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Genome-wide multilocus analysis of intraspecific differentiation in *Oryza rufipogon* Griff. from China and the influence of introgression from *O. sativa* L.

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ABSTRACT. Twenty-five populations of *Oryza rufipogon* from China and 144 cultivars of *Oryza sativa* were selected for this study. Based on the DNA fragment of *Ehd1-4* and subspecies-specific sequence-tagged site markers in different chromosomes, intraspecific differentiation in *O. rufipogon* from China was analyzed. The introgression from *O. sativa* to *O. rufipogon* was also analyzed based on simple sequence repeat markers. The results revealed that the DNA fragment of *Ehd1-4* could distinguish the *O. sativa* subspecies *japonica* and *indica*. Furthermore, although significant *indica-japonica* differentiation did

not occur in most *O. rufipogon* populations from China, *O. rufipogon* varieties from Hainan Island and from the mainland of China showed differentiation tendencies. *Japonica*-like *O. rufipogon* varieties were predominant in Mainland China. However, more *indica*-like *O. rufipogon* varieties were found in Hainan Island. Finally, although cultivar-specific alleles were found in most of the *O. rufipogon* varieties from Hainan Island and Guangdong Province, some varieties remain pure and non-introgressive.

Key words: *Oryza rufipogon*; *Oryza sativa*; Intra-differentiation; *Ehd1-4*; Introgression

INTRODUCTION

Oryza sativa is an essential staple crop for billions of people throughout the world and has been produced on every continent with arable land (Khush, 1997). Several studies have suggested that *Oryza rufipogon*, also known as common wild rice, is the ancestor of *O. sativa*, and represents a rich genetic resource (Kovach et al., 2007). However, 30-40% of its genetic variation was lost during rice domestication (Sun et al., 2001). This suggests that the most valuable source of genetic variation today remains the wild germplasm itself, and useful variations could be rapidly identified by genomic techniques, which can then be exploited with the use of transgenic technology (Kovach et al., 2007). China represents the largest genetic diversity center of *O. rufipogon* (Wang et al., 2004), containing rich *O. rufipogon* resources (Wang et al., 2008). *O. rufipogon* grows in eight Provinces of China, namely, Guangdong, Guangxi, Jiangxi, Hunan, Fujian, Yunnan, Hainan, and Taiwan (Gao et al., 2000).

Knowledge concerning the genetic differentiation of *O. sativa* and its wild relatives forms the basis for clarifying biosystematic relationships that will facilitate the use of germplasm resources for rice breeding (Sun et al., 2002). Numerous genetic differentiation studies using molecular markers or DNA sequence information have demonstrated that differentiation between two subspecies of *O. sativa*, *indica* and *japonica*, has occurred in *O. rufipogon*, with the *japonica*-like type dominant in Chinese *O. rufipogon* and the *indica*-like type as the major type in South or Southeast Asia (Sun et al., 1995, 2002; Wang et al., 1992, 2008). However, different studies have shown conflicting results of the genetic differentiation in a certain *O. rufipogon* population (Sun et al., 2002; Wang et al., 2008), which further contributes to the controversy related to the history of rice domestication (Kovach et al., 2007). Different samples and methods were most likely the primary reasons leading to different conclusions. In addition, introgression from *O. sativa*, which might influence the genetic structure of *O. rufipogon* (Song et al., 2006), was neglected in most studies on rice genetic differentiation. In addition, Hainan, being an island, has not always been well considered in most rice genetic studies, although a recent study indicated that Hainan Island was a likely candidate as the original center of domestication (Wang et al., 2008).

The early heading date 1 (*Ehd1*) gene is located on chromosome 10 (Doi and Yoshimura, 1998) and encodes a B-type response regulator that does not appear to have an ortholog in the *Arabidopsis* genome (Doi et al., 2004). *Ehd1* confers short-day promotion

of flowering independently of *Hd1* in rice. It also promotes flowering by inducing flowering locus T-like gene expression only under short-day conditions (Doi et al., 2004).

A sequence-tagged site (STS) is a short unique sequence that can be detected by polymerase chain reaction (PCR) and can be identified at known locations on a chromosome (Tragoonrung et al., 1992). Chin et al. (2007) developed subspecies-specific (SS) STS markers and estimated their scores to preliminarily elucidate reproductive barriers between *japonica* and *indica*.

In this study, intraspecific differentiation of *O. rufipogon* from China was investigated based on the DNA fragment of *Ehd1* and SS STS markers. Furthermore, introgression from *O. sativa* to *O. rufipogon* was detected using simple sequence repeat (SSR) markers. The objectives of this study were to analyze and identify genetic differentiation of *O. rufipogon* from China, especially Hainan Island, and to elucidate the factors most likely affecting intraspecific differentiation in *O. rufipogon*.

MATERIAL AND METHODS

Collection of materials

Twenty-five *O. rufipogon* varieties from China were selected for this study. Eight varieties were obtained from Hainan Island Province and 17 varieties were obtained from six inland Provinces: Guangdong (N = 4), Guangxi (N = 9), Yunnan (N = 1), Hunan (N = 1), Fujian (N = 1), and Jiangxi (N = 1) (Table 1). *O. rufipogon* seeds from these varieties were collected in our laboratory. Ten seeds per cultivar/variety were germinated, and the seedlings were harvested for DNA extraction.

One hundred and forty-four *O. sativa* cultivars from around the world were selected for this study; 66 cultivars were from various provinces in China, and 78 cultivars were from other countries. Among them, 64 cultivars were *O. sativa japonica* and 80 cultivars were *O. sativa indica* (Table 1). Ten seeds of each of these varieties were collected for DNA extraction. Seeds of these cultivars were provided by the Crop Science Research Institute and the China National Rice Research Institute at the Chinese Academy of Agricultural Sciences (CAAS).

