province, China	*O. sativa*	Seeds	10
Shuangqing	Jiangsu Province, China	*O. sativa*	Seeds	10
Nanjing 11	Jiangsu Province, China	*O. sativa*	Seeds	10

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Table 1. Continued.

Code	Origin	Taxa	Samples type	Number of samples
Nannong 4008	Jiangsu Province, China	*O. sativa*	Seeds	10
9311	Jiangsu Province, China	*O. sativa*	Seeds	10
Shuangfeng 1	Shanghai Province, China	*O. sativa*	Seeds	10
Yingtoujing	Zhejiang Province, China	*O. sativa*	Seeds	10
Zheli 1	Zhejiang Province, China	*O. sativa*	Seeds	10
Zaoxianmi	Zhejiang Province, China	*O. sativa*	Seeds	10
Zaoshu 691	Hubei Province, China	*O. sativa*	Seeds	10
3635	Hubei Province, China	*O. sativa*	Seeds	10
Xiangjing 2	Hunan Province, China	*O. sativa*	Seeds	10
Muguanuo	Hunan Province, China	*O. sativa*	Seeds	10
Xiangzaoxian	Hunan Province, China	*O. sativa*	Seeds	10
86-106	Hunan Province, China	*O. sativa*	Seeds	10
Gaoyuanjing 1	Sichuan Province, China	*O. sativa*	Seeds	10
Meihuanuo	Sichuan Province, China	*O. sativa*	Seeds	10
Hongmangdazu	Sichuan Province, China	*O. sativa*	Seeds	10
Chuanmi 2	Sichuan Province, China	*O. sativa*	Seeds	10
Jiushizao	Sichuan Province, China	*O. sativa*	Seeds	10
Gannongwanjing 2	Jiangxi Province, China	*O. sativa*	Seeds	10
4434	Jiangxi Province, China	*O. sativa*	Seeds	10
Nante	Jiangxi Province, China	*O. sativa*	Seeds	10
Jinxibai	Jiangxi Province, China	*O. sativa*	Seeds	10
Sanbaili	Jiangxi Province, China	*O. sativa*	Seeds	10
Hongwei 1	Fujian Province, China	*O. sativa*	Seeds	10
Minghui 63	Fujian Province, China	*O. sativa*	Seeds	10
Dijiaowujian	Fujian Province, China	*O. sativa*	Seeds	10
Xixuan 4	Fujian Province, China	*O. sativa*	Seeds	10
Jinxingdanuo	Guangdong Province, China	*O. sativa*	Seeds	10
Guangluai 4	Guangdong Province, China	*O. sativa*	Seeds	10
GD-5S	Guangdong Province, China	*O. sativa*	Seeds	10
Kuyexiangnuo	Guangxi Province, China	*O. sativa*	Seeds	10
Baise 1	Guangxi Province, China	*O. sativa*	Seeds	10
Tainan 6	Taiwan, China	*O. sativa*	Seeds	10
Gaoxiongyu 122	Taiwan, China	*O. sativa*	Seeds	10
Jianongxianyu 31	Taiwan, China	*O. sativa*	Seeds	10
Taizhongxianxuan 220	Taiwan, China	*O. sativa*	Seeds	10
Menjiagao	Hainan Province, China	*O. sativa*	Seeds	10
Tonghong'ai	Hainan Province, China	*O. sativa*	Seeds	10
Xianzhan	Hainan Province, China	*O. sativa*	Seeds	10
Qiuqihong	Hainan Province, China	*O. sativa*	Seeds	10
Qiuguang	Japan	*O. sativa*	Seeds	10
Nipponbare	Japan	*O. sativa*	Seeds	10
Changyeyu	Japan	*O. sativa*	Seeds	10
Beijin	Japan	*O. sativa*	Seeds	10
Youliujiannuo	Japan	*O. sativa*	Seeds	10
Jinuo	Japan	*O. sativa*	Seeds	10
Shuiyuan 354	Korea	*O. sativa*	Seeds	10
Shuiyuan 380	Korea	*O. sativa*	Seeds	10
Lili 372	Korea	*O. sativa*	Seeds	10
Chizhenzhu	Korea	*O. sativa*	Seeds	10
JINBU 9	Korea	*O. sativa*	Seeds	10
Jiexiaonuo	Korea	*O. sativa*	Seeds	10
Starbonnet CI9584	USA	*O. sativa*	Seeds	10
Sunbonnet	USA	*O. sativa*	Seeds	10
CALROSF	USA	*O. sativa*	Seeds	10
EDITH	USA	*O. sativa*	Seeds	10
BU189	Brazil	*O. sativa*	Seeds	10
BU342	Brazil	*O. sativa*	Seeds	10
BU349	Brazil	*O. sativa*	Seeds	10
BU412	Brazil	*O. sativa*	Seeds	10
Balilla	Italy	*O. sativa*	Seeds	10
Angke	Indonesia	*O. sativa*	Seeds	10
Bp205f-Kn-78-1	Indonesia	*O. sativa*	Seeds	10
BP1356-1g-Kn-4	Indonesia	*O. sativa*	Seeds	10
CR60	Cambodia	*O. sativa*	Seeds	10

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Table 1. Continued.

