Comparison of DNA marker analysis of the blast resistance genes *Pib* and *Pita* in IRRI-bred rice varieties with gene estimation by conventional genetic analysis

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Abstract

Rice blast resistance genes are important for rice (*Oryza sativa* L.) improvement programs. DNA markers linked to resistance genes are a powerful tool to detect the presence of genes and are widely used in the selection of breeding materials through marker-assisted selection. This study was conducted to evaluate the detection ability of DNA markers for the rice blast resistance genes *Pib* and *Pita* in IRRI-bred rice varieties. Forty-two Indica-type varieties, which have been previously analyzed for the presence of *Pib* and *Pita* by conventional genetic analysis using a differential system involving standard blast isolates (*Pyricularia oryzae* Cavara) from the Philippines, were tested. To detect for the presence of *Pib* and *Pita*, previously reported PCR-based dominant markers were used. DNA fragments of *Pib* using the DNA marker Sub3-5 were amplified in 40 out of the 42 varieties examined. Additionally, DNA fragments of *Pita* using three gene specific markers were amplified in 27 or 28 varieties out of the 42 examined, depending on the marker used. The results of DNA marker analysis of 42 IRRI-bred rice varieties were largely consistent with those of previous gene estimations of *Pib* and *Pita* by a differential system. These results suggest that the efficiency of detecting blast resistance genes through use of DNA markers is dependent on the rice variety and the DNA markers. The proper markers for the *Pita* gene provide a basis for stacking other blast resistance genes into high-yielding and good-quality advanced breeding rice lines.

Keywords: DNA marker, IRRI-bred rice variety (*Oryza sativa* L.), *Pib*, *Pita*, resistance gene

Introduction

Rice blast resistance genes are important in rice (*Oryza sativa* L.) improvement programs. The resistance to blast disease is governed by a gene-for-gene relationship between the resistance gene in the host and the avirulence gene in the blast pathogen *Pyricularia oryzae* Cavara. (Silue et al. 1992). Around 40 blast resistance genes have been identified by genetic studies and five resistance genes, *Pib*, *Pita*, *Piz-5*(*Pi2*), *Piz-t*, and *Pi9*, have been isolated (Wang et al. 1999; Qu et al. 2006; Zhou et al. 2006). *Pib* and *Pita*, two major resistance genes, introgressed from Indica cultivars, encode predicted nucleotide binding proteins (Wang et al. 1999; Bryan et al. 2000). Specific gene markers are a powerful tool to detect the presence of genes and are widely used to select breeding materials through marker-assisted selection. Several gene specific markers of *Pib* and *Pita* have been developed using the genomic sequences of these genes in resistant and susceptible varieties (Wang et al. 1999; Jia et al. 2002; Fjellstrom et al. 2004).

Genetic studies of blast resistance were limited by the lack of a suitable differential system for blast resistance genes. The 12 Japonica-type differential varieties for blast resistance were selected by Yamada et al. (1976) and Kiyosawa (1981), but they carried additional gene(s) to tropical blast isolates, and these masked the reaction of the targeted gene (Noda et al. 1999). To bypass this limitation, the pathogenicities of blast isolates from the Philippines were determined using differential varieties and

lines (Yanoria et al. 2000). Several blast isolates with distinct pathogenicities were selected and studied in detail using a set of monogenic lines that contain 23 types of single resistance genes with the genetic background of a Chinese variety Lijiang-xin-tuan-heigu (Tsunematsu et al. 2000). Using this system, a total of seven resistance genes, *Pi20*, *Pita*, *Pik*† (one of *Pik* alleles, *Pik*, *Pik-h*, *Pik-m*, or *Pik-p*), *Pib*, *Pik-s*, *Piz-t*, and *Pii* or *Pi3*(t), in 42 IRRI (International Rice Research Institute) -bred rice varieties were targeted and 42 IRRI-bred varieties were classified into seven groups based mainly on the presence of *Pi20*, *Pita*, and one of *Pik*† allele gene (Ebron et al. 2004). To confirm the presence of resistance genes in IRRI-bred varieties, conventional genetic analysis, which comprise segregation analyses and allelism tests, were performed using BC_1F_2 and F_2 populations, respectively (Ebron et al. 2005).