Screening of flowering time-controlling genes in 9311 (*O. sativa indica*) and Nipponbare (*O. sativa japonica*)

Previous studies have identified the genes controlling flowering time in rice (Shinozuka et al., 1999; Izawa et al., 2003; Doi et al., 2004; Lee et al., 2005; Lu et al., 2006; Kim et al., 2007; Tamaki et al., 2007). The DNA sequences of these genes in strain 9311 of *indica* and strain Nipponbare of *japonica* were acquired by a rice genome search in the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/), the BGI-RIS (http://rise.genomics.org.cn/rice/index2.jsp), and the GRAMENE (http://www.gramene.org/genome_browser/index.html) databases. Differences in the sequences of these genes between 9311 and Nipponbare were analyzed with the Vector NTI advance 10 software. The gene showing the greatest divergence between 9311 and Nipponbare was selected for further study.

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

Code	Origin	Taxa
DA	Dong'ao, Hainan Province, China	*O. rufipogon*
DZP	Dazhipo, Hainan Province, China	*O. rufipogon*
HQ	Danzhou, Hainan Province, China	*O. rufipogon*
WDL	Donglu, Hainan Province, China	*O. rufipogon*
ZY	Zhongyuan, Hainan Province, China	*O. rufipogon*
DL	Dalu, Hainan Province, China	*O. rufipogon*
FC	Fuchen, Hainan Province, China	*O. rufipogon*
CM	Chengmai, Hainan Province, China	*O. rufipogon*
PS	Pengshan, Guangdong Province, China	*O. rufipogon*
ST	Shatian, Guangdong Province, China	*O. rufipogon*
TL	Tanlu, Guangdong Province, China	*O. rufipogon*
DAL	Daling, Guangdong Province, China	*O. rufipogon*
LS	Lingshan, Guangxi Province, China	*O. rufipogon*
GX	Guixian, Guangxi Province, China	*O. rufipogon*
WM	Wuming, Guangxi Province, China	*O. rufipogon*
WX	Wuxuan, Guangxi Province, China	*O. rufipogon*
ZZ	Zongzuo, Guangxi Province, China	*O. rufipogon*
LA	Long'an, Guangxi Province, China	*O. rufipogon*
SL	Shanglin, Guangxi Province, China	*O. rufipogon*
HX	Hexian, Guangxi Province, China	*O. rufipogon*
LC	Liucheng, Guangxi Province, China	*O. rufipogon*
YJ	Yuanjiang, Yunnan Province, China	*O. rufipogon*
ZP	Zhangpu, Fujian Province, China	*O. rufipogon*
CL	Chaling, Hunan Province, China	*O. rufipogon*
DX	Dongxiang, Jiangxi Province, China	*O. rufipogon*
Wanjing 1	Anhui Province, China	*O. sativa japonica*
Anxuan 4	Anhui Province, China	*O. sativa indica*
Liushizao	Anhui Province, China	*O. sativa indica*
6017	Beijing, China	*O. sativa indica*
Zhonghua 11	Beijing, China	*O. sativa japonica*
Zhonghua 8	Beijing, China	*O. sativa japonica*
Zhongzuo 8604	Beijing, China	*O. sativa japonica*
Dijiaowujian	Fujian Province, China	*O. sativa indica*
Minghui 63	Fujian Province, China	*O. sativa indica*
Xixuan 4	Fujian Province, China	*O. sativa indica*
Hongwei 1	Fujian Province, China	*O. sativa japonica*
Hucongnuo	Fujian Province, China	*O. sativa japonica*
GD-5S	Guangdong Province, China	*O. sativa indica*
Guangluai 4	Guangdong Province, China	*O. sativa indica*
Jinxingdanuo	Guangdong Province, China	*O. sativa japonica*
Baise 1	Guangxi Province, China	*O. sativa indica*
Kuyexiangnuo	Guangxi Province, China	*O. sativa japonica*
Qiannong 5782	Guizhou Province, China	*O. sativa indica*
Qiuqihong	Hainan Province, China	*O. sativa indica*
Tonghong'ai	Hainan Province, China	*O. sativa indica*
Xianzhan	Hainan Province, China	*O. sativa indica*
Kenyu 16	Hebei Province, China	*O. sativa japonica*
Mudanjiang 19	Heilongjiang Province, China	*O. sativa japonica*
Xinyang 14	Henan Province, China	*O. sativa indica*
Zhengdao 5	Henan Province, China	*O. sativa japonica*
Zaoshu 691	Hubei Province, China	*O. sativa indica*
3635	Hubei Province, China	*O. sativa japonica*
86-106	Hunan Province, China	*O. sativa indica*
Xiangzaoxian	Hunan Province, China	*O. sativa indica*
Muguanuo	Hunan Province, China	*O. sativa japonica*
Xiangjing 2	Hunan Province, China	*O. sativa japonica*
9311	Jiangsu Province, China	*O. sativa indica*
Nanjing 11	Jiangsu Province, China	*O. sativa indica*
Nannong 4008	Jiangsu Province, China	*O. sativa indica*
Shuangqing	Jiangsu Province, China	*O. sativa japonica*
Wuyujing	Jiangsu Province, China	*O. sativa japonica*
4434	Jiangxi Province, China	*O. sativa indica*

Continued on next page

Table 1. Continued.

