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Full Length Research Paper

Chloroplast DNA genetic diversity between Asian cultivated rice (*Oryza Sativa* L.) and different types of cytoplasmic male sterile rice

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The chloroplast DNA (cpDNA) genetic diversity in *Oryza sativa* and different types of cytoplasmic male sterility (CMS) lines was elucidated by comparing the polymorphic sites in nine highly variable regions. A total of 151 polymorphic bases were detected among 29 materials; of which 131 bases were identified with *indica-japonica* characteristics. 17 CMS lines were divided into 3 cytoplasmic types: CMS-WA (wild-abortive), CMS-HL (Honglian) and CMS-BT (Boro II). CMS-WA contained V20A, Jin23A, GuangyeA, Chuangxiang29A, Zhenshan97A, T98A, Yong 6A, II 32A, ZhongjiuA, You1A, K17A, CIRAD496A and Gang46A; CMS-HL consisted of YuetaiA; CMS-BT contained T1A, 6A and 199A. All these three CMS types could be distinguished using these sequences: CMS-WA lines uniquely had a "GTTGAG" sequence at the position 220 to 225 of rps16 intron; CMS-HL lines contained a specific "G" base at position 595 of rps16 intron; and CMS-BT lines were also distinguishable by using their *indica* specific sequences "GCTT" in *ccsA* gene under a *japonica* cpDNA background. On the other hand, it was proved that some genetic variations occurred in cpDNA of CMS, so, the cytoplasm resources of CMS lines were not ordinary, but some variants. However, further study is needed to reveal the relationship between these variations and pollen sterility.

Key words: Genetic diversity; cytoplasmic male sterility; chloroplast DNA; *indica-japonica* differentiation.

INTRODUCTION

Cytoplasmic male sterility (CMS) is a maternally inherited characteristic found in many (more than 150) plant species. CMS/restoration systems are useful tools for hybrid seed production, and are ideal models for studying the interactions between nuclear and cytoplasm genomes. Asian cultivated rice (*Oryza sativa* L.) is a major source of nutrition for more than half the global population (Vaughan et al., 2003). The success utilization of cytoplasmic male sterility in rice is regarded as a significant breakthrough in the last century, greatly contributing to the food supply. The earliest CMS line reported was CMS-BT which was bred by crossing indica rice Chinsurah Boro II as the female parent and Taichung 65 as the male parent (Shinjyo, 1969). Later, a group of scientists headed by Yuan Longping in China successfully bred CMS-WA (wild-abortive) lines by using *Oryza rufipogon* cytoplasm of Hainan, which was regarded as the most significant step in three-line hybrid rice breeding (Lin and Yuan, 1980). To date, several CMS systems defined by different CMS cytoplasm have been bred successfully. They are CMS-GA (with *indica* cytoplasm of cultivar Gambiaca), CMS-Yinshui (with *indica* cytoplasm of cultivar Indonesian Paddy No.6), CMS-K (with *japonica* cytoplasm of cultivar K52), CMS-HL (with *O. rufipogon* cytoplasm of Hainan Red Awn CWR), and so on (Yuan, 2002; Zhu, 2000). These systems have been widely used for hybrid rice breeding in China and other Asian countries.

Some researchers believe that CMS is mostly related

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Abbrevations: CMS, Cytoplasmic male sterility; **cpDNA,** chloroplast DNA; **WA,** wild-abortive; **HL,** Honglian; **BT,** Boro II; **mtDNA,** mitochondria DNA;**ORF,** open reading frame.

Table 1. Typical *indica/japonica* cultivars and common wild rice controls used in this study.

No.	Material	Origin/ cytoplasm	Provider
1	9311*	IRRI	Hunan Normal University
2	IR36*	Jiangsu, China	Hunan Normal University
3	Nanjing-3*	Jiangsu, China	Hunan Normal University
4	Guangluai-4*	Guangdong, China	Hunan Normal University
5	Kasalath*	India	Hunan Normal University
6	Basmati*	India	Hunan Normal University
7	Nippoonbare**	Japan	Hunan Normal University
8	*Bairizao***	Sichuan, China	Hunan Normal University
9	Akihikari**	Japan	Hunan Normal University
10	Aizinuo**	Yunnan, China	Hunan Normal University
11	Hainan CWR***	Hainan, China	Hunan Normal University
12	Chaling CWR***	Hunan, China	Hunan Normal University

*, indicating typical *indica* rice; **, indicating typical *japonica* rice; ***, indicating common wild rice.

to mitochondria and several CMS related genes are mapped in mitochondria DNA (mtDNA) during the past decades (Kadowaki and Harada, 1989; Iwahashi et al., 1984; Yao et al., 2001; Lin et al., 2000; Liu, 2009). However, the molecular mechanism of cytoplasmic effect has not been studied in details. In particular, little study on cpDNA genetic diversity of these CMS types has been reported. Hirai et al. (1985) constructed the first physical map of rice cpDNA; Hiratsuka et al. (1989) published the rice chloroplast genome sequence from *Nipponbare*. Five chloroplast genome sequences of rice are available at present. Furthermore, ORF100 has been reported as an efficient marker to determine whether a rice chloroplast genome belongs to *indica* or *japonica* type: the ORF100 bands of *japonica* rice lags behind those of *indica* rice because of a 69 bp deletion in the *indica* ORF100 region (Kanno et al., 1993). Chen et al. (1993, 1994) classified 137 cultivated rice into *indica* and *japonica* based on the ORF100 marker, and the results corresponded to the classification by morphological and physiological characters. Tang et al. (2004) found that compared with *japonica, indica* has a 32 bp insertion in the ORF29-TrnC^{GCA} (tRNA-Cys^{GCA}) spacer, leading to a lag of the *indica* bands. Therefore, ORF29-TrnC^{GCA} can be regarded as another marker to distinguish the *indica* chloroplast genome from that of *japonica*. Shaw et al. (2005) found that the intron of ribosomal protein s16 (rps16) and the spacer of TrnT^{UGU}-TrnL^{UAA} [tRNAThr(UGU)- tRNA-Leu(UAA)] are highly variable regions in plant chloroplast genomes. Some other chloroplast genes such as psbB (photosystem II 47 kDa protein), rpoc2 (RNA polymerase beta'' chain), ccsA (cytochrome c biogenesis protein), ndhH (NADH dehydrogenase 49 kDa subunit) and psbA (photosystem II protein D1) are important genes involved in the process of rice photosynthesis and the variable regions of these genes also provide effective sources for polymorphic study of closely related species.

Therefore, in this study, we performed a more comprehensive comparison of the cpDNA of *O. sativa* and different types of CMS lines by using 3 cpDNA non-coding regions and 6 coding regions, thereby exploring the cpDNA genetic diversity in *O. sativa* and CMS lines. The results will deepen our understanding on the cytoplasm resources of CMS lines and ultimately accelerate the genetic improvement of CMS lines.

MATERIALS AND METHODS

Plant materials

17 different types of CMS lines were used in this study. Meanwhile, six *indica* cultivars, four *japonica* cultivars and two lines of common wild rice were used as controls (Tables 1 and 2).

Chloroplast DNA extraction

Chloroplast DNA was extracted from the fresh leaves of rice using the method mentioned by Rogers and Bendic (1988).

PCR amplification and agarose-gel electrophoresis

Chloroplast genome primers cp1 to cp11 were designed by primer design software Primer Premier 5.0 based on the chloroplast genome sequence of *9311* (Table 3). PCR reaction system included 2.5 µl 10× PCR buffer (Tiangen Biotech CO., Ltd, Beijing, China), 0.5 µl forward and reverse primers (synthetized by Sangon Biotech CO., Ltd, Shanghai, China), 0.3 µl 10 mM dNTPs (Tiangen Biotech CO., Ltd, Beijing, China), 1 U Taq (Tiangen Biotech CO., Ltd, Beijing, China), 40 ng template DNA, and complementary ultrapure water to 25 µl. PCR amplification was performed as pre-denaturation at 95°C for 3 min, then 95°C for 30 s, 50 to 55°C for 30 s, and 72°C for 50 to 90 s; the cycle was repeated 30 times, and then 72°C for 10 min. The PCR products were fractionated on 1% agarose-gel (Gene Company Ltd, Hongkong, China) which contained 0.5 µlg/ml EB, then observed with Kodak Gel Logic 100 ultraviolet gel imaging system (Eastman Kodak Company,

Table 2. 17 different types of CMS lines used in this study.

