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DNA Isolation and Optimization of ISSR-PCR Reaction System in *Oryza sativa* L.

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Abstract*— Inter simple sequence repeats (ISSRs) have been utilized widely for molecular markers in analyzing the genetic diversity and phylogenetic and regions in the genome flanked by microsatellite sequences. PCR amplification of these regions using a single primer yields multiple amplification products that can be used as a dominant multilocus marker system for the study of genetic variation in various organisms. For this study provides, DNA isolation, adjusting in six factors (Buffer, $MgCl_2$, dNTPs, ISSR primers, Template DNA and *Taq polymerase*) at six levels, and optimization of PCR temperature for the ISSR reaction was 60-45 °C, primers screening on indica rice (*Oryza sativa*). In this research, simple method of DNA isolation by using seedling. The objective of the present investigation was to assess the optimizations and quantification. Has been shown that stalk enhanced the maximum value of genomic. The results show that 100 ISSR primers were examined as well as, 56 ISSR primers was productively amplified. Optimum components for PCR reactions were 5.0 µl of 5X PCR Buffer, 1.5 µl of 25mM $MgCl_2$, 1 µl of 10 mM dNTP, 1 µl of 10 Mm ISSR primers, 2 µl Template DNA, and 0.1 µl of 5 units/ml *Taq polymerase*. Based on this study, has brought out some information on the relationship between these ISSR primers will be applied further for molecular profiling as well as response evaluation in rice varieties.

***Keywords*— DNA isolation; optimal PCR condition; ISSR screening; plant genetics.**

I. INTRODUCTION

Rice (*Oryza sativa* L.) is the record primary food crop in the global [1]. It role is an important cereal crop for world food security and is used to food more than 3 billion persons on an everyday calorie intake of between 50 to 80% [2]. However, the United Nations has been reported the request for rice increments for the populace which is probably to increase by a more 38% within 30 years, [3]. It's also nutrition, particularly for developing countries and Asian cultured rice is an essential as a staple food crop [4]. The cultivable area under rice needs to be increased to improve the production demands.

ISSR is a microsatellite-based multilocus marker method, which is useful and straightforward for genetic assessment diversity in various crop plants [5]. ISSR markers are highly polymorphic polymorphism of bands and are beneficial in researches on genetic diversity, genome mapping, evolutionary biology, phylogeny, and gene tagging [6]. Also notes Reddy et al., [7] investigated the ISSR-PCR detection, which affects, the utilize of microsatellite sequences as primers in PCR to produce multilocus markers. The markers from ISSR are useful and extremely polymorphic in evaluation on genome mapping, evolutionary biology, phylogeny, gene tagging and genetic diversity. Also ISSR, their capacity to detect variation without any prior sequence information. The technique and its application in plant breeding and genetics in a wide range of crop plants [7]. Blair et al. [8], investigated the distinguished rice genotypes (indica and japonica), applying ISSR polymorphism data. ISSR marker availability technology an effect on for the

production of possible fingerprinting assessment marker for, cultivars, species, and genomes, moreover phylogenetic investigation on the basis of the ISSR technique, resulting phenogram holds polyphyletic development in the genus *Oryza*, wherein multiple heredities undergo independent deviation later than division from a general ancestor [9].

However, none of these studies have focused on optimize and selected of ISSR primers as a part of the venture on a biological indicator to increase the molecular tools available to obtain genetic information in rice. The principal objective of this research was to examine the extraction procedure for DNA rice and to assessment ISSR primer in indica rice based on optimization annealing temperatures. Our research includes DNA isolation, quantification, PCR optimization, primers selection, and detection for Malaysian local rice MRQ74 rice.

II. Materials and Methods

The MRQ74 genotype of indica rice used in this research to optimum annealing temperature for ISSR primers was conducted in the Malaysian Nuclear Agency Laboratory during 2015.

A. DNA Isolation

For DNA extraction, leaf tissue was collected various pots of MRQ74 variety (Figure 1). The genomic DNA rice varieties were isolated using freshly collected young leaves. The white powder form of fresh seedling rice from green colored to a fine powder from mortar was got when ground in liquid nitrogen (-196°C) was used for DNA extraction. To extract genomic DNA the CTAB technique was used with some modifications. One gram of freshly harvested young leaves was grounded into powder using a mortar and pestle in liquid nitrogen. The powder was then directly transferred into Eppendorf tube size 50 ml containing 15 ml preheated extraction buffer made up of [1.4 M NaCl, 100 mM Tris-HCI, 20 mM EDTA (pH 8), 3% CTAB, and 2% PVP]. Then it was incubated in a water bath with the addition of 30 µL Mercaptoethanol solution at 60^{o}C for 1 h in water bath, the tube was gently shaken at 10 min intervals. Then one volume of chloroform/isoamyl alcohol (24/1) v/v mixture was added in the same tube after incubation and inverted. This followed a centrifugation at 5000 rpm at 25°C for 10 minute. The supernatant was put in a new Eppendorf tubes (Volume 1.5 ml), its DNA was precipitated through and supplemented with cold isopropanol (0.6 volume) then placed in ice for 30 min. DNA precipitate was fished out and placed into Eppendorf tube following a centrifugation at 12000 rpm for 10 min. The DNA pellet was collected and washed using 200 µL of ethanol (70%). The mixtures were pulsed spin by centrifuged at 12000 rpm once and the ethanol discarded. However, to collected for the DNA pellet was air-dried and re-suspended in 300 µL TE buffer then incubated at 4^{o}C overnight and continued with DNA precipitation.

The DNA pellet obtained from the previous step was later supplemented with 3 M sodium acetate (1/10 volume) and cold absolute ethanol (2 volume). Gently mix by inverting Eppendoft tube and incubate on ice for 30 min then centrifuged at 12000 rpm for 10 min at 4°C for DNA precipitation and the supernatant was removed. The DNA pellet was rinsed using ethanol 100 µL (70%) then centrifuged at 12000 rpm for 1 min at 4°C, the process was repeated two times. DNA was left to dry at room temperature and 100 µL TE buffer was added to each tube. DNA was kept in -20°C freezer overnight and then 1 µL RNase was added, DNA was left to incubate for 1hour at 37°C. DNA was then ready to be utilized in agarose gel electrophoresis [10].

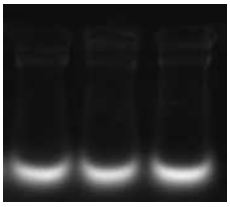

Fig. 1. Gel electrophoresis image of DNA quality extracted using CTAB method at 1.0 % agarose, 70 volts, 20 minutes

B. DNA Electrophoresis and Quantification

The DNA concentrations were determined using Nanodrop Spectrophotometer model (Nanodrop 1000 brand Thermo Scientific). Loading 1 µl of each DNA sample was put on Nanodrop was used for DNA quantification.
The purity of recovered total DNA (Table 1), was used measuring the absorbance ratio at (260-280) nm [11].

TABLE I
Amount of genomic DNA extracted using CTAB method

Sample	Average DNA concentration (ng/ µl)	Average OD260/280
MRQ74 Rice seedlings	70.20±2.6	1.9±0.15

C. PCR Master Mix Optimization

Different volumes of PCR reagent parameter [IS32 (TG)8G (17 mer)] such as a buffer, Primer $MgCl_2$, *Taq polymerase*, dNTP, and DNA template [12], were optimized as in (Table 2) by giving total volumes of 25 µl for each reaction.

D. Primers Selection and PCR Optimization

A set of 100 ISSR primers were obtained from the Malaysian Nuclear Agency and screened for their repeatable amplification used in this study are presented in (Table 1). Fresh leaