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Identification of *Indica-Japonica* Subspecies Rice by Duplex Fluorescence PCR Detection for Chloroplast DNA and Endogenous *gos* Gene

Biying Shao, Tingyu Miao, Juan Peng, Bin Chen, Wenbing Chen*

Technology Center of Fuzhou Customs District, Fuzhou, China

Email address:
byshao@souho.com (Biying Shao), miaoty419@163.com (Tingyu Miao), 617572834@qq.com (Juan Peng), 621213wbc@163.com (Wenbing Chen)

*Corresponding author

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Abstract: Asia cultivated rice are classified into two subspecies, *indica* and *japonica* rice (*hsien* and *keng* rice called in China, separately). It is necessary to establish a convenient and effective method to identify two subspecies because of the different quality characteristics between the rice products of them, which lead to different processing uses and commodity values. The identification method of PCR based on the difference of chloroplast DNA is convenient and effective, to be attempted to establish. Based on 69 bp fragment deletion of chloroplast DNA (cpDNA) found in ORF100 region nucleotides within the cpDNA Pst I-12 fragment in *indica* rice but not in *japonica*, the primer pair and a probe located on the specific cpDNA fragment in *japonica* rice were designed to identify *indica* and *japonica* rice. Another primer pair and probe used to detect endogenous gene *gos* in rice were combined with above primer pair and probe for detecting cpDNA to establish duplex fluorescence PCR to amplify cpDNA and *gos* gene for improving detection accuracy, to avoid the false negative results caused by DNA extraction error. The duplex fluorescence PCR detection method was established using typical *japonica* rice (pearl rice) and typical *indica* rice (Taixian 11). The accuracy of the method was validated with 547 samples including 177 samples of rice seed and eaten rice known as conventional *japonica* varieties and *japonica* type hybrid combinations and 370 samples of rice seed and eaten rice known as conventional *indica* rice varieties and *indica* type hybrid combinations. In 177 *japonica* samples, 170 samples with both positive results of cpDNA marker and *gos* gene, a coincidence rate of 96.05% agreement *japonica* rice, and 7 samples with positive results of *gos* gene and negative of cpDNA marker, not agreement *japonica* rice with non-coincidence rate of 3.95%, were detected. Of the 370 samples of *indica* rice seed and eaten rice, with positive results of *gos* gene and negative of cpDNA marker were detected in 340 samples, the rate of coincidence to *indica* rice was 91.89%. Other 30 samples with both positive results of cpDNA marker and *gos* gene, were detected, and the non-coincidence rate with *indica* rice was 8.11%. These identification results were in good agreement with known *indica* and *japonica* varieties, and the coincidence rate of *japonica* varieties was higher than *indica*. This method can be used to identify *indica* and *japonica* subspecies rice, especially suitable to identify conventional rice varieties.

Keywords: *Indica* (*hsien*) Rice Seed/Eaten Rice, *japonica* (*keng*) Rice/Eaten Rice, Chloroplast DNA (cpDNA), Endogenous *gos* Gene, Duplex Fluorescence PCR, Identification

1. Introduction

Cultivated rices in Asia were divided into *Oryza sativa* L. subspecies *indica* and *Oryza sativa* L. subspecies *japonica* [1], *hsien and keng* subspecies also called in China for thousands of years [2, 3]. The traditional identification methods of them are mainly morphological identification and biochemical index classification methods [1-7]. These methods have the defects of intermediate transition type and can not be clearly divided into two categories. With the development of molecular biology technology, more and more molecular markers, such as isozyme markers [7-8] and

DNA markers including RFLP [9-11], RAPD [12, 13], InDel (insertion/deletion, *japonica* specific insertion deletion) [14-18], cytoplasmic molecular marker [7, 19-23], were used to study the origin and the differentiation of *indica-japonica* subspecies in Asian cultivated rice (*Oryza sativa* L.).