No.	Material	Origin/ cytoplasm	Provider
13	V20A	Hainan CWR	Hunan Normal University
14	Jin23A	Hainan CWR	Hunan Normal University
15	GuangyeA	Hainan CWR	Hunan Normal University
16	Chuanxiang29A	Hainan CWR	Hunan Normal University
17	Zhenshan97A	Hainan CWR	Hunan Normal University
18	T98A	Hainan CWR	Hunan Normal University
19	Yong6A	Hainan CWR	Hainan Shennongdafeng Seeds CO., ltd
20	II 32A	Indonesian Paddy No.6	Hunan Normal University
21	ZhongjiuA	Indonesian Paddy No.6	Hunan Normal University
22	You1A	Indonesian Paddy No.6	Hunan Normal University
23	K17A	K52	Hunan Normal University
24	CIRAD496A	IRRI	hunan yahua seeds co., ltd
25	Gang46A	Gambiaca	Hunan Normal University
26	YuetaiA	Hainan Red Awn CWR	Wuhan University
27	T1A	Chinsurah Boro II	National Hybrid Rice Research Center Of Tianjin Branch Center
28	6A	Chinsurah Boro II	National Hybrid Rice Research Center Of Tianjin Branch Center
29	199A	Chinsurah Boro II	National Hybrid Rice Research Center Of Tianjin Branch Center

Table 3. Primer sequence and target fragment of chloroplast DNA.

Primer	Forword sequence 5'—3'	Reverse sequence 5'—3'	Target fragment
cp1	GTGGACCTGACTCCTTGAA	AGCCGAGGTCGTGGTAA	ORF100
cp2	GCAGCCCAAGCGAGACT	AAGGCTCGGCGATACTG	ORF29-*TrnC*^{GCA}
cp3	TTTTCTCCTCATACGGCT	TAGTCTGTTCTATTCGTCCC	*rps16* gene intron
cp4	AGTGGGCTTACATAACAGAAA	ACCAAGGCTCAATACAATCA	*TrnT*^{UGU}-*TrnL*^{UAA}
cp5	AGTCTTTGCCAATGCGATAA	GTTGCTGACCCATACCAC	psbB
cp6	CTATGTGGTATGGGTCAGC	AAAGAAGGGATGGGAGAT	psbB
cp7	TAGCAACACTTTTCCCACA	GCTCATTCCAATCCTCAA	rpoc2
cp8	TGAGGATTGGAATGAGCG	TTTTGGGAGATGGTGGA	rpoc2
cp9	TCAAGCATCTCGGTTCG	CGCCTCATTAGCCCATAC	ccsA
cp10	TTCCCAGTAAGAGGGTCAA	CTTCTCACTTGTTATGGCTTG	ndhH
cp11	AGAGGGAAGTTGTGAGCATT	CGTCTGGGTATGCGTCCT	psbA

America).

DNA sequencing and data analysis

The target fragments were isolated from the agarose gel under UV radiation, reclaimed and purified with the reagent kit (*Tiangen* minipurification kit, Beijing, China), and then directly sequenced by ABI3730 sequencer (Applied Biosystems Inc, America). Sequence analysis on these polymorphic fragments was all performed by MEGA 4 (Tamura et al., 2007).

RESULTS

CpDNA genetic diversity analysis of *O. sativa*

151 polymorphic bases (bp) were detected in these 3 non-coding regions and 6 coding regions among 29 rice species (Table 4). There existed 141 polymorphic bases in these cpDNA fragments within *O. sativa*; of which, 131 *indica-japonica* characteristic bases involved in 13 sites were found (marked with "*" in Table 4). 6 *indica* and 4 *japonica* were classified clearly at these *indica-japonica* specific sites and their cpDNA classification results coincided with length polymorphic results of ORF100 and ORF29 -TrnC^{GCA} fragments (Figures 1 and 2). As a result, ORF100 and ORF29-TrnC^{GCA} fragments should be reliable molecular markers to classify the *indica/ japonica* type of *O. sativa* cpDNA.

CpDNA genetic diversity analysis of different types of CMS lines

124 polymorphism bases were found among the 17 CMS

Table 4. Sequence divergence of the 9 cpDNA regions.

Material	Gene and its base site No.															
	ORF100	ORF29-TrnCGCA			rps16 intron			TrnTUGU-TrnLUAA								
	248-316 *	130-131 *	163-194 *	220-225	267-273 *	309-312	595	322-326 *	413*	463	479	520	572	599	635	774-779 *
9311	D-69	AA	I-32	-	CTTTATC	-	T	-	-	G	A	A	G	T	A	-
IR36	D-69	AA	I-32	-	CTTTATC	-	T	-	-	G	A	A	G	T	A	-
Nanjing-3	D-69	AA	I-32	-	CTTTATC	-	T	-	-	G	C	G	A	T	A	-
Guangluai-4	D-69	AA	I-32	-	CTTTATC	CTTT	T	-	-	G	A	A	G	T	A	-
Kasalath	D-69	AA	I-32	-	CTTTATC	CTTT	T	-	-	G	A	A	G	T	A	-
Basmati	D-69	AA	I-32	-	CTTTATC	CTTT	T	-	-	G	A	A	G	T	A	-
Nipponbare	I-69	GG	D-32	-	-	-	T	TATAT	T	G	A	A	G	T	A	AGAAAA
Bairizao	I-69	GG	D-32	-	-	-	T	TATAT	T	G	A	A	G	T	A	AGAAAA
Akihikari	I-69	GG	D-32	-	-	-	T	TATAT	T	A	A	A	G	C	T	AGAAAA
Aizinuo	I-69	GG	D-32	-	-	-	T	TATAT	T	G	A	A	G	T	A	AGAAAA
Hainan CWR	D-69	AA	I-32	-	-	-	T	-	-	G	A	A	G	T	A	AGAAAA
Chaling CWR	I-69	GG	D-32	-	-	-	T	TATAT	T	G	A	A	G	T	A	AGAAAA
V20A	D-69	AA	I-32	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	AGAAAA
Jin23A	D-69	AA	I-32	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	AGAAAA
GuangyeA	D-69	AA	I-32	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	AGAAAA
Chuanxiang29A	D-69	AA	I-32	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	AGAAAA
Zhenshan97A	D-69	AA	I-32	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	AGAAAA
T98A	D-69	AA	I-32	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	AGAAAA
II 32A	D-69	AA	I-32	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	AGAAAA
ZhongjiuA	D-69	AA	I-32	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	AGAAAA
You1A	D-69	AA	I-32	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	AGAAAA
K17A	D-69	AA	I-32	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	AGAAAA
CIRAD496	D-69	AA	I-32	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	AGAAAA
Gang46A	D-69	AA	I-32	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	AGAAAA
Yong6A	D-69	AA	I-32	GTTGAG	-	-	T	-	-	G	A	A	G	T	A	AGAAAA
YuetaiA	D-69	AA	I-32	-	-	-	G	-	-	G	A	A	G	T	A	AGAAAA
T1A	I-69	GG	D-32	-	-	-	T	TATAT	T	G	A	A	G	T	A	AGAAAA
6A	I-69	GG	D-32	-	-	-	T	TATAT	T	G	A	A	G	T	A	AGAAAA
199A	I-69	GG	D-32	-	-	-	T	TATAT	T	G	A	A	G	T	A	AGAAAA

"*" indicates *indica-japonica* characteristic sites; "I" indicates insertion, "D" indicating deletion. All the genbank accession No. of these sequences were listed in Supplementary Table 1.

lines and no polymorphism was found within the CMS lines of the same type in these regions. Furthermore, CMS-Yinshui, CMS-GA and CMS-K lines had the same sequences with CMS-WA lines, and they all contained a specific sequence of "GTTGAG" at the position of 220 to 225 of

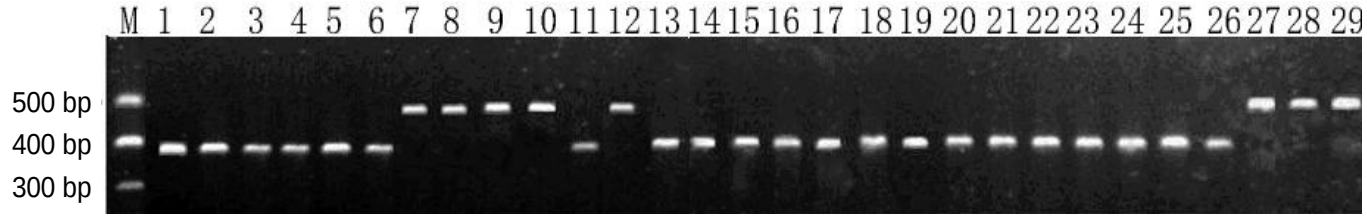

Figure 1. Amplification fragments length analysis of ORF100. M: Marker; 1 to 29: different materials according to Tables 1 and 2.

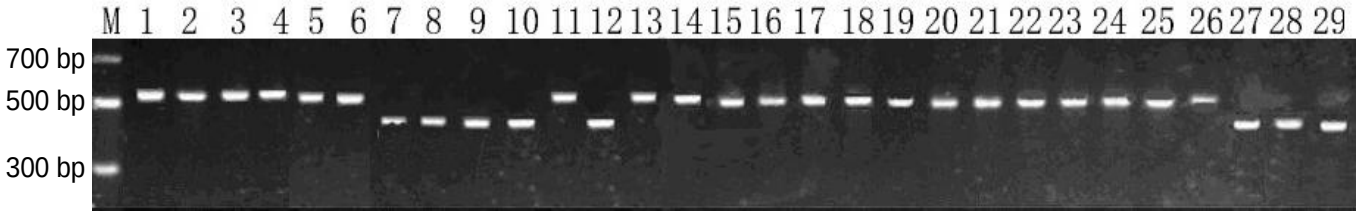

Figure 2. Amplification fragments length analysis of ORF29 -TrnC^{GCA}; M: marker, 1 to 29: different materials according to Tables 1 and 2.

rps16 *intron*. CMS-HL lines also had distinguishable base "G" at the position of 595 of rps16 *intron*. While the CMS-BT lines were nearly identical with typical *japonica* except an *indica* specific sequence "GCTT" at nucleotide sites 540 to 543 of *ccsA* gene. Based on this result, the 17 CMS lines were reclassified into 3 types: CMS-WA, CMS-HL and CMS-BT in our research: CMS-WA contained V20A, Jin23A, GuangyeA, Chuangxiang29A, Zhenshan-97A, T98A, Yong 6A, II 32A, ZhongjiuA, You1A, K17A, CIRAD496A and Gang46A; CMS-HL type consisted of YuetaiA; and CMS-BT contained T1A, 6A and 199A.

DISCUSSION

CpDNA genetic diversity and molecular markers of *indica* and *japonica*

Indica and *japonica* are two important subspecies of *O. sativa*. There exist marked differences in agronomic characters and ecological adaptabilities between them; and these characteristic associations of traits have been explained by the hybrid sterility or reproductive barriers (Sato et al., 1988; Zhang et al., 1997; Fukuoka et al., 1998; Harushima et al., 1998; Hiroshi, 2009). The nuclear genetic diversity between *indica* and *japonica*, especially some nuclear genetic differences related to important agronomic traits, were investigated widely in recent decades (Second, 1985; Cai and Wang, 1993; Mochizuki et al., 1993; Chen et al., 1994; Fukui et al., 1994；Garris et al., 2005; Wang and Tanksley, 1989; Zhang et al., 1992). However, our understanding of cytoplasmic genetic diversity between them as well as the relationship among this genetic diversity, agronomic characters and phylogeny is still limited (Chen et al., 1993, 1994; Kanno et al., 1993; Sun et al., 1997; Shaw J et al., 2005). In our study, 131 *indica-japonica* characteristic bases spread across 13 sites were identified (marked with "*" in Table 4). Six *indica* and four *japonica strains* were classified clearly at these *indica-japonica* specific sites and their cpDNA classification results coincided with length polymorphic results of ORF100 and ORF29 -TrnC^{GCA} fragments (Figures 1 and 2). Hence, the ORF100 and ORF29-TrnC^{GCA} fragments are reliable molecular markers that can be used to classify the *O. sativa* cpDNA into the *indica/japonica* types. The results also show abundant polymorphism between the *indica* and *japonica* subspecies not only in the non-coding regions, but also in the coding regions. The polymorphic bases in the encoding regions can lead to changes in the amino acids. However, whether these amino acid changes are related to the different phenotypes of these two subspecies, remains to be determined.