Code	Origin	Taxa
Jinxibai	Jiangxi Province, China	*O. sativa indica*
Nante	Jiangxi Province, China	*O. sativa indica*
Sanbaili	Jiangxi Province, China	*O. sativa indica*
Gannongwanjing 2	Jiangxi Province, China	*O. sativa japonica*
Ji 91-2605	Jilin Province, China	*O. sativa japonica*
Liao 201	Liaoning Province, China	*O. sativa japonica*
86XW-17	Ningxia Province, China	*O. sativa japonica*
Lujing 1	Shandong Province, China	*O. sativa japonica*
Shuangfeng 1	Shanghai Province, China	*O. sativa japonica*
Xinong 8116	Shangxi Province, China	*O. sativa indica*
91-13-11	Shangxi Province, China	*O. sativa japonica*
Laolongxu	Shangxi Province, China	*O. sativa japonica*
Jinxi 870441	Shanxi Province, China	*O. sativa japonica*
Chuanmi 2	Sichuan Province, China	*O. sativa indica*
Jiushizao	Sichuan Province, China	*O. sativa indica*
Meihuanuo	Sichuan Province, China	*O. sativa indica*
Gaoyuanjing 1	Sichuan Province, China	*O. sativa japonica*
Hongmangdazu	Sichuan Province, China	*O. sativa japonica*
Shanjiugu	Sichuan Province, China	*O. sativa japonica*
Jianongxianyu 31	Taiwan, China	*O. sativa indica*
Taizhongxianxuan 220	Taiwan, China	*O. sativa indica*
Gaoxiongyu 122	Taiwan, China	*O. sativa japonica*
Tainan 6	Taiwan, China	*O. sativa japonica*
Putaohuang	Tianjing, China	*O. sativa japonica*
Yinfang	Tianjing, China	*O. sativa japonica*
9011	Xinjiang Province, China	*O. sativa japonica*
Zaoxianmi	Zhejiang Province, China	*O. sativa indica*
Zheli 1	Zhejiang Province, China	*O. sativa indica*
Yingtoujing	Zhejiang Province, China	*O. sativa japonica*
71011	Australia	*O. sativa indica*
80A86YR72	Australia	*O. sativa japonica*
80A90YR73	Australia	*O. sativa japonica*
80A97YR30	Australia	*O. sativa japonica*
80A97YR74	Australia	*O. sativa japonica*
Aus257	Bengal	*O. sativa indica*
BR061-2B-25	Bengal	*O. sativa indica*
BR319-1-HR28	Bengal	*O. sativa indica*
UGEY MAP	Bhutan	*O. sativa indica*
BU189	Brazil	*O. sativa japonica*
BU342	Brazil	*O. sativa japonica*
BU349	Brazil	*O. sativa japonica*
BU412	Brazil	*O. sativa japonica*
C.Costo	Burma	*O. sativa indica*
FAON11	Burma	*O. sativa indica*
Manan Thukho	Burma	*O. sativa indica*
Mya-1	Burma	*O. sativa indica*
Mya-2	Burma	*O. sativa indica*
CR60	Cambodia	*O. sativa indica*
CR64	Cambodia	*O. sativa indica*
CR65	Cambodia	*O. sativa indica*
Matant MF	India	*O. sativa indica*
Mulant of dwarf	India	*O. sativa indica*
PSRM1-17	India	*O. sativa indica*
RP1667-301-1196-1562	India	*O. sativa indica*
RP1670-1418-2205-1582	India	*O. sativa indica*
Toga	India	*O. sativa indica*
Angke	Indonesia	*O. sativa indica*
BP1356-1g-Kn-4	Indonesia	*O. sativa indica*
Bp205f-Kn-78-1	Indonesia	*O. sativa indica*
IR67406-6-3-2-3	IRRI	*O. sativa indica*
IR70416-53-2-2	IRRI	*O. sativa indica*
IR70445-146-3-3	IRRI	*O. sativa indica*

Continued on next page

Table 1. Continued.

Code	Origin	Taxa
Balilla	Italy	*O. sativa japonica*
Beijin	Japan	*O. sativa japonica*
Changyeyu	Japan	*O. sativa japonica*
Jinuo	Japan	*O. sativa japonica*
Nipponbare	Japan	*O. sativa japonica*
Qiuguang	Japan	*O. sativa japonica*
Youliujiannuo	Japan	*O. sativa japonica*
Chizhenzhu	Korea	*O. sativa japonica*
Jiexiaonuo	Korea	*O. sativa japonica*
JINBU 9	Korea	*O. sativa japonica*
Lili 372	Korea	*O. sativa japonica*
Lixiaonuo	Korea	*O. sativa japonica*
Shuiyuan 354	Korea	*O. sativa japonica*
Shuiyuan 380	Korea	*O. sativa japonica*
Khao Toum	Laos	*O. sativa indica*
SLK 2-21-4	Laos	*O. sativa indica*
Mack Kouk	Laos	*O. sativa japonica*
HNR-17	Madagascar	*O. sativa indica*
HNR-2	Madagascar	*O. sativa indica*
HNR-5	Madagascar	*O. sativa indica*
NO.1	Madagascar	*O. sativa indica*
Mollika	Nepal	*O. sativa indica*
NR10068-60-5-2	Nepal	*O. sativa indica*
NR10073-167-3-1-1	Nepal	*O. sativa indica*
NR10078-76-1-1	Nepal	*O. sativa indica*
AZUCENA	Philippine	*O. sativa japonica*
Bg300	Sri Lanka	*O. sativa indica*
Bg304	Sri Lanka	*O. sativa indica*
Bg305	Sri Lanka	*O. sativa indica*
Bg358	Sri Lanka	*O. sativa indica*
Bg359	Sri Lanka	*O. sativa indica*
CNTLR85033-9-3-1-1	Thailand	*O. sativa indica*
SPR85163-5-1-2	Thailand	*O. sativa indica*
CALROSF	USA	*O. sativa japonica*
EDITH	USA	*O. sativa japonica*
Starbonnet CI9584	USA	*O. sativa japonica*
Sunbonnet	USA	*O. sativa japonica*
1v-139	Vietnam	*O. sativa indica*
NR11	Vietnam	*O. sativa indica*
VR345	Vietnam	*O. sativa indica*
VR349	Vietnam	*O. sativa indica*
VR350	Vietnam	*O. sativa indica*
VR7	Vietnam	*O. sativa indica*
VR340	Vietnam	*O. sativa japonica*
VR347	Vietnam	*O. sativa japonica*

DNA extraction, PCR amplification, sequencing of *Ehd1-4*, and correlation analysis

Total DNA was extracted from leaves with the modified CTAB method (Xie et al., 1999) and used for PCR amplification. Because there is higher conservation in exons than in introns between species or subspecies (Palumbi and Baker, 1994), the primers for PCR amplification were located in exons and designed using published sequence information of *Ehd1* in Nipponbare and 9311. The primer sequences were: 5'-GGCAGTTCCAAAGAAGATACT-3' and 5'-TTGTCTGAATCCCATCGG-3'. The amplified region comprised two parts of exons and one intron, and was named *Ehd1-4*. PCR amplification was performed in a total volume of 50 μL, which contained 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM each

primer, 0.025 U/μL *Pfu* polymerase (Promega) and Ex *Taq* DNA polymerase (Takara) mixture (1:1), and 5 ng/μL template DNA. Amplification was carried out as follows: 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 54°C, 80 s at 72°C, and a final extension at 72°C for 10 min. Amplification products were separated by electrophoresis on 1.2% agarose gel stained with ethidium bromide (EB), and photographs were taken using a gel imaging system (GBox, Gene Company). The PCR fragments purified with a DNA gel extraction kit (Axygen Biosciences) were cloned into the pEASY-T3 cloning vector (Transgen Biotech). Independent plasmids were selected randomly, and at least five positive clones were sequenced individually. Sequences were aligned and analyzed using the Vector NTI advance 10 software.