It is necessary to establish a DNA identification method to distinguish *indica* rice and *japonica* rice because of their different commercial value. Kanno et al. [23] found that *indica* rice had a 69 bp deletion in the ORF100 region of Pst I-12 fragment of chloroplast DNA (cpDNA, same below) compared with *japonica* rice. Because of long-term natural or artificial hybridization, frequent communication of genetic information and complex DNA components in nucleus, it is difficult to identify *indica-japonica* subspecies based on differential markers of nuclear DNA. As a complicated method for the identification of *indica-japonica* subspecies, the previously published methods for the differentiation and identification of *indica-japonica* rice based on the PCR amplification polymorphism of nuclear DNA and cpDNA were presented, there are some problems such as expensive cost and low accuracy. This technique only detects one stable cpDNA site, which is accurate, efficient, economical and simple. Duplex fluorescence real-time PCR were widely used to detect the pathogenic bacteria and viruses in food [24-26] and plants [27], also the animal-derived components and genetically modified components in food [28-30]. There was no report on the identification of *indica-japonica* subspecies by duplex fluorescence real-time PCR. The primer pair selected (Chen. et al) and a probe located on the 69 bp fragment in cpDNA of *japonica* rice were designed to identify *indica* and *japonica* rice [31]. Another primer pair and probe used to detect endogenous gene *gos* [32] of rice were combined with above primer pair and probe detecting cpDNA to establish duplex fluorescence PCR for cpDNA and *gos* gene for improving detection accuracy.

The accuracy of the method was validated with 177 samples of rice seed and eaten rice of *japonica* varieties and *japonica* hybrid combinations and 370 samples of rice seed and eaten rice of conventional *indica* rice varieties and *indica* hybrid combinations, and the reasons for non-coincidence were analyzed.

2. Materials and Methods

2.1. Materials

Rice seed and eaten rice were mainly collected from rice seeds consigned for inspection, eaten rice from local supermarkets in Fuzhou city, and local rice varieties and hybrid rice combinations from institutions such as Fujian Academy of Agricultural Sciences, Guangxi Academy of Agricultural Sciences, Yunnan Agricultural University and Kaili College in Guizhou Province, a total of 547 samples. The typical *japonica* pearl rice from Northeast China and typical *indica* rice Taixian 11 from Taiwan Province of China were used to sequence the specific cpDNA fragments of *indica-japonica* subspecies and to establish the duplex fluorescence PCR method.

2.2. DNA Extraction and Duplex Fluorescence PCR

2.2.1. DNA Extraction

100 g rice seeds or eaten rice were ground into powder, 1.0 g of powder was added into 50 mL centrifuge tube, then CTAB method [31] was used to extract DNA. 2 μL DNA solution were taken in the ultra-micro nucleotides determinator for examining, dilute to 100 ng/μL with TE solution (pH 8.0), keep at -20°C.

2.2.2. Primer and Probe

The sequence of primers and probes are shown in Table 1, the probe to be designed basing on the sequences on *indica-japonica* specific cpDNA fragment of rice in Figure 1. The PCR amplification system with 2 short target fragments and 5 ratios of primer dosage are shown in Table 2.

Table 1. *The sequences of primers and probes in duplex fluorescence PCR for cpDNA marker and endogenous gos gene to identify indica-japonica rice.*

Target gene fragment	Primers	Primer sequences (5'- -3')	Length of PCR products (bp)
indica-japonica specific cpDNA segment of rice	Rice-F4 forward primer [31]	AATCGCAACCCCTTTCCGC	204
	Rice-R1 reverse primer [31]	TTGAGGATTATTCCATGATTCC	
	probe	HEX-ATGCAATAGAGAGCGAGTGG-BHQ1	
Endogenous *gos* gene fragment in rice [32]	gos9-F forward primer	TTAGCCTCCCGCTGCAGA	68
	gos9-R1 reverse primer	AGAGTCCACAAGTGCTCCCG	
	gos9-P probe	FAM-CGGCAGTGTGGTTGGTTTCTTCGG-TAMRA	

Table 2. *The different proportion of two set primers and probes in duplex fluorescence PCR system for cpDNA marker and endogenous gos gene to identify indica-japonica rice.*