During the 1970s and early 1980s, many IRRI-bred varieties were released and distributed worldwide, and cultivated directly in farmar's filed or used as important parental varieties in breeding programs. IR8, which was released in 1966, triggered the Green revolution in tropical countries of Asia (Hossain 1995). In the 1970s, IR36 was utilized for rice production in several Asian countries. IR64, which was released in 1985, has been widely accepted as a high-quality rice variety in many countries (Khush 1987). A more recently developed variety, IR72, displays a high yield potential, shorter growth duration, and enhanced resistance to several diseases and insect pests. IRRI-bred varieties and their progenies are now planted in rice crops worldwide. Therefore, it is important to understand blast resistance genes in IRRI-bred varieties for rice breeding.

In this study, the detection ability of gene specific markers for *Pib* and *Pita* was evaluated. Forty-two IRRI-bred varieties were analyzed using four PCR-based

Table 1. Gene identification by conventional genetic analysis for two resistance genes *Pib* and *Pita* in 42 IRRI-bred varieties

Variety group	IRRI-bred variety	*Pib*			*Pita*		
		Reaction pattern[1)]	BC_1F_2 analysis[2)]	Allelism test[2)]	Reaction pattern[1)]	BC_1F_2 analysis[2)]	Allelism test[2)]
VG 1a	IR20, IR28, IR30, IR45, IR66	+	-	-	-	-	-
VG 1b	IR29, IR34	+	+ (IR34)	+ (IR34)	-	-	-
VG 2a	IR8, IR22, IR24, IR26, PSBRc30	+	+ (IR24)	+ (IR24)	-	-	-
VG 2b	IR43	+	-	-	-	-	-
VG 2c	PSBRc2	+	-	-	-	-	-
VG 3	IR5, IR32, IR36, IR38, IR40, IR42, IR44, IR50, IR52, IR54, IR58, IR60, IR62, IR65, IR68, IR72, PSBRc4	+	+ (IR36, IR60)	+ (IR36, IR60)	+	+ (IR36, IR60)	+ (IR36)
VG 4	PSBRc1	unknown	-	-	-	-	-
VG 5	IR74	unknown	+	-	-	-	-
VG 6	IR56, IR70	unknown	-	-	+	-	+
VG 7a	IR46, IR48, IR64, PSBRc28	+	+ (IR46, IR64)	+ (IR64)	+	+ (IR46, IR64)	+ (IR64)
VG 7b	PSBRc10, PSBRc18, PSBRc20	+	-	-	+		

1) Classification of IRRI-bred varieties and genes *Pib* and *Pita* determined using a differential system involving Philippine blast isolates of *P. grisea* obtained and modified from Ebron et al. (2004).

2) Classification of IRRI-bred varieties and genes *Pib* and *Pita* determined using genetic analysis and allelism test referred from the data of Ebron et al. (2005).

+ or - indicates presence or absence of genes *Pib* and *Pita*, respectively.

dominant markers that were designed from the sequences of the blast resistance genes *Pib* and *Pita*. The results for detection of DNA fragments by PCR were compared with those of gene estimation for *Pib* and *Pita* by conventional genetic analysis.

Materials and methods

IRRI-bred rice varieties

A total of 42 Indica-type varieties at IRRI, which have been previously analyzed by conventional genetic methods, were used. Thirty-four out of the 42 Indica-type varieties are IR varieties, including IR8, IR24, IR36, IR64, and IR72, all of which are widely distributed and used in many countries. Eight out of the 42 Indica-type varieties, PSBRc1, PSBRc2, PSBRc4, PSBRc10, PSBRc18, PSBRc20, PSBRc28, and PSBRc30, were developed by IRRI and designated by the Philippine Seed Board. Six other varieties, Nipponbare, Yashiromochi, BL1, IRBLb-B (monogenic line for *Pib* derived from BL1), K1, and IRBLta-K1 (monogenic line for *Pita* derived from K1), were used as controls. The 42 IRRI-bred varieties were classified into seven groups, VG 1 to VG 7, based on a differential system (Table 1). In some cases, subgroups within a group were also identified because the variety reacted differentially to a particular isolate. VG 1, VG2, and VG 7 were further divided into two, three, and two subgroups, respectively.