Correlations between insertion/deletions (InDels) of *Ehd1-4* and intraspecific differentiation were analyzed by Spearman's correlation, Kendall's method of nonparametric correlations, and the Mann-Whitney non-parametric test in SPSS 13.0 (Chicago, IL, USA).

Selection and amplification of SS STS markers

Nine SS STS markers, which were located on different chromosomes and showed perfect SS scores, were selected for PCR amplification. No SS STS markers were selected from chromosomes 5 and 8 due to non-perfect SS scores (Chin et al., 2007). The information of the nine SS STS markers is summarized in Table 2. The thermocycler profile was: 5 min at 94°C; 35 cycles each of 30 s at 94°C, 30 s at 50°C, 52°C, or 56°C, 30 s at 72°C; and 10 min at 72°C for a final extension. Amplified products were resolved by electrophoresis on 3% agarose gels stained with EB.

Table 2. Subspecies-specific STS markers that perfectly distinguished *japonica* from *indica*.

Chrom.	Marker name	Primers	Product size *japonica*/*indica* (bp)	Annealing temperature (°C)
2	S02026	tggtccatcatattgccaac / tcctctcagatccgattttca	167/180	50
3	S03041	gctgacattgtccgaggttt / ccgacgtccaacctaagc	192/201	52
4	S04128	tcacgggaaagctttggtat / aacttatgcagccaccatcc	163/181	52
6	S06001	agctcaatatcaggcaagcag / aaatgacacagttgacctttgaa	231/248	50
7	S07011	ctggatccaaggcatcattc / cttcgctctcaccatcaaca	229/205	52
9	S09026B	gggaggcagagggaactact / ttatcaggccaggtcctttg	207/182	56
10	S10003A	ataagacggacggtcaaacg / atctcttgtgggctttgtgg	234/246	52
11	S11004A	tctctggccttctactcatgg / ttgtgtttctacttggactcttttt	173/157	52
12	S12011B	tgggggagttctgaaatctg / ttaagttcggtgccccataa	156/178	52

SSR analysis

Three SSR markers (RM44, RM212, and RM215) that could detect cultivar-specific alleles in common wild rice populations (Song et al., 2006) were selected for this study. Detailed information about these SSR markers is provided in Table 3. The program of PCR amplification was 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 50°-56°C, 15 s at 72°C, and a final extension at 72°C for 10 min. The PCR products were separated on 4% high-resolution agarose gel (Agarose SFR, Amresco) stained with EB, and photographs were taken using a gel imaging system (GBox, Gene Company). Possible introgression from *O. sativa* to *O. rufipogon* could be confirmed based on their respective allele frequencies.

Table 3. SSR primer pairs used in the study.

Primer code	Chromosome location	Primer sequence	Annealing temperature (°C)
RM44	8	acgggcaatccgaacaacc / tcgggaaaacctaccctacc	54
RM212	1	ccactttcagctactaccag / cacccatttgtctctcattatg	54
RM215	9	caaaatggagcagcaagagc / tgagcacctccttctctgtag	56

RESULTS

Screening for genes controlling flowering time

Eighteen flowering time-controlling genes were selected according to published references (Shinozuka et al., 1999; Izawa et al., 2003; Doi et al., 2004; Lee et al., 2005; Lu et al., 2006; Kim et al., 2007; Tamaki et al., 2007). The sequences of these genes in Nipponbare (*japonica*) and 9311 (*indica*) were acquired by a search of rice genome in public databases (NCBI, BGI-RIS, and GRAMENE) and were aligned using Vector NTI advance 10 (data not shown). As a result, the *Ehd1* gene on chromosome 10 was selected for further study based on a 20-bp InDel mutation between 9311 and Nipponbare. The InDel mutation was located at the fourth intron of *Ehd1* in Nipponbare. A continuous 20-bp deletion occurred in *Ehd1-4* of Nipponbare, whereas a continuous 20-bp insertion was found in *Ehd1-4* of 9311.

Correlation between InDels of *Ehd1-4* and *O. sativa* subspecies

Ehd1-4 of 144 *O. sativa* cultivars were amplified with PCR. The sequence alignment of *Ehd1-4* among the 144 *O. sativa* cultivars indicated that a continuous 20-bp deletion [Del(20)] occurred in *Ehd1-4* of 62 cultivars, while a continuous 20-bp insertion [In(20)] was found in *Ehd1-4* of the other cultivars. All 62 cultivars with Del(20) were individuals of the *japonica* subspecies. Among the 82 cultivars with In(20), 80 cultivars were *indica* and two were *japonica* (Table 4). Both Pearson and non-parametric correlation analyses indicated that InDel(20) in *Ehd1-4* was significantly correlated with *O. sativa* subspecies ($r = 0.972$, $P < 0.01$).

Indica-japonica differentiation of *O. rufipogon* in *Ehd1-4* and SS STS markers

All samples from the 25 *O. rufipogon* varieties were analyzed based on *Ehd1-4*. The results indicated three variants (In(20), Del(20), and InDel(20)) of *Ehd1-4* in *O. rufipogon* (Table 4). The *Ehd1-4* in the In(20)-type *O. rufipogon* was similar to *O. sativa indica*, and the Del(20)-type *O. rufipogon* was similar to *O. sativa japonica*. *Ehd1-4* in the InDel(20)-type *O. rufipogon* contained both the In(20) and Del(20) types. Similar results were found using the nine SS STS markers. The *O. rufipogon* varieties showed different degrees of *indica-japonica* differentiation at every SS STS locus analyzed.