Composition	Amount of addition (μL)				
	1:1	1:2	1:3	1:4	1:5
ddH_2O	8	7.5	7	6.5	6
2×Mix	10	10	10	10	10
gos-F	0.2	0.2	0.2	0.2	0.2
gos-R	0.2	0.2	0.2	0.2	0.2
gos-P	0.1	0.1	0.1	0.1	0.1
Rice-F4	0.2	0.4	0.6	0.8	1.0
Rice-R1	0.2	0.4	0.6	0.8	1.0
Rice-P	0.1	0.2	0.3	0.4	0.5
DNA (200 ng/μL)	1	1	1	1	1

```
                F4
japonica  AATCGCAACCCCTTTCCGCTACACATAAAAATGGATTTCGAAAGTCAATTTTTCTTTTCA   60
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
indica    AATCGCAACCCCTTTCCGCTACACATAAAAATGGATTTCGAAAGTCAATTTTTCTTTTCA
                                                              P
japonica  ATATCTTTACTTTTTTTCAGAATCCTATTTTTGTTCTTATACCCATGCAATAGAGAGCGA   120
          ||||||||||||||||||
indica    ATATCTTTACTTTTTTTCA.....................................  ...

japonica  GTGGGAAAAGGGAGGTTACTTTTTTTCATTTTTCCCTTAAAAAATAGGCTTTCTTGGAAA   180
                                    ||||||||||||||||||||||||||||||||
indica    ............................TTTTTCCCTTAAAAAATAGGCTTTCTTGGAAA
                R1
japonica  TAGGAATCATGGAATAATCCTGAA   204
          ||||||||||||||||||||||||
indica    TAGGAATCATGGAATAATCCTGAA
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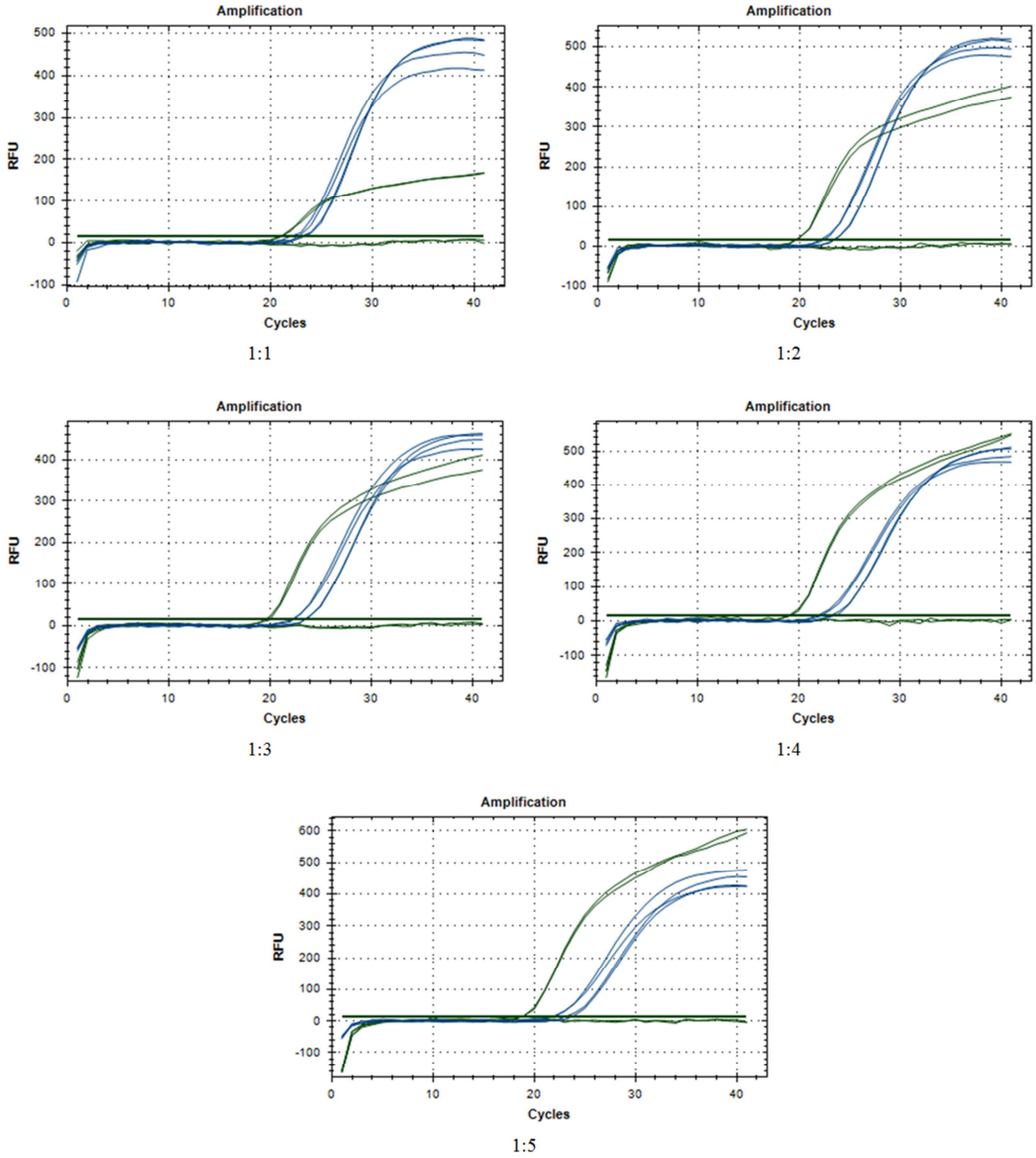
Figure 1. *The position of Taqman fluorescence PCR probe in specific cpDNA fragment of japonica rice.*



(RFU: Relative Fluorescence Unit)

Figure 2. *The curve diagram of duplex fluorescence PCR.*

Table 3. *The Ct value and fluorescence intensity of duplex fluorescence PCR for specific cpDNA in japonica rice and endogenous gos gene.*

Ratio of primer and probe in duplex fluorescence PCR mixture	*Indica*/*japonica* rice sample	*Gos* gene in rice		Specific cpDNA in *japonica* rice	
		Ct value	Fluorescence intensity	Ct value	Fluorescence intensity
1:1	*indica*	22.67	400	-	-
		21.94	440	-	-
	japonica	22.99	480	21.04	165
		23.28	480	21.00	165
1:2	*indica*	22.28	480	-	-
		22.01	500	-	-
	japonica	23.37	510	19.60	380
		23.28	510	19.51	400
1:3	*indica*	22.32	420	-	-
		22.19	460	-	-
	japonica	23.45	460	19.53	400
		23.47	450	20.04	370
1:4	*indica*	21.78	480	-	-
		22.17	470	-	-
	japonica	23.49	500	19.13	540
		23.12	500	19.03	540
1:5	*indica*	21.86	420	-	-
		22.02	470	-	-
	japonica	23.16	450	18.84	600
		23.77	420	18.85	600

2.2.3. Duplex Fluorescence PCR Procedure

Initial denaturing at 95°C for 30 s; 40 cycles with two steps: 95°C for 5 s, 60°C for 34 s.

3. Results

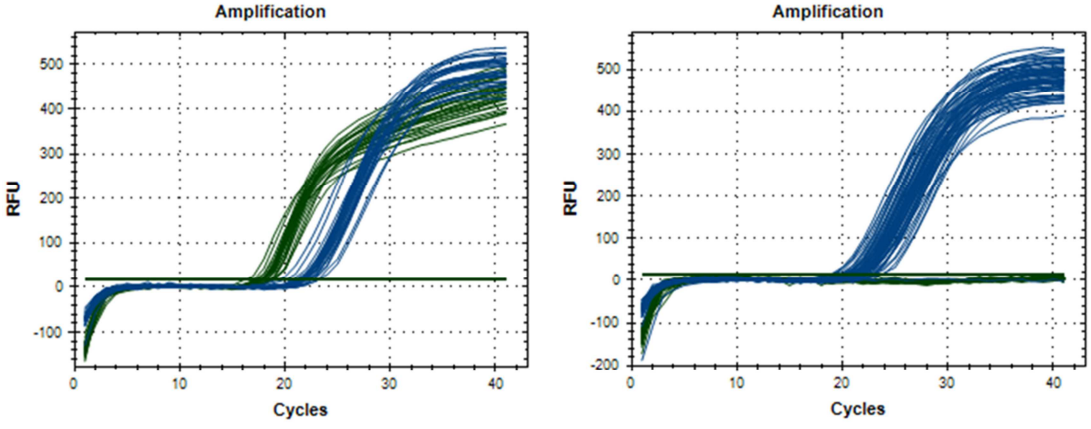
3.1. Optimization and Establishment of Duplex Fluorescence PCR

According to the results of optimization, two primers dosage proportions 1:3 and 1:4 were the best, and 1:3 was chosen to save primers within the five proportions design of primers dosage from 1:1 to 1:5 as shown in figure 2 and table 3. The other compositions and PCR thermal cycles are as shown in table 2 and 2.2.3., to establish the method of duplex fluorescence PCR.

3.2. Identification of Indica and Japonica Rice by Duplex Fluorescence PCR

The results of duplex fluorescence PCR of cpDNA marker and endogenous *gos* gene indicated that single curve results with positive of *gos* gene in *indica* rice and two curve results with positive of cpDNA marker and *gos* gene in *japonica* rice shown as Figure 3.