Code	Origin	Taxa	Samples type	Number of samples
1v-139	Vietnam	*O. sativa*	Seeds	10
NR11	Vietnam	*O. sativa*	Seeds	10
VR345	Vietnam	*O. sativa*	Seeds	10
VR349	Vietnam	*O. sativa*	Seeds	10
VR350	Vietnam	*O. sativa*	Seeds	10
VR340	Vietnam	*O. sativa*	Seeds	10
VR347	Vietnam	*O. sativa*	Seeds	10
Matant MF	India	*O. sativa*	Seeds	10
Mulant of dwarf	India	*O. sativa*	Seeds	10
PSRM1-17	India	*O. sativa*	Seeds	10
RP1667-301-1196-1562	India	*O. sativa*	Seeds	10
RP1670-1418-2205-1582	India	*O. sativa*	Seeds	10
Toga	India	*O. sativa*	Seeds	10
CNTLR85033-9-3-1-1	Thailand	*O. sativa*	Seeds	10
SPR85163-5-1-2	Thailand	*O. sativa*	Seeds	10
C. Costo	Burma	*O. sativa*	Seeds	10
Mya-1	Burma	*O. sativa*	Seeds	10
Mya-2	Burma	*O. sativa*	Seeds	10
Manan Thukho	Burma	*O. sativa*	Seeds	10
FAON11	Burma	*O. sativa*	Seeds	10
AZUCENA	Philippines	*O. sativa*	Seeds	10
IR67406-6-3-2-3	IRRI	*O. sativa*	Seeds	10
IR70416-53-2-2	IRRI	*O. sativa*	Seeds	10
IR70445-146-3-3	IRRI	*O. sativa*	Seeds	10
Bg300	Sri Lanka	*O. sativa*	Seeds	10
Bg304	Sri Lanka	*O. sativa*	Seeds	10
Bg305	Sri Lanka	*O. sativa*	Seeds	10
Bg358	Sri Lanka	*O. sativa*	Seeds	10
Bg359	Sri Lanka	*O. sativa*	Seeds	10
NO.1	Madagascar	*O. sativa*	Seeds	10
HNR-2	Madagascar	*O. sativa*	Seeds	10
HNR-5	Madagascar	*O. sativa*	Seeds	10
HNR-17	Madagascar	*O. sativa*	Seeds	10
Khao Toum	Laos	*O. sativa*	Seeds	10
Mack Kouk	Laos	*O. sativa*	Seeds	10
SLK 2-21-4	Laos	*O. sativa*	Seeds	10
Mollika	Nepal	*O. sativa*	Seeds	10
NR10073-167-3-1-1	Nepal	*O. sativa*	Seeds	10
NR10068-60-5-2	Nepal	*O. sativa*	Seeds	10
NR10078-76-1-1	Nepal	*O. sativa*	Seeds	10
Aus257	Bengal	*O. sativa*	Seeds	10
BR061-2B-25	Bengal	*O. sativa*	Seeds	10
BR319-1-HR28	Bengal	*O. sativa*	Seeds	10
UGEY MAP	Bhutan	*O. sativa*	Seeds	10
80A86YR72	Australia	*O. sativa*	Seeds	10
80A90YR73	Australia	*O. sativa*	Seeds	10
80A97YR74	Australia	*O. sativa*	Seeds	10
80A97YR30	Australia	*O. sativa*	Seeds	10
71011	Australia	*O. sativa*	Seeds	10

The materials from Hainan and Guangdong, China, were collected by our laboratory. The other materials were provided by the Crop Science Research Institute and China Rice Research Institute at the Chinese Academy of Agricultural Science or the Academy of Agricultural Science in Guangdong Province.

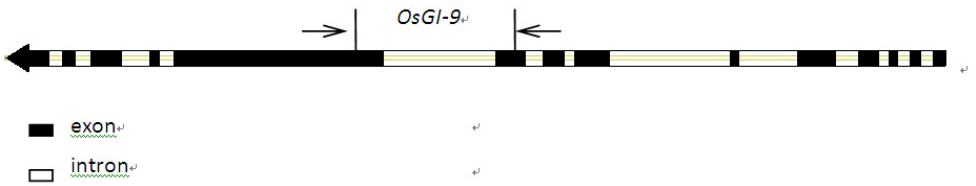

Figure 2. Scheme of the *OsGI* gene. *OsGI-9* was the amplified region in the study.

Phylogenetic analysis of heterozygous alleles

As both *O. rufipogon* and *O. sativa* are diploid plants, *O. rufipogon* and *O. sativa* phylogeny were analyzed in the following manner. The haplotypes were first aligned using ClustalX 2.0 and exported in PHYLIP format (Larkin et al., 2007). The haplotype alignment was then transformed into a haplotype distance matrix using DNADIST in the PHYLIP 3.69 software (Felsenstein, 2008), transformed into an individual distance matrix by the host-associate method using the PBC software (Göker and Grimm, 2008), and the phylogenetic organism network was reconstructed by the NeighborNet method using the Splitstree4.0 software (Bryant and Moulton, 2004; Huson and Bryant, 2006).

Seed dormancy and cold tolerance evaluation

All of the *O. sativa* seeds used in this study were stored at 4°C to maintain dormancy (Wang et al., 2009), and 100 seeds of each cultivar were selected to determine the germination rate (GR) and germination rate index (GRI). GR is the percentage of germinated seeds and GRI reflects the strength of seed germination. The seeds were soaked in 75% alcohol for 10 min, washed with sterile distilled water and arranged on 9-cm Petri dishes covered with 2 layers of wet filter paper. The seeds were divided into 2 groups; one group was cultured at 14°C and the other at 30°C. Both groups were kept moist with a 12-h light/12-h dark cycle and GR and GRI were determined after 25 days of culture. Seed dormancy was divided into 5 levels according to the GR at 30°C: GR >80%, non-dormant; 50 to ≤80%, mild dormancy; 30 to ≤50%, moderate dormancy; 5 to ≤30%, strong dormancy; ≤5%, very strong dormancy. Seed cold tolerance was estimated according to the difference between GR and GRI at 14° and 30°C (Cao et al., 2001; Miura et al., 2004; Wang et al., 2009) as follows:

$$GR\ (\%) = (\text{number of generated seeds} / \text{total number of seeds}) \times 100$$

$$GRI = G_1/T_1 + G_2/T_2 + \ldots + G_{n-1}/T_{n-1} + G_n/T_n$$

where G_1 is the number of germinated seeds on day T_1, G_n is the number of germinated seeds on day T_n; T_1 is the day number at the first count, and T_n is the day number at the last count.

Statistical analysis

Data were analyzed using SPSS 13.0 (Chicago, IL, USA) using Spearman's correlation and Kendall's method of non-parametric correlations for correlation analyses, and Mann-Whitney non-parametric tests.

RESULTS

OsGI-9 in *O. rufipogon* and *O. sativa*

The *OsGI-9* regions in 516 individuals from 38 populations were amplified using PCR. Electrophoresis indicated the presence of 3 different band types in *O. rufipogon*: S-type (1.2 kb), F-type (0.9 kb), and FS-type (1.2 and 0.9 kb; Figure 3). FS-type individuals were

present in 18 *O. rufipogon* populations, including 7 populations from Hainan Island and 11 populations from inland China. S-type individuals were present in 19 *O. rufipogon* populations, including 4 populations from Hainan Island and 15 populations from inland China. F-type individuals were present in 9 *O. rufipogon* populations, including 5 populations from Hainan Island and 4 populations from inland China (Table 2).

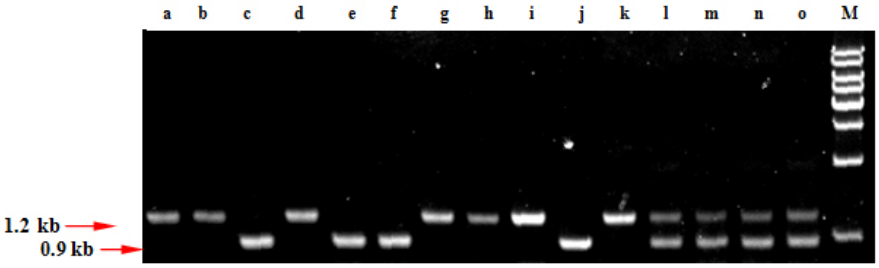

Figure 3. Band types of OsGI-9 in Oryza rufipogon individuals from the DZP population. *Lanes a-o* = different individuals in the DZP population. *Lanes a*, *b*, *d*, *g*, *h*, *i*, *k* = 1.2 kb; *lanes c*, *e*, *f*, *j* = 0.9 kb; *lanes l*, *m*, *n*, *o* = 0.9 and 1.2 kb; *lane M* = molecular marker.