The overall analysis, which was based on 10 loci (*Ehd1-4* and nine SS STS loci) on different chromosomes in *O. rufipogon*, showed that there were four different alleles at one locus, namely, *japonica*-like, *indica*-like, both *japonica*- and *indica*-like, and none (Table 5). Most of the alleles were *japonica*-like, *indica*-like, or both *japonica*-like and *indica*-like. Based on the whole genome multi-loci analysis, no significant *indica-japonica* differentiation was found in most *O. rufipogon* varieties from China, although some *O. rufipogon* varieties were significantly *japonica*-like (YJ, DX, CL, HX, SL, ZZ) or *indica*-like (WDL).

Table 4. InDel in *Ehd1-4* of *Oryza sativa* and *O. rufipogon.*

Code	Taxa	*Ehd1-4* (bp)
Wanjing 1	*O. sativa japonica*	Del(20)[a]
Anxuan 4	*O. sativa indica*	In(20)[b]
Liushizao	*O. sativa indica*	In(20)
6017	*O. sativa indica*	In(20)
Zhonghua 11	*O. sativa japonica*	Del(20)
Zhonghua 8	*O. sativa japonica*	Del(20)
Zhongzuo 8604	*O. sativa japonica*	Del(20)
Dijiaowujian	*O. sativa indica*	In(20)
Minghui 63	*O. sativa indica*	In(20)
Xixuan 4	*O. sativa indica*	In(20)
Hongwei 1	*O. sativa japonica*	Del(20)
Hucongnuo	*O. sativa japonica*	Del(20)
GD-5S	*O. sativa indica*	In(20)
Guangluai 4	*O. sativa indica*	In(20)
Jinxingdanuo	*O. sativa japonica*	Del(20)
Baise 1	*O. sativa indica*	In(20)
Kuyexiangnuo	*O. sativa japonica*	Del(20)
Qiannong 5782	*O. sativa indica*	In(20)
Qiuqihong	*O. sativa indica*	In(20)
Tonghong'ai	*O. sativa indica*	In(20)
Xianzhan	*O. sativa indica*	In(20)
Kenyu 16	*O. sativa japonica*	Del(20)
Mudanjiang 19	*O. sativa japonica*	Del(20)
Xinyang 14	*O. sativa indica*	In(20)
Zhengdao 5	*O. sativa japonica*	Del(20)
Zaoshu 691	*O. sativa indica*	In(20)
3635	*O. sativa japonica*	Del(20)
86-106	*O. sativa indica*	In(20)
Xiangzaoxian	*O. sativa indica*	In(20)
Muguanuo	*O. sativa japonica*	Del(20)
Xiangjing 2	*O. sativa japonica*	Del(20)
9311	*O. sativa indica*	In(20)
Nanjing 11	*O. sativa indica*	In(20)
Nannong 4008	*O. sativa indica*	In(20)
Shuangqing	*O. sativa japonica*	Del(20)
Wuyujing	*O. sativa japonica*	Del(20)
4434	*O. sativa indica*	In(20)
Jinxibai	*O. sativa indica*	In(20)
Nante	*O. sativa indica*	In(20)
Sanbaili	*O. sativa indica*	In(20)
Gannongwanjing 2	*O. sativa japonica*	Del(20)
Ji 91-2605	*O. sativa japonica*	Del(20)
Liao 201	*O. sativa japonica*	Del(20)
86XW-17	*O. sativa japonica*	Del(20)
Lujing 1	*O. sativa japonica*	Del(20)
Shuangfeng 1	*O. sativa japonica*	Del(20)
Xinong 8116	*O. sativa indica*	In(20)
91-13-11	*O. sativa japonica*	Del(20)
Laolongxu	*O. sativa japonica*	Del(20)
Jinxi 870441	*O. sativa japonica*	Del(20)
Chuanmi 2	*O. sativa indica*	In(20)
Jiushizao	*O. sativa indica*	In(20)
Meihuanuo	*O. sativa indica*	In(20)
Gaoyuanjing 1	*O. sativa japonica*	Del(20)
Hongmangdazu	*O. sativa japonica*	Del(20)
Shanjiugu	*O. sativa japonica*	Del(20)
Jianongxianyu 31	*O. sativa indica*	In(20)
Taizhongxianxuan 220	*O. sativa indica*	In(20)
Gaoxiongyu 122	*O. sativa japonica*	Del(20)
Tainan 6	*O. sativa japonica*	Del(20)
Putaohuang	*O. sativa japonica*	Del(20)
Yinfang	*O. sativa japonica*	In(20)

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Table 4. Continued.