The accuracy of the duplex fluorescence PCR method was validated by 547 samples of rice seed and eaten rice. In 177 of *japonica* samples, 170 samples with both positive results of cpDNA marker and *gos* gene (Figure 3/A), a coincidence rate of 96.05% with *japonica* rice, and 7 samples with positive results of *gos* gene and negative of cpDNA marker, and non-coincidence rate of 3.95% with *japonica* rice, were detected. Of the 370 samples of *indica* rice seed and eaten rice, with positive results of *gos* gene and negative of cpDNA marker (Figure 3/B) were detected in 340 samples, the rate of coincidence to *indica* rice was 91.89%. Other 30 samples with both positive results of cpDNA marker and *gos* gene, were detected, and the non-coincidence rate with *indica* was 8.11%. Above results were shown as Table 4.



A: Two curve results (japonica type) B: Single curve results (indica type)

Figure 3. *The results of duplex fluorescence PCR of cpDNA marker and gos endogenous gene for identification of indica-japonica rice.*

Table 4. *Identification results of indica and japonica subspecies by duplex fluorescence PCR in 547 rice samples.*

Subspecie type	cpDNA deletion type (%)	cpDNA non-deletion type (%)	Total samples
indica	340 (91.89%)	30 (8.11%)	370
japonica	7 (3.95%)	170 (96.05%)	177

4. Discussions

In this paper, based on the difference of 69 bp length between *indica* rice and *japonica* rice in the ORF100 region of PstI-12 fragment of cpDNA [23], a duplex fluorescence PCR method was established by using a previously designed primers [31]/a newly designed probe for PCR amplification of 204 bp cpDNA fragment from *japonica* rice and primers/probe [32] for rice endogenous *gos* gene amplification, to avoid the false negative results caused by DNA extraction error and to improve the accuracy of the detection results. The method was validated in the identification of 547 rice samples of known *indica* and *japonica* varieties, and the results were highly consistent with those of *indica* and *japonica* varieties, and the coincidence rate of *japonica* subspecies was higher than that of *indica* rice. The results showed that the original local varieties of *japonica* rice from high altitude and remote areas such as Guangxi Province, Yunnan Province, Guizhou Province had the highest coincidence rate with cpDNA, almost 100%. The lowest coincidence rate was found in hybrid combinations of *indica* rice with complex genetic background. According to the database of China Rice Data Center, 22 of the 30 samples with non-delection cpDNA fragments expected to be *indica* rice had the maternal lineage of *japonica* rice. The results of fluorescence PCR identification of cpDNA did not accord with the expected subspecies of *indica* or *japonica* rice. The possible reasons are the traditional method of false name, natural hybridization, artificial hybridization and multi-generation backcross for nucleus replacement to