Table 2. *OsGI*-9 band types in *Oryza rufipogon*.

Code	*OsGI-9* band types		
	F (0.9 kb)	S (1.2 kb)	FS (0.9 and 1.2 kb)
DZP	4/18*	10/18	4/18
LHL	-	4/7	3/7
SG	-	-	3/3
HQ	-	-	14/14
WDL	-	-	14/14
ZY	3/13	-	10/13
DL	-	-	16/16
HA	4/4	-	-
HL	3/3	-	-
FC	-	4/4	-
CM	-	7/7	-
DA	128/128	-	-
YJ	-	18/18	-
PS	-	13/16	3/16
ST	15/15	-	-
TL	3/17	2/17	12/17
DAL	-	-	13/13
FUS		4/6	2/6
YD_4-24	-	52/52	-
$N6^4$	-	10/10	-
B8		10/10	-
YD_2-154	10/10	-	-
YD_2-204	-	-	10/10
YD_2-320	-	10/10	-
YD_2-555	-	-	10/10
YD_2-675	-	10/10	-
YD_2-714	-	-	10/10
YD_2-758	-	-	10/10
YD_2-790	-	10/10	-
YD_2-810	-		10/10
YD_2-950	-	10/10	-
YD_2-1015	-	-	10/10
YD_2-1065	-	10/10	-
YD_2-1092	-	10/10	-
YD_2-1390	-		10/10
YD_2-1445	-	10/10	-
YD_2-1	10/10		
YD_6-66	-	10/10	-

*Means that 4 individuals among 18 samples from DZP have F-type of *OsGI-9*.

S-type and FS-type *O. rufipogon* individuals were predominant in the inland region of China, with a slightly larger number of S-type individuals. *O. rufipogon* individuals from Yuanjiang, Yunnan Province (YJ), which are thought to be a typical representation of *O. rufipogon* (Sun et al., 2002; Tan et al., 2008), and Dongxiang, Jiangxi province (YD4-24), the northernmost range of *O. rufipogon* in China (Yang et al., 2007; Xia et al., 2010), were both S-type. Although the *O. rufipogon* from Guangdong and Guangxi Provinces were mainly S-type and FS-type, a few F-type individuals were observed in this population. Compared to inland China, 3 *O. rufipogon* types were observed in Hainan Island, with FS-type *O. rufipogon* being predominant. However, notably, all of the individuals from the largest *O. rufipogon* population in Hainan Island (DA) were F-type (Table 2).

Although most *O. rufipogon* populations were composed of a single type (F, S, or FS), some populations contained 2 or 3 types. For example, 2 *O. rufipogon* types were present in the LHL, ZY, and PS populations and 3 *O. rufipogon* types were present in the DZP and TL populations. Further analysis indicated that these populations contained S and FS individuals, F and FS individuals, or S, F, and FS individuals; however, no single population was composed of only S- and F-type individuals (Table 2).

Sequence alignment revealed that *OsGI-9* in S-type *O. rufipogon* contained two 255-bp fragment repeats, *OsGI-9* in F-type *O. rufipogon* contained a single 255-bp fragment repeat, and *OsGI-9* in FS-type *O. rufipogon* contained both the single and the double 255-bp fragment repeats (Figure 4). A small number of single base mutation sites were also observed in *OsGI-9* (Figure 4).

PCR amplification of *OsGI-9* in 139 *O. sativa* cultivars indicated the presence of S-type and F-type individuals; however, no FS-type individuals were observed. A total of 62 cultivars were S-type (1.2 kb) and 77 cultivars were F-type (0.9 kb). The *OsGI-9* in all individuals from each cultivar were the same type (Table 3). Sequence analysis showed that *OsGI-9* in S-type *O. sativa* had two 255-bp repeat fragments and F-type *O. sativa* had a single 255-bp repeat fragment, identical to S-type and F-type *O. rufipogon* individuals (Figure 4).