Code	Taxa	*Ehd1-4* (bp)
9011	*O. sativa japonica*	Del(20)
Zaoxianmi	*O. sativa indica*	In(20)
Zheli 1	*O. sativa indica*	In(20)
Yingtoujing	*O. sativa japonica*	Del(20)
71011	*O. sativa indica*	In(20)
80A86YR72	*O. sativa japonica*	Del(20)
80A90YR73	*O. sativa japonica*	Del(20)
80A97YR30	*O. sativa japonica*	Del(20)
80A97YR74	*O. sativa japonica*	Del(20)
Aus257	*O. sativa indica*	In(20)
BR061-2B-25	*O. sativa indica*	In(20)
BR319-1-HR28	*O. sativa indica*	In(20)
UGEY MAP	*O. sativa indica*	In(20)
BU189	*O. sativa japonica*	Del(20)
BU342	*O. sativa japonica*	Del(20)
BU349	*O. sativa japonica*	Del(20)
BU412	*O. sativa japonica*	Del(20)
C.Costo	*O. sativa indica*	In(20)
FAON11	*O. sativa indica*	In(20)
Manan Thukho	*O. sativa indica*	In(20)
Mya-1	*O. sativa indica*	In(20)
Mya-2	*O. sativa indica*	In(20)
CR60	*O. sativa indica*	In(20)
CR64	*O. sativa indica*	In(20)
CR65	*O. sativa indica*	In(20)
Matant MF	*O. sativa indica*	In(20)
Mulant of dwarf	*O. sativa indica*	In(20)
PSRM1-17	*O. sativa indica*	In(20)
RP1667-301-1196-1562	*O. sativa indica*	In(20)
RP1670-1418-2205-1582	*O. sativa indica*	In(20)
Toga	*O. sativa indica*	In(20)
Angke	*O. sativa indica*	In(20)
BP1356-1g-Kn-4	*O. sativa indica*	In(20)
Bp205f-Kn-78-1	*O. sativa indica*	In(20)
IR67406-6-3-2-3	*O. sativa indica*	In(20)
IR70416-53-2-2	*O. sativa indica*	In(20)
IR70445-146-3-3	*O. sativa indica*	In(20)
Balilla	*O. sativa japonica*	Del(20)
Beijin	*O. sativa japonica*	Del(20)
Changyeyu	*O. sativa japonica*	Del(20)
Jinuo	*O. sativa japonica*	Del(20)
Nipponbare	*O. sativa japonica*	Del(20)
Qiuguang	*O. sativa japonica*	Del(20)
Youliujiannuo	*O. sativa japonica*	Del(20)
Chizhenzhu	*O. sativa japonica*	Del(20)
Jiexiaonuo	*O. sativa japonica*	Del(20)
JINBU 9	*O. sativa japonica*	Del(20)
Lili 372	*O. sativa japonica*	Del(20)
Lixiaonuo	*O. sativa japonica*	Del(20)
Shuiyuan 354	*O. sativa japonica*	Del(20)
Shuiyuan 380	*O. sativa japonica*	Del(20)
Khao Toum	*O. sativa indica*	In(20)
SLK 2-21-4	*O. sativa indica*	In(20)
Mack Kouk	*O. sativa japonica*	In(20)
HNR-17	*O. sativa indica*	In(20)
HNR-2	*O. sativa indica*	In(20)
HNR-5	*O. sativa indica*	In(20)
NO.1	*O. sativa indica*	In(20)
Mollika	*O. sativa indica*	In(20)
NR10068-60-5-2	*O. sativa indica*	In(20)
NR10073-167-3-1-1	*O. sativa indica*	In(20)
NR10078-76-1-1	*O. sativa indica*	In(20)

Continued on next page

Table 4. Continued.

Code	Taxa	*Ehd1-4* (bp)
AZUCENA	*O. sativa japonica*	Del(20)
Bg300	*O. sativa indica*	In(20)
Bg304	*O. sativa indica*	In(20)
Bg305	*O. sativa indica*	In(20)
Bg358	*O. sativa indica*	In(20)
Bg359	*O. sativa indica*	In(20)
CNTLR85033-9-3-1-1	*O. sativa indica*	In(20)
SPR85163-5-1-2	*O. sativa indica*	In(20)
CALROSF	*O. sativa japonica*	Del(20)
EDITH	*O. sativa japonica*	Del(20)
Starbonnet CI9584	*O. sativa japonica*	Del(20)
Sunbonnet	*O. sativa japonica*	Del(20)
1v-139	*O. sativa indica*	In(20)
NR11	*O. sativa indica*	In(20)
VR345	*O. sativa indica*	In(20)
VR349	*O. sativa indica*	In(20)
VR350	*O. sativa indica*	In(20)
VR7	*O. sativa indica*	In(20)
VR340	*O. sativa japonica*	Del(20)
VR347	*O. sativa japonica*	Del(20)
DA	*O. rufipogon*	In(20)
DZP	*O. rufipogon*	Del(20)
HQ	*O. rufipogon*	Del(20)
WDL	*O. rufipogon*	In(20)
ZY	*O. rufipogon*	Del(20)
DL	*O. rufipogon*	Del(20)
FC	*O. rufipogon*	InDel(20)[c]
CM	*O. rufipogon*	Del(20)
PS	*O. rufipogon*	InDel(20)
ST	*O. rufipogon*	InDel(20)
TL	*O. rufipogon*	InDel(20)
DAL	*O. rufipogon*	In(20)
LS	*O. rufipogon*	Del(20)
GX	*O. rufipogon*	Del(20)
WM	*O. rufipogon*	Del(20)
WX	*O. rufipogon*	Del(20)
ZZ	*O. rufipogon*	Del(20)
LA	*O. rufipogon*	InDel(20)
SL	*O. rufipogon*	Del(20)
HX	*O. rufipogon*	Del(20)
LC	*O. rufipogon*	InDel(20)
YJ	*O. rufipogon*	Del(20)
ZP	*O. rufipogon*	In(20)
CL	*O. rufipogon*	Del(20)
DX	*O. rufipogon*	Del(20)

[a]Continuous 20-bp deletion in *Ehd1-4* region of a sample. [b]Continuous 20-bp insert. [c]Both continuous 20-bp insert and deletion occur in two haplotypes of a sample. For code abbreviations, see Table 1.

Although significant *indica-japonica* differentiation was not observed in most *O. rufipogon* varieties from China, *O. rufipogon* from Hainan Island and from the mainland of China revealed different differentiation tendencies. Most *O. rufipogon* varieties from Hainan Island contained more *indica*-like alleles than *japonica*-like alleles, whereas there were relatively more *japonica*-like alleles in most *O. rufipogon* varieties from Mainland China (Table 5). For example, WDL from Hainan Island was the only variety with a significantly higher frequency of *indica*-like alleles in China. The DA population, which is the largest population of *O. rufipogon* in Hainan Island, contained relatively more *indica*-like alleles. The DX population, representing the most northern *O. rufipogon* population in Mainland China, was significantly

japonica-like. The same result was also found in the YJ population, which has been accepted as the typical *O. rufipogon* in China (Pang et al., 1995; Sun et al., 2002; Li et al., 2006; Tan et al., 2008). Some varieties (HX, SL, ZZ) from Guangxi in Mainland China also showed a significantly high frequency of *japonica*-like alleles (Table 5).

Table 5. Intraspecies differentiation of *Oryza rufipogon* in China by *Ehd1-4* and 9 SS STS loci.