DNA markers and genotyping

Whole genomic DNA was extracted from fresh leaves using the CTAB method (Murray and Thompson 1980). Primer set for *Pib*, Sub3-5 (5′- AGGGAAAAA TGGAAATGTGC -3′ and 5′-AGTAACCTTCTGCTGC CCAA-3′), was used to detect *Pib*, and primer sets for *Pita*, $Pita_{440}$ (YL153: 5′-CAACAATTTAATCATACA CG-3′ and YL154: 5′-ATGACACCCTGC-GATGCAA-3′), $Pita_{1042}$ (YL155: 5′-AGCAGGTTATAAGCTAGG CC-3′ and YL87: 5′-CTACCAACAAGTTCATCAAA-3′), and $Pita_{403}$ (YL100: 5′-CAATGCCGAGTGTGCAA AGG-3′ and YL102: 5′-TCAGGTTGAAGATGCATA GC-3′) were used for *Pita* in a previous study (Wang et al. 1999; Jia et al. 2002). The genotypes of *Pib* and *Pita* using these primers were then determined by PCR amplification in a DNA engine dyad Peltier thermal cycler (Bio-Rad). The 15 µl PCR reaction mixtures contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 200 µM dNTP, 0.2 µM primer, and 5-10 µg/ml of genomic DNA as template. The thermal cycler was programmed for a first denaturation step of 5 min at 95 ℃ , followed by 35 cycles, each of 30 s at 95 ℃, 30 s at 55 ℃ for three markers, Sub 3-5, $Pita_{440}$ (YL153/YL154), and $Pita_{1042}$ (YL155/YL87), and at 64.5 ℃ for Pita403 (YL100/YL102), and 30 s at 72 ℃ . The SSR products were resolved in 1.0% agarose gel by electrophoresis at 200 V for 1 h in 0.5 X TBE buffer. The gels were stained with ethidium bromide and photographed under ultraviolet light.

Results

Genotyping of Pib and Pita using DNA markers

BL1 and IRBLb-B carrying *Pib* were used as positive controls, while Yashiromochi and Nipponbare were used as negative controls. The presence and absence of the *Pib* genes in 42 IRRI-bred rice varieties and control varieties were confirmed using the DNA marker Sub3-5 (Fig. 1, Table 2). The DNA fragments of positive control varieties, BL1 and IRBLb-B carrying *Pib*, were amplified, while those of the negative control, Yashiromochi and Nipponbare, were not amplified. DNA fragments using Sub3-5 were amplified in all but two (PSBRc18 and IR54) of the 42 IRRI-bred rice varieties. Similarly, K1, IRBLta-K1, and Yashiromochi carrying *Pita* were used as positive controls, while Nipponbare was used as a negative control. The presence or absence of the *Pita* genes in 42 IRRI-bred rice varieties and control varieties was confirmed using three DNA markers, $Pita_{440}$ (YL153/YL154), $Pita_{1042}$ (YL155/YL87), and $Pita_{403}$ (YL100/YL102) (Fig.1, Table 2). The DNA fragments of positive control varieties, K1, IRBLta-K1, and Yashiromochi carrying *Pita* were amplified, while those of negative control, Nipponbare, were not amplified. Of the 42 IRRI-bred rice varieties examined, DNA fragments using either the $Pita_{440}$ or $Pita_{1042}$ markers were detected in 28 varieties. Using the $Pita_{403}$ marker, DNA fragments were amplified in 27 of the 42 IRRI-bred rice varieties examined.

Comparison between conventional genetic analysis and DNA marker analysis

The detection of DNA fragments for *Pib* in positive and negative control varieties was compared with the presence or absence of *Pib*. The DNA fragments of Sub3-5 in positive control varieties, BL1 and IRBLb-B, carrying *Pib* were detected while those of negative control varieties, Yashiromochi and Nipponbare, were not detected. For control varieties, the results of DNA fragments were consistent with the presence or absence of *Pib*. The detection of DNA fragments for *Pib* in 37 IRRI-bred rice varieties was consistent with the results of gene estimation for *Pib* using conventional genetic analysis, while those in two varieties were different from the results of gene estimation for *Pib* (Table 2). Although the two varieties, IR54 and PSBRc18, were found carrying *Pib* using conventional genetic analysis, DNA fragments for *Pib* were not detected. Three varieties, IR56, IR70, and PSBRc1, had not been previously examined for *Pib* using conventional genetic analysis. In summary, the results of DNA fragment detection for *Pib* in 42 IRRI-bred varieties were generally consistent with those of gene estimation by conventional genetic analysis, expected for IR54 and PSBRc18.