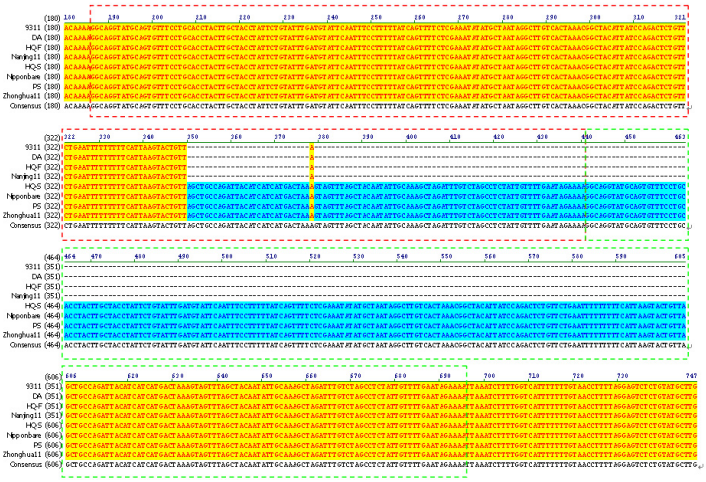

Figure 4. Sequence alignment of *OsGI-9* in partial *Oryza rufipogon* and *O. sativa*. 9311 and Nanjing11 were *O. sativa indica* varieties; Nipponbare and Zhonghua11 were *O. sativa japonica* varieties; DA, PS, and HQ were *O. rufipogon* from different populations, in which HQ-F and HQ-S meant the sequences of F- and S-band in one HQ individual. Red and green dashed boxes meant two 255-bp repeated fragments.

Table 3. *OsGI-9* band types in 139 *Oryza sativa* cultivars.

Code	*OsGI-9* band type	
	S (1.2 kb)	F (0.9 kb)
Mudanjiang 19	√	
Ji 91-2605	√	
Liao 201	√	
86XW-17	√	
9011	√	
91-13-11	√	
Laolongxu	√	
Xinong 8116		√
Qiannong 5782		√
Jinxi 870441	√	
Kenyu 16	√	
Zhonghua 11	√	
Zhonghua 8	√	
Zhongzuo 8604	√	
6017		√
Yinfang	√	
Putaohuang	√	
Lujing 1	√	
Zhengdao 5	√	
Xinyang 14		√
Wanjing 1	√	
Anxuan 4		√
Liushizao		√
Wuyujing	√	
Shuangqing	√	
Nanjing 11		√
Nannong 4008		√
9311		√
Shuangfeng 1	√	
Yingtoujing	√	
Zheli 1		√
Zaoxianmi		√
Zaoshu 691		√
3635		√
Xiangjing 2	√	
Muguanuo	√	
Xiangzaoxian		√
86-106	√	
Gaoyuanjing 1	√	
Meihuanuo		√
Hongmangdazu	√	
Chuanmi 2		√
Jiushizao		√
Gannongwanjing 2	√	
4434		√
Nante		√
Jinxibai		√
Sanbaili		√
Hongwei 1	√	
Minghui 63		√
Dijiaowujian		√
Xixuan 4		√
Jinxingdanuo	√	
Guangluai 4		√
GD-5S		√
Kuyexiangnuo	√	
Baise 1		√
Tainan 6	√	
Gaoxiongyu 122	√	
Jianongxianyu 31		√

Continued on next page

Table 3. Continued.

Code	*OsGI-9* band type	
	S (1.2 kb)	F (0.9 kb)
Taizhongxianxuan 220		√
Menjiagao		√
Tonghong'ai		√
Xianzhan		√
Qiuqihong		√
Qiuguang	√	
Nipponbare	√	
Changyeyu	√	
Beijin	√	
Youliujiannuo	√	
Jinuo	√	
Shuiyuan 354	√	
Shuiyuan 380	√	
Lili 372	√	
Chizhenzhu	√	
JINBU 9	√	
Jiexiaonuo	√	
Starbonnet CI9584	√	
Sunbonnet	√	
CALROSF	√	
EDITH	√	
BU189	√	
BU342	√	
BU349	√	
BU412	√	
Balilla	√	
Angke		√
Bp205f-Kn-78-1		√
BP1356-1g-Kn-4		√
CR60		√
1v-139		√
NR11		√
VR345	√	
VR349		√
VR350		√
VR340		√
VR347	√	
Matant MF		√
Mulant of dwarf		√
PSRM1-17		√
RP1667-301-1196-1562		√
RP1670-1418-2205-1582		√
Toga		√
CNTLR85033-9-3-1-1		√
SPR85163-5-1-2		√
C. Costo		√
Mya-1		√
Mya-2		√
Manan Thukho		√
FAON11		√
AZUCENA	√	
IR67406-6-3-2-3		√
IR70416-53-2-2		√
IR70445-146-3-3		√
Bg300		√
Bg304		√
Bg305		√
Bg358		√
Bg359		√
NO.1		√
HNR-2		√
HNR-5		√
HNR-17		√
Khao Toum		√

Continued on next page

Table 3. Continued.