Code	*japonica* loci	*indica* loci	Coexistent *japonica* and *indica* loci	Non-*japonica* or -*indica* loci	Biased *japonica* (%)	Biased *indica* (%)
DA	3	5	2	0	30.0	50.0
DZP	4	5	1	0	40.0	50.0
HQ	4	3	2	1	40.0	30.0
WDL	4	6	0	0	40.0	60.0
ZY	4	4	1	1	40.0	40.0
DL	5	3	1	1	50.0	30.0
FC	4	3	3	0	40.0	30.0
CM	5	5	0	0	50.0	50.0
PS	3	2	5	0	30.0	20.0
ST	2	3	5	0	20.0	30.0
TL	4	2	4	0	40.0	20.0
DAL	5	4	1	0	50.0	40.0
LS	4	2	4	0	40.0	20.0
GX	5	3	1	1	50.0	30.0
WM	5	2	3	0	50.0	20.0
WX	5	2	3	0	50.0	20.0
ZZ	6	4	0	0	60.0	40.0
LA	3	4	3	0	30.0	40.0
SL	6	3	1	0	60.0	30.0
HX	6	4	0	0	60.0	40.0
LC	3	2	5	0	30.0	20.0
YJ	7	3	0	0	70.0	30.0
ZP	4	5	1	0	40.0	50.0
CL	6	2	2	0	60.0	20.0
DX	7	3	0	0	70.0	30.0

For code abbreviations, see Table 1.

Introgression from *O. sativa* to *O. rufipogon*

One hundred and forty-four *O. sativa* cultivars, and all *O. rufipogon* varieties from Hainan Island and Guangdong Province in China (Table 1) were selected to detect the introgression from *O. sativa* to *O. rufipogon* using three of the selected SSR markers (RM44, RM212, RM215).

Cultivar-specific alleles were found in most *O. rufipogon* varieties from Hainan Island and Guangdong Province, except in the varieties DA, PS, and TL (Table 6). However, different *O. rufipogon* varieties were likely affected by the introgression from different *O. sativa* subspecies. That is, some *O. rufipogon* varieties were likely affected by *O. sativa indica*, whereas others were likely affected by *O. sativa japonica* or both *indica* and *japonica*. In Hainan Island, no cultivar-specific alleles were found in the largest population, DA, while the other varieties were clearly affected by introgression from *O. sativa*. In Guangdong Province, cultivar-specific alleles were found in the varieties ST and DAL, whereas PS and TL were not affected by introgression from *O. sativa* (Table 6). Therefore, introgression from *O. sativa* to *O. rufipogon* was a general phenomenon, although some *O. rufipogon* populations appear to remain pure and non-introgressive.

Table 6. Detection of cultivar-specific alleles in *Oryza rufipogon* from Hainan Island and Guangdong Province in China by 3 SSR markers.

	RM44 (bp)		RM212 (bp)		RM215 (bp)
	130	100	115	130	160
japonica	1[a]	-	1	-	1
indica	-	1	0.89[b]	0.11	1
DA	-	-	-	-	-
DZP	-	-	-	-	-
HQ	-	-	-	-	-
WDL	-	-	-	-	-
ZY	-	-	-	-	-
DL	-	-	-	-	-
FC	-	-	-	-	-
CM	-	-	-	-	-
PS	-	-	-	-	-
ST	-	-	-	-	-
TL	-	-	-	-	-
DAL	-	-	-	-	-

[a]1 means that all *japonica* cultivars detected had the allele of RM44 with 130 bp. [b]0.89 means that 89% of the *indica* cultivars detected had the allele of RM212 with 115 bp. For abbreviations, see Table 1.

DISCUSSION

Identification of *O. sativa indica* or *japonica* with *Ehd1-4*

In the process of *O. sativa* domestication and cultivation, a series of genetic changes occurred in order to meet environmental challenges, which resulted in differentiation of the subspecies *O. sativa indica* and *O. sativa japonica* (Chang, 1976; Morishima et al., 1992; Johns and Mao, 2007). This differentiation between *indica* and *japonica* resulted from genome-wide DNA changes such as DNA rearrangements, base substitutions, InDels, translocations, and inversions (Feng et al., 2002; Gao et al., 2005). The completion of the whole genome sequences of *O. sativa japonica* Nipponbare and *indica* 9311 (Goff et al., 2002; Yu et al., 2002) revealed a large number of InDels and InDel polymorphisms between *indica* and *japonica* (Shen et al., 2004; Gao et al., 2005; Mei et al., 2007). Shen et al. (2004) indicated that 90% of InDels between Nipponbare and 9311 could be used as molecular markers and that 68 to 89% of these InDel markers showed polymorphisms between three *japonica* cultivars (Nipponbare, 9522, and Zhonghua 11) and three *indica* cultivars (9311, GLA4, and Longtepu B). Mei et al. (2007) used 46 InDel markers that were polymorphic between Nipponbare and 9311 to detect 46 *indica* and 47 *japonica* cultivars and found that these markers often represented inter-subspecific diversity, although some particular cultivars or marker loci were exceptions. The results of the present study also indicated that some InDel fragments could be used for *indica* and *japonica* cultivars. For example, *Ehd1-4* (an InDel fragment) that was polymorphic between Nipponbare and 9311 was used to detect 142 other cultivars (63 *japonica* cultivars and 79 *indica* cultivars). These cultivars are not only involved in breeding, but are also local cultivars. These results demonstrated that *Ehd1-4* could accurately identify *indica* or *japonica* with a rate of 97.2%.