CpDNA genetic diversity and cytoplasmic origin analysis of CMS lines

According to the more comprehensive sequences comparison of these 9 variable cpDNA regions, so-called CMS-Yinshui, CMS-GA and CMS-K lines had the same sequences with CMS-WA lines, which suggested that the chloroplast genetic background of these CMS lines is the same. Some researches (Li et al., 2006) and production practice also proved CMS lines of these four types can be restored by the same restorer as well as maintained by the same maintainer. Consequently, these four CMS types were all redefined as CMS-WA in our study. This reflected to some extent that genetic background of CMS is more single. So, it is becoming more and more important to search for the lines that contain novel cytoplasmic sterile source, which is a crucial step for increasing the biological diversity of rice as well as a key

direction of maintaining biological safety in agriculture.

Furthermore, all the 3 CMS types could be distinguished from *O. sativa* and *O. rufipogon* strains using these regions. CMS-WA lines uniquely had a "GTTGAG" sequence at the position 220 to 225 of rps16 *intron*. CMS-HL lines contained a specific "G" base at position 595 of rps16 *intron*, and CMS-BT lines had the same cpDNA sequences with *japonica*, except an *indica* specific sequence "GCTT" in *ccsA* gene in our study. So, they were also distinguishable by using their *indica* specific sequences under the *japonica* cpDNA background. All these results indicate that some genetic variations must have been occurred in the cpDNA of CMS lines, thus, a corresponding DNA fingerprinting system for them was preliminarily established by using these genetic variations. However, whether these variations affect the pollen sterility or not, and if they do, the role they play needs further study.

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Supplementary Table 1. Genbank accession No. of these sequences.

Material	rps16 intron	$TrnT^{UGU}$-$TrnL^{UAA}$	psbB	rpoc2	ccsA	ndhH	psbA
9311	AY522329	AY522329	AY522329	AY522329	AY522329	AY522329	AY522329
IR36	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Nanjing-3	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Guangluai-4	GQ150219	GQ150239	GQ848581	GU001685	GQ150197	GQ150219	GQ150239
Nippoonbare	AY522330	AY522330	AY522330	AY522330	AY522330	AY522330	AY522330
Kasalath*	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Basmati*	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Bairizao	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Akihikari	GQ848577	GQ150256	GQ848587	GU001691	GQ150215	GQ848577	GQ150256
Aizinuo	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Hainan CWR	GQ150222	GQ150242	GQ848591	GU001688	GQ150200	GQ150222	GQ150242
Chaling CWR	GQ848579	GQ150243	GQ848584	GU001689	GQ150203	GQ848579	GQ150243
V20A	GQ150231	GQ150247	GQ848597	GU001700	GQ150207	GQ150231	GQ150247
Jin23A	GQ150229	GQ150245	GQ848595	GU001698	GQ150205	GQ150229	GQ150245
GuangyeA	GQ150230	GQ150246	GQ848596	GU001699	GQ150206	GQ150230	GQ150246
Chuanxiang29A	GQ150232	GQ150248	GQ848598	GU001701	GQ150208	GQ150232	GQ150248
Zhenshan97A	GQ150233	GQ150249	GQ848599	GU001702	GQ848576	GQ150233	GQ150249
T98A	GQ150234	GQ150250	GQ848600	GU001703	GQ150209	GQ150234	GQ150250
II 32A	GQ150235	GQ150251	GQ848601	GU001704	GQ150210	GQ150235	GQ150251
ZhongjiuA	GQ150236	GQ150252	GQ848602	GU001705	GQ150211	GQ150236	GQ150252
You1A	GQ150237	GQ150253	GQ848603	GU001706	GQ150212	GQ150237	GQ150253
K17A	GQ150238	GQ150254	GQ848604	GU001707	GQ150213	GQ150238	GQ150254
CIRAD496	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Gang46A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Yong6A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
YuetaiA	GQ150223	GQ150255	GQ848586	GU001708	GQ150214	GQ150223	GQ150255
T1A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
6A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
199A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

“N/A” indicating not submitted yet.