Code	*OsGI-9* band type	
	S (1.2 kb)	F (0.9 kb)
Mack Kouk	√	
SLK 2-21-4		√
Mollika		√
NR10073-167-3-1-1		√
NR10068-60-5-2		√
NR10078-76-1-1		√
Aus257		√
BR061-2B-25		√
BR319-1-HR28		√
UGEY MAP		√
80A86YR72	√	
80A90YR73	√	
80A97YR74	√	
80A97YR30	√	
71011	√	

Phylogeny of *O. rufipogon* and *O. sativa* based on *OsGI-9*

The phylogenetic organism network (not haplotype) based on *OsGI-9* indicated that all *O. rufipogon* and *O. sativa* individuals could be divided into 5 groups: 3 *O. rufipogon* groups (F-type, S-type, and FS-type) and 2 *O. sativa* groups (F-type and S-type; Figure 5).

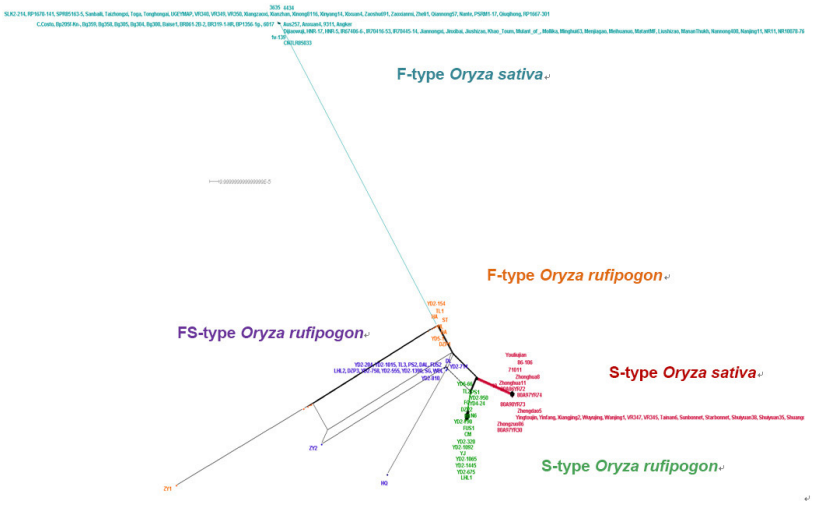

Figure 5. Phylogenetic network (NeighborNet) of the individuals from *Oryza rufipogon* and *O. sativa* based on *OsGI-9*.

The F-type and S-type *O. sativa* groups were located at the poles of the network, with the 3 *O. rufipogon* groups distributed between them (Figure 5), suggesting that the genetic distance between the *O. rufipogon* groups is less than the genetic distance between the 2 *O. sativa* groups, and indicating that *O. rufipogon* is less differentiated than *O. sativa*. In the phylogenetic network, the FS-type *O. rufipogon* group was located in the middle of the *O. rufipogon* groups, and far from the *O. sativa* groups. F-type *O. sativa* was closest to F-type *O. rufipogon*,

and S-type *O. sativa* was closest to S-type *O. rufipogon* (Figure 5). These results suggest that the 2 *O. sativa* groups are more closely related than are the different *O. rufipogon* groups.

In addition, the *O. rufipogon* cultivars from HQ (FS-type), ZY1 (F-type), and ZY2 (FS-type) were located far from their respective groups (Figure 5), probably due to the presence of other *OsGI-9* mutations apart from the 255-bp InDel in these individuals. Taken together, these results indicate that the *OsGI-9* 255-bp InDel variation was retained during the domestication of *O. rufipogon* to *O. sativa*.

Correlation between *OsGI-9* and phenotype

The *OsGI-9* 255-bp InDel variation is common in *O. sativa* cultivars, implying that it may be significant to the adaptability of cultivars. Current studies indicate that *OsGI* and orthologous genes play an important role in photoperiod regulation, seed dormancy, and cold tolerance (Chandler, 1992; Stewart, 2009).

The GR of *O. sativa* at 30ºC reflects its degree of seed dormancy (Cao et al., 2001; Miura et al., 2004; Wang et al., 2009). The correlation between *OsGI-9* in *O. sativa* and GR at 30°C was significant (Keudall's r = 0.279, Spearman's r = 0.316, P < 0.01; Table 4). The degree of dormancy varied in S-type *O. sativa*, while most F-type *O. sativa* were not dormant (Figure 6). The GR of S-type *O. sativa* was significantly lower than that of F-type *O. sativa* at 30ºC (Z = -3.707, P < 0.01 non-parametric test; Table 5), demonstrating that the dormancy of S-type *O. sativa* is stronger than that of F-type *O. sativa*.