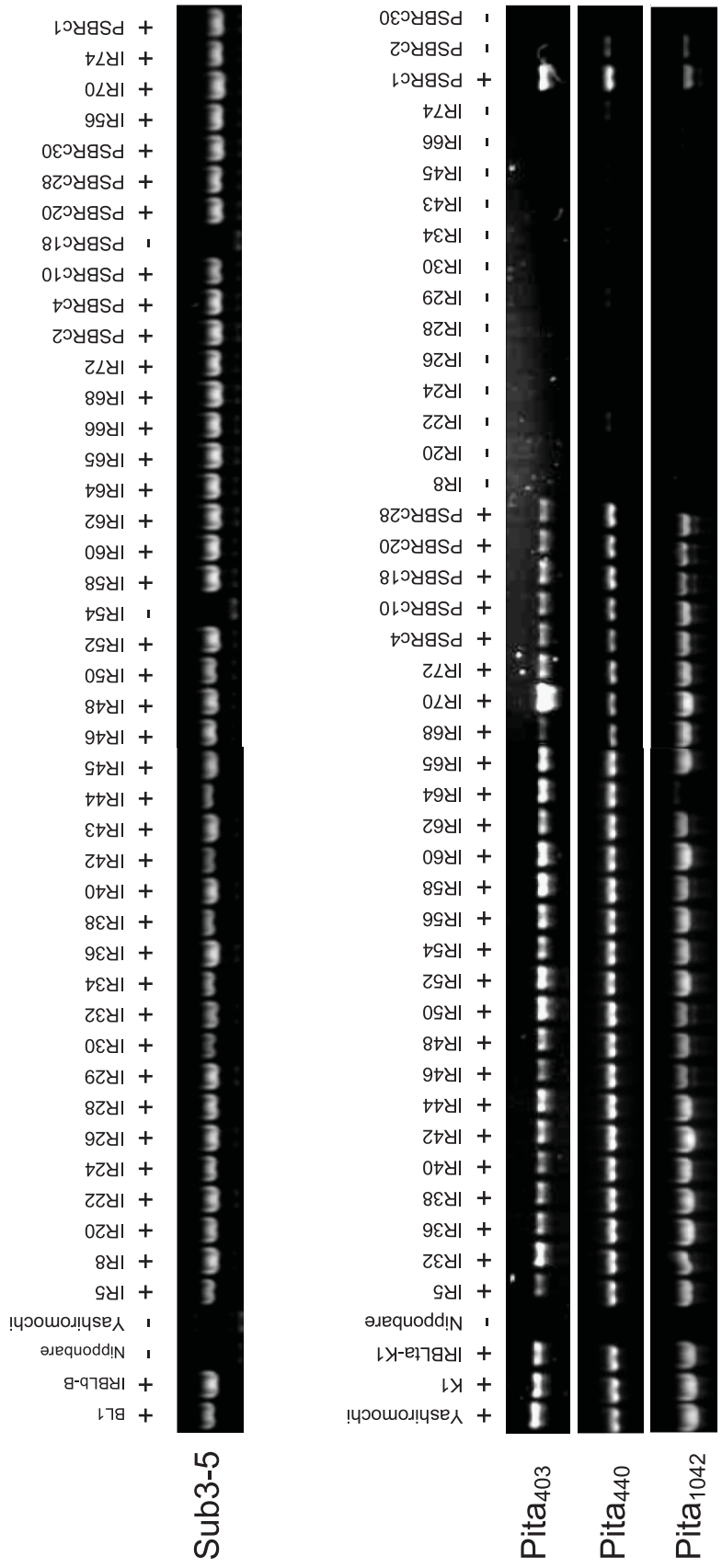

Fig. 1. Electorophoretic profiles of DNA markers, Sub3-5, $Pita_{440}$, $Pita_{1042}$, and $Pita_{403}$, used for detection of *Pib* and *Pita* in control varieties and 42 IRRI-bred varieties