improve varieties, but the cytoplasm of *indica* and *japonica* rice remains basically unchanged. In addition, it is also related to the different geographical distribution of *indica* and *japonica* subspecies. *indica* rice is cultivated in a wide area, with various varieties and frequent exchanges of genetic information. Especially, it is common to breed female sterile lines for hybrid rice seed production through *indica-japonica* crossing. *japonica* rice is mainly distributed in high altitude, high latitude cold and relatively closed area [14, 17], which leads to less communication of genetic information and low mutation frequency, so their coincidence rate of cpDNA detection by PCR is higher. Cai X. X. et al.(2006) had ever studied on *indica-japonica* differentiation of rice by DNA Insertion/Deletions of differential fragments. Their results indicated that introgression hybridization was found between *indica* and *japonica* subspecies in the area of mixed cultivation of two subspecies rice, some *indica* varieties has the genetic lineage of *japonica* rice, although the *indica* rice cultivars showed obvious *indica* phenotypic traits, they still retained some characteristic loci of *japonica* rice at the molecular level [15]. These results are consistent with the results of present paper. Many to hundreds of DNA loci had been used to detect in the published PCR method based on nuclear DNA [9, 18] and chloroplast and mitochondrial DNA [21-23], their accuracy is not ideal, usually between 80-90%, and is also better in *japonica* than in *indica* subspecies. The method of *indica-japonica* rice identification established in this paper has a high accuracy for local conventional varieties, with more economical, more accurate and more efficient and stable, because of detecting only one cpDNA locus, but there is still much room for improvement for hybrid combinations, which needs technical improvement. Due to genetic introgression between *indica* and *japonica* rice, it is very difficult to identify with 100% accuracy using only one gene locus. So it is expected to further screen the specific key gene sites of *indica-japonica* subspecies for PCR amplification, and complement the method based on chloroplast DNA markers to improve the accuracy of identification results.

5. Conclusions

The duplex fluorescence PCR method to identify two subspecies of *indica* and *japonica* in Asia cultivated rice was developed. The accuracy of the duplex fluorescence PCR method was validated by 547 samples of rice seed and eaten rice. The coincidence rate of 96.05% was agreement with *japonica* rice in both fluorescence PCR positive results of cpDNA marker and *gos* gene. In the results with positive of *gos* gene and negative of cpDNA marker detected, the coincidence rate of 91.89% was agreement with *indica* rice. Above duplex fluorescence PCR method can be used to identify *indica* and *japonica* subspecies rice, especially suitable for the identification of conventional rice varieties.

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