Table 4. Non-parametric correlations between *OsGI-9* type and indices of germination in *Oryza sativa*.

		GR		GRI		GR-D	GRI-D
		30°C	14°C	30°C	14°C		
Keudall's	*OsGI-9*	R = 0.279**	R = 0.272**	R = 0.258**	R = 0.254**	0.069	-0.214**
Spearman's	*OsGI-9*	R = 0.316**	R = 0.311**	R = 0.311**	R = 0.302**	0.080	-0.260**

GR = mean germination rate; GRI = mean germination rate index; GR-D = mean difference between GR at 14° and 30°C; GRI-D = mean difference between GRI at 14° and 30°C; **P < 0.01 (two-tailed).

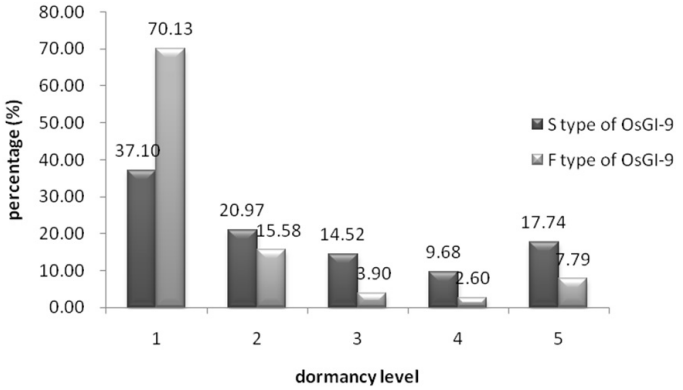

Figure 6. Frequency distribution of seed dormancy phenotype in 139 *Oryza sativa* cultivars. 1-5 at horizontal axis indicates GR > 80%, 80% ≥ GR > 50%, 50% ≥ GR > 30%, 30% ≥ GR>5%, and 5% ≥ GR > 0, respectively. Numbers above columns account for the proportion of the total number of cultivars in the same *OsGI-9* type.

Table 5. Variation in the germination indices of 139 *Oryza sativa* cultivars.

OsGI-9 type	Number of cultivars	30°C		14°C		GRI-D
		GR (%)	GRI	GR (%)	GRI	
S	62	59.5	1.9	42.4	0.4	-1.5
F	77	80.6**	2.8**	68.5**	0.8**	-2.7**

For abbreviations, see legend to Table 4. **P < 0.01 (two-tailed).

The cold tolerance of *O. sativa* was evaluated by determining the difference in GR (GR-D) or GRI (GRI-D) at 14°C and 30°C. A significant correlation was observed between *OsGI-9* in *O. sativa* and GRI-D (Keudall's r = -0.214, Spearman's r = -0.260, P < 0.01; Table 4). Although cold could significantly reduce the GR and GRI of *O. sativa* seeds, the influence of cold on GRI in S-type *O. sativa* was significantly less than in F-type *O. sativa* (Z = -3.06, P < 0.01; Table 5), indicating that S-type *O. sativa* have increased cold tolerance.

DISCUSSION

Phylogenetic analysis of intra-individual allele heterozygotes

Phylogeny plays an important role in reflecting evolutionary relationships among species, and for reconstructing the evolutionary process (Lessa, 1992). Due to a faster evolutionary rate, ease of amplification, near neutrality, and other evolutionary characteristics, introns have become a widely used nuclear genetic marker, and have significant potential for use in interspecific and intraspecific phylogenetic analyses (Dalebout et al., 2008). IIAHs provide a rich source of phylogenetic information and can reveal potential introgression between species or populations during the analysis of phylogenetic relationships between related species or the genetic reconstruction of populations. However, it is common practice to randomly select one haplotype from heterozygous alleles for such analyses (Göker and Grimm, 2008; Nakagome et al., 2008).

Heterozygous FS-type *OsGI-9* was observed in many *O. rufipogon* individuals in this study. In order to determine the evolutionary relationship between heterozygous and homozygous *O. rufipogon* individuals, the genetic distance between haplotypes was converted into the genetic distance between individuals, and the individual phylogenetic network was constructed. The evolutionary relationships of heterozygous individuals cannot be analyzed using a haplotype phylogenetic tree, but can be reflected in an individual (or organism) phylogenetic network.

Identification of the primitive *O. rufipogon* type

Throughout their evolution, many types of *O. rufipogon* with rich morphological phenotypes and genetic diversity emerged in complex ecological environments (Pang and Chen, 2002; Dong et al., 2010). It is not known which types are primitive and which are derived, or how *O. rufipogon* evolved precisely. Such knowledge could help to better characterize the origin and differentiation of *O. sativa*, and promote efficient utilization of *O. rufipogon* resources.