Table 2. Gene identification by conventional genetic analysis and DNA marker for two resistance genes for *Pib* and *Pita* in 42 IRRI-bred varieties

IRRI-bred variety		Target resistance gene					
		Pib		*Pita*			
		Method of gene identification					
		Conventional genetic analysis	DNA marker Sub3-5	Conventional genetic analysis	DNA marker $Pita_{440}$	DNA marker $Pita_{1042}$	DNA marker $Pita_{403}$
1a[1)]	IR20	+	+	-	-	-	-
	IR28	+	+	-	-	-	-
	IR30	+	+	-	-	-	-
	IR45	+	+	-	-	-	-
	IR66	+	+	-	-	-	-
1b	IR29	+	+	-	-	-	-
	IR34	+	+	-	-	-	-
2a	IR8	+	+	-	-	-	-
	IR22	+	+	-	-	-	-
	IR24	+	+	-	-	-	-
	IR26	+	+	-	-	-	-
	PSBRc30	+	+	-	-	-	-
2b	IR43	+	+	-	-	-	-
2c	PSBRc2	+	+	-	+	+	-
3	IR5	+	+	+	+	+	+
	IR32	+	+	+	+	+	+
	IR36	+	+	+	+	+	+
	IR38	+	+	+	+	+	+
	IR40	+	+	+	+	+	+
	IR42	+	+	+	+	+	+
	IR44	+	+	+	+	+	+
	IR50	+	+	+	+	+	+
	IR52	+	+	+	+	+	+
	IR58	+	+	+	+	+	+
	IR60	+	+	+	+	+	+
	IR62	+	+	+	+	+	+
	IR65	+	+	+	+	+	+
	IR68	+	+	+	+	+	+
	IR72	+	+	+	+	+	+
	PSBRc4	+	+	+	+	+	+
	IR54	+	-	+	+	+	+
4	PSBRc1	/	+	-	+	+	+
5	IR74	+	+	-	-	-	-
6	IR56	/	+	+	+	+	+
	IR70	/	+	+	+	+	+
7a	IR46	+	+	+	+	+	+
	IR48	+	+	+	+	+	+
	IR64	+	+	+	+	+	+
	PSBRc28	+	+	+	+	+	+
7b	PSBRc10	+	+	+	+	+	+
	PSBRc20	+	+	+	+	+	+
	PSBRc18	+	-	+	+	+	+
Control	BL1	+	+	+	+	+	+
	IRBLb-B	+	+	+	+	+	+
	Yashiromochi	-	-	+	+	+	+
	Nipponbare	-	-	-	-	-	-

1) Variety group based on differential system.

'+ or - indicates presence or absence of *Pib* and *Pita*, respectively.

The DNA fragments of positive and negative control varieties for *Pita* were compared with the presence and absence of *Pita*. The DNA fragments of $Pita_{440}$, $Pita_{1042}$, and $Pita_{403}$ in positive control varieties, K1, IRBLta-K1, and Yashiromochi, carrying *Pita* were detected, while those of the negative control variety, Nipponbare, were not detected. For control varieties, the results of DNA fragment detection were consistent with the presence or absence of Pita. The detection of DNA fragments for Pita using two DNA markers, $Pita_{440}$ and $Pita_{1042}$, was consistent with the results of gene estimation for Pita using conventional genetic analysis in 40 out of the 42 IRRI-bred rice varieties (Table 2). Although PSBRc1 and PSBRc2 were not found carrying *Pita* by conventional genetic analysis, DNA fragments for *Pita* in these varieties were detected using $Pita_{440}$ and $Pita_{1042}$. The detection of DNA fragments for *Pita* using $Pita_{403}$ was consistent with the results of gene estimation for *Pita* using conventional genetic analysis in 41 out of the 42 IRRI-bred rice varieties. Although PSBRc1 was found not to carry *Pita* by conventional genetic analysis, DNA fragments for Pita were detected in PSBRc1. In summary, the results of DNA fragment detection for Pita in 42 IRRI-bred varieties were consistent with the results of gene estimation using conventional genetic analysis, expect for PSBRc1.