Indica-japonica differentiation of *O. rufipogon* in China

The *indica-japonica* differentiation generally accepted in cultivated rice was also de-

tected in *O. rufipogon* using isozyme and DNA markers (Wang et al., 1992; Sun et al., 2002). Some researchers have indicated that most *O. rufipogon* in China is more similar to the *japonica*-type, while others are more similar to the *indica*-type (Sun et al., 1995), and that the *indica*-like type dominates among South and Southeast Asian *O. rufipogon* varieties (Sun et al., 2002). Although both *indica*-like and *japonica*-like *O. rufipogon* was observed in Guangxi and Guangdong Provinces of China, the *japonica*-like type was nonetheless more common than the *indica*-like type. *O. rufipogon* from Jiangxi and Hunan Provinces, China, represented the wild-type species. Among four strains from Yuanjiang, Yunnan Province, China, two strains were wild type and two others were *japonica*-like type (Sun et al., 2002). Wang et al. (2008) also suggested that both *japonica*-like and *indica*-like variants of *O. rufipogon* occur in China. The frequency of *japonica*-specific alleles was found to significantly increase with increasing latitude (from south to north) in China. *O. rufipogon* from Jiangxi and Hunan Provinces, China, were all clearly *japonica*-like. Other populations of *O. rufipogon* in China showed no significant divergence between *indica* and *japonica*. Both *japonica*- or *indica*-like alleles and haplotypes indicated that there were both *japonica*-like and *indica*-like variants in Guangdong, Guangxi, Fujian, and Hainan Provinces, China. Sun et al. (1997) found that *O. rufipogon* from China was mainly the *japonica*-like type. Furthermore, *japonica*-like *O. rufipogon* was found to dominate in Guangdong and Guangxi Provinces, China, whereas both wild type and *japonica*-like types were detected in Yuanjiang, Yunnan Province, China. Li et al. (2006) reported that *O. rufipogon* in Yuanjiang showed *indica-japonica* differentiation and the *japonica*-like type was predominant. In addition, recent studies revealed that *indica-japonica* differentiation has occurred not only in the nuclear genome, but also in cytoplasmic genomes (mitochondrial and chloroplast genomes) of *O. rufipogon* (Sun et al., 2002).

Most of the results of the present study are in accordance with previous results. All studies showed that only minor *indica-japonica* differentiation was evident in *O. rufipogon* from China, although differentiation tendencies were observed in different *O. rufipogon* populations. Many researchers have indicated that *japonica*-like *O. rufipogon* was the major type in China, although both *japonica*-like and *indica*-like types have been found in *O. rufipogon* from China (see above). However, results of the present study revealed that *japonica*-like *O. rufipogon* is predominant in the mainland of China, and that the *indica*-like type is the dominant type among Hainan Island *O. rufipogon*. Furthermore, Hainan Island has been suggested as a likely candidate to understand rice diversification (Wang et al., 2008). Therefore, we inferred that there are likely two different *O. rufipogon* varieties between the mainland and Hainan Island, and *O. rufipogon* from Hainan Island is likely more similar to South and Southeast Asian *O. rufipogon* varieties. These results suggest that China has relatively more abundant *O. rufipogon* resources than other countries with *O. rufipogon*. Some results of the present study contradict previous studies. Only *indica*-like type *O. rufipogon* was found in samples from Fujian Province in our study, whereas Wang et al. (2008) reported both *japonica*-like and *indica*-like types in Fujian *O. rufipogon*. We sampled from a lesser number of sites in Fujian Province, which likely caused the disagreement between the results of these two studies.

What caused the *indica-japonica* differentiation of *O. rufipogon*? Do different natural conditions, influence of gene flow from cultivated rice, or other factors? Which of the two subspecies was primarily dynamic? In our opinion, the *indica-japonica* differentiation of *O. rufipogon* was mainly caused by adaptation to different ecological and geographical environments. Much of the evidence concerning the history of rice domestication supports

this suggestion. *O. sativa indica* and *O. sativa japonica* are more closely related to certain *O. rufipogon* varieties than they are to each other (Kovach et al., 2007). In addition, molecular clock evidence showed that the divergence time of *indica* and *japonica* was earlier than that of rice domestication (Vitte et al., 2004; Zhu and Ge, 2005; Kovach et al., 2007). Furthermore, *O. rufipogon* grows over a broad region (from South China to South and Southeast Asia), and geographical isolation likely played a major role in genetic differentiation between populations of *O. rufipogon* (Sun et al., 2002; Zhou et al., 2003; Kovach et al., 2007). All of the above indicates that ancestral *O. rufipogon* was differentiated before rice domestication, likely as a result of broad adaptation to diverse climates. In addition, *O. rufipogon* of Yuanjiang, Yunnan Province is located on a hillside with an altitude of 780 m, and no *O. sativa* has been cultivated within a thousand meters (Li et al., 2006; Tan et al., 2008). Therefore, because no gene flow from *O. sativa* is possible in the Yuanjiang population, it has generally been accepted as representing typical *O. rufipogon* in China (Pang et al., 1995; Sun et al., 2002; Tan et al., 2008). Both our results and those of previous studies (Sun et al., 1997, 2002; Li et al., 2006; Wang et al., 2008) found that the *O. rufipogon* of Yuanjiang was the *japonica*-like type. Analogously, the *O. rufipogon* of Dong'ao, which has no cultivar-specific alleles, was found to be relatively more *indica*-like. These results further support the hypothesis that the primary factor of *indica-japonica* differentiation of *O. rufipogon* is due to ecological and geographical environments, and not due to introgression from cultivated rice.

Nevertheless, introgression from *O. sativa* to *O. rufipogon* is a common occurrence in regions where they are sympatric (Chen et al., 2004; Song et al., 2006; Vaughan et al., 2008), which has also likely influenced the divergence of *O. rufipogon*. Therefore, the influence of introgression from *O. sativa* on intraspecific differentiation of *O. rufipogon* might not reflect simple positive or negative action, but instead is likely related to characteristic divergence patterns of different *O. rufipogon* varieties and historical cultivars of sympatric *O. sativa*.

Conservation of *O. rufipogon* from Hainan Island

In many studies concerning *O. rufipogon* in China, Hainan Island has received little attention. However, results of the present study showed that *O. rufipogon* from Hainan Island differs from that of the mainland with respect to the *indica-japonica* differentiation pattern (Table 5), and the Hainan Island population is a good candidate for understanding diversification of *O. rufipogon* in China (Wang et al., 2008). Furthermore, the population of Dong'ao, the largest population in Hainan Island, remains pure and non-introgressive (Table 6). Therefore, the implementation of scientific conservation strategies in Hainan Island is of high priority.

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