Primitive *O. rufipogon* share few traits with *O. sativa*, having a prostrate morphology, purple leaf sheath, smaller and shorter flag, longer anther (>5 mm), purple and exposed stigma, red and long awn, easier seed shatter, more slender grain (length/width >3.5), black or brown hulls, and red seeds (Pang and Chen, 2002). *O. rufipogon* commonly grow in perennial

swamps or rivers, and mainly reproduce asexually with a low capacity for sexual reproduction. *O. rufipogon* populations are generally larger; however, the morphology of individuals within an *O. rufipogon* population are identical and offspring from self-crossing rarely separate. *O. rufipogon* habitats are mostly distinct from *O. sativa* habitats, which has prevented the influence of hybridization or introgression. Compared with primitive *O. rufipogon*, many morphological changes have been observed in derived *O. rufipogon*, whose morphology is more or less similar to that of *O. sativa* (Pang and Chen, 2002).

In this study, individuals from the *O. rufipogon* YJ population in Yuanjiang, Yunnan Province, China, and YD4-24 individuals from the northern most *O. rufipogon* population in China, that are accepted to be primitive (Sun et al., 2002; Yang et al., 2007; Tan et al., 2008; Xia et al., 2010), all possessed S-type *OsGI-9* (Table 2). The DA population is the largest *O. rufipogon* population in Hainan Island and grows in a perennial swamp. Individuals from this population are identical and possess the majority of primitive *O. rufipogon* morphological characteristics (Dong et al., 2010). Our results indicate that all *O. rufipogon* DA individuals are F-type (Table 2). On the contrary, individuals from the HQ, DL, and WDL populations do not possess primitive characteristics (Dong et al., 2010) and are all FS-type (Table 2). These results imply that F-type and S-type *O. rufipogon* are more primitive than the FS-type. The FS-type is located between F-type and S-type in the phylogenetic network (Figure 5), and FS-type individuals were always observed in populations containing both F-type and S-type individuals (Table 2), suggesting that the FS-type individuals are a hybrid of F- and S-type *O. rufipogon*.

O. rufipogon is commonly accepted to be the progenitor of *O. sativa*. Although *O. sativa* was domesticated from *O. rufipogon*, the process was so complex that, as yet, there is no consensus view as to how domestication occurred (Sweeney and McCouch, 2007; Sang and Ge, 2007a,b). Two diverging opinions have been proposed. One supports a multiple origin of rice domestication, suggesting that initial *O. sativa* cultivars were domesticated from divergent *O. rufipogon* populations with different sets of alleles, and introgression between independently domesticated cultivars fixed a similar set of alleles that were critical for domestication. The other opinion considers a single rice origin, suggesting that some critical domestication alleles were fixed in the initial cultivars, and then introgression between different populations of *O. rufipogon* to *O. sativa* increased cultivar diversity (Vaughan et al., 2008a,b). The phylogenetic network analysis in this study indicated that F-type *O. sativa* are closest to F-type *O. rufipogon* and that S-type *O. sativa* are closest to S-type *O. rufipogon* (Figure 5), supporting the multiple origin hypothesis. The presence of non-FS-type individuals in *O. sativa* cultivars is probably the result of continued domestication and breeding in different climatic conditions.

OsGI 9 intron InDel

The role of introns and intergenic sequences has received increasing attention in recent studies of gene function. Current studies indicate that introns can be divided into 3 groups. Group I and Group II introns are mainly found in organelles and bacteria and are self-cutting, although their structural features vary. Group III introns are pre-mRNA introns that are found in most eukaryotic cells (Cousineau et al., 2000). Research has indicated that intron base mutations and InDels are related to many human diseases and variation in biological characteristics (Reszka et al., 2006).

In this study, we observed a 255-bp InDel in the ninth *OsGI* intron (*OsGI-9*). *Arabidopsis GI*, the *OsGI* rice homolog, which regulates photoperiod-mediated flowering and the circadian clock, can affect fruit setting and cold tolerance (Fowler et al., 1999; Brock et al., 2007). In this study, *OsGI-9* variation in *O. sativa* was associated with seed dormancy and cold tolerance (Tables 4 and 5), but was not associated with photoperiod (data not shown). Most S-type *O. sativa* cultivars had strong seed dormancy and high cold tolerance and most F-type *O. sativa* cultivars had poor seed dormancy and low cold tolerance (Figures 1 and 6; Tables 3 and 5). Therefore, we speculate that the 255-bp *OsGI-9* InDel is correlated with seed dormancy or cold tolerance in *O. sativa*.

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