Discussion

The use of gene specific markers enabled the detection of the *Pib* and *Pita* genes in most IRRI-bred rice varieties that have been previously identified to contain these genes by an independent system. The *Pib* gene was previously found in IR54 and PSBRc18 by conventional genetic analysis (Ebron et al. 2004), but could not be detected using a DNA marker in this study (Table 2). For these varieties, further DNA analysis using other gene specific markers for *Pib*, such as Pibdom, which was developed by Fjellstrom et al. (2004), will be needed. Although three varieties, IR56, IR70, and PSBRc1, were not found to contain *Pib* by conventional genetic analysis, DNA fragments using gene specific marker were detected in three varieties. In previous analysis, IR56, IR70, and PSBRc1 were not found to carry *Pib* using conventional genetic analysis due to the narrow spectra of resistance in *Pib*. *Pib* showed a narrow spectra of resistance to blast isolates from the Philippines and this reaction pattern might be completely masked by the presence of $Pik^{\dagger}$. Therefore, the results of DNA analysis suggest that IR56, IR70, and PSBRc1 may contain the *Pib* gene.

Similarly, the gene specific markers, $Pita_{440}$, $Pita_{1042}$, and $Pita_{403}$, were useful and effective for *Pita* gene identification because results of DNA fragments in all IRRI-bred varieties, with the exception of PSBRc1 and PSBRc2, were the same as those obtained by conventional genetic analysis. Although the DNA fragments of *Pita* in PSBRc1 and PSBRc2 were detected, the results of the reaction pattern using conventional genetic analysis showed that PSBRc1 and PSBRc2 did not have the *Pita* gene. Additionally, allelism testing between IR56 carrying *Pita* and $Pik^{\dagger}$ and PSBRc1 carrying $Pik^{\dagger}$ showed that PSBRc1 did not contain the *Pita* gene. Thus, the sequence of the primer sites of $Pita_{440}$, $Pita_{1042}$ and $Pita_{403}$, (markers made from the Yashiromochi sequence) in PSBRc1 and PSBRc2 are almost identical to those in the Yashiromochi sequence. Parts of the *Pita* gene sequence, outside of these primer sites may differ between the Yashiromochi and the PSBRc1 and PSBRc2 varieties, because they were bred using different donors in Japan and Philippines. The new DNA marker for *Pita*, which can identify and differentiate *Pita* between Yashiromochi and the two IRRI-bred varieties, PSBRc1 and PSBRc2 will need based on the detail analysis in sequence level.

The utility of gene specific markers was demonstrated in this study, and gene detection using DNA analysis was in agreement with that of conventional genetic analysis. IR74 was not found with *Pib* using conventional genetic analysis because *Pib* was completely masked by the presence of $Pik^{\dagger}$, but IR74 was identified carrying *Pib* using segregation analysis (Table 1). The DNA fragments of gene specific markers for *Pib* in IR74 were also detected. Although *Pib* in IR74 was not detected using conventional genetic analysis, the amplification of DNA using *Pib* specific DNA marker were observed. IR74 did not harbor the functional *Pib* gene, but might remain the base sequence of the resistance gene with some modifications. This result suggests that precise gene detection was achieved using DNA analysis and conventional genetic analysis in the case of narrow resistance spectra. Three resistance genes, *Pi2*, *Piz-t*, and *Pi9*, have been cloned and sequenced (Qu et al. 2006; Zhou et al. 2006). Based on this information, gene specific markers for these genes will be developed to identify the genotypes of *Pi2*, *Piz-t*, and *Pi9*. Therefore, various varieties, which carry multiple resistance genes and show complex reactions, will be able to be identified using gene specific markers.

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Appendix 1. Protocol for DNA extraction using CTAB

CTAB extraction in small scale

1. Cut freeze-dried leaf (0.1 g: 1 cm x 5 cm) using scissors. Put small leaf pieces in 2.0 ml tube with three beads.
2. Break into pieces by bead shocker (1200 rpm, 2 m, 1 or 2 times). Remove the beads and add 750 µl of 1.5x CTAB extraction buffer and mix well.
3. Incubate in a water bath at 56℃ for 20-60 min
4. Add 600 µl of chloroform/isoamyl alcohol (24:1).
5. Gently agitate the tube in a shaker (40 rpm, 20 min)
6. Centrifuge the tube at 10000 rpm for 12 min.
7. Transfer the supernatant solution from the top phase to a new clean tube (1.5ml) using a 1 ml pipette.
8. Add 800 µl of CTAB precipitation buffer and slowly mix well.
9. Maintain for one day to two weeks (a longer period is better).
10. Centrifuge the tube at 13000 rpm for 10 min.
11. Gently pour off the supernatant and add 400 µl of high-salt TE-RNase. Mix well and remove pellet.
12. Maintain at 56 ℃ for 60 min.
13. After confirming pellet has resuspended completely, add 400 µl of 2-propanol and gently mix well.
14. Centrifuge the tube at 13000 rpm for 10 min and pour off the supernatant gently. Wash the precipitate by soaking with 400 µl of 70% ethanol.
15. Centrifuge the tube at 13000 rpm for 10 min.
16. Pour off the supernatant and invert the tubes. Completely dry the tubes.
17. Re-hydrate in 200 µl 1x TE buffer and check the scale of DNA concentration and electrophoresis.

Solutions for DNA extraction using the CTAB method

1.5 X CTAB EXTRACTION BUFFER

Components:		m.w./stock
1.5% CTAB		15 g
75 mM Tris- HCl	(1M, pH8.0)	75 ml
15 mM EDTA	(0.5M, pH8.0)	30 ml
1.05 M NaCl	(58.4)	61.4 g
Distilled water		to 1 liter

CTAB= Cetyl Trimethy Ammonium Bromide, $CH3(CH2)15(CH3)3NBr$ MW = 364.4

CTAB PRECIPITATION BUFFER

Components:		m.w./stock
1.0% CTAB		10 g
50 mM Tris-HCl	(1M, pH 8.0)	50 ml
10mM EDTA	(0.5M, pH 8.0)	20 ml
Distilled water		to 1 liter

Appendix 2. Protocol for PCR using SSR markers

A. Polymerase Chain Reaction to amplify the SSR marker

Note: Use miniscale extracted DNA (1:40 dilution).

Prepare the PCR reaction in a 96-well PCR plate. The components are in the table below.

Table. PCR cocktail components for SSR markers

Components of cocktail	Stock conc.	Final conc.	1 reaction 10µl, for SSR
Distilled water (dH_2O)	-	-	1.75 µl
PCR buffer (10XTB)	10X	1X	1 µl
dNTP	1 mM	0.1 mM	1 µl
Primer Forward	50 ng	2.5 ng	0.5 µl
Primer Reverse	50 ng	2.5 ng	0.5 µl
Taq polymerase	4 U/µl	1 U/10 µl	0.25 µl
Template DNA	5 ng/µl	25 ng/10µl reaction	5.0 µl
Total	-	-	10.0 µl

PCR profile for SSR markers

Turn on the thermocycler, place the 96-well PCR plate inside the block, close the heating lid and set the following program conditions:

1. 95 ℃ for 5 min (initial denaturation)
2. 35 cycles of the following steps
   95 ℃ for 30 s (denaturation)
   55 ℃ for 30 s (primer annealing, maybe adjusted)
   72 ℃ for 30 s (extension)
3. 20 ℃ untimed (storage)

B. Electrophoresis using 4.0% agarose and TBE buffer to check PCR product

1. Add 2-3 µl 10x loading buffers to the remaining PCR product prior to loading.
2. Load about 8 µl of sample in each well.

DNA size marker, e.g. 100 bp DNA ladder (50ng/µl), may be used for size determination.

PCR COMPONENTS

10x loading buffer

50% glycerol	25 ml
0.4% bromophenol blue	0.2 g
0.4% xylene cyanole	0.2 g

10x PCR buffer (1 liter)

dH_20	200 ml
1M Tris HCl (pH8.4)	500 ml
5M KCl	200 ml
150mM MgCl2	100 ml
Gelatin	0.1 g

1mM dNTP (4X 25 uM)

250 µl each dNTPs (100 mM)
24 ml dH_2O

Primers

100 mM stock,
5 mM working concentration:
Dilute 100 mM stock by adding 99 µl dH_2O for every 1 µl of 100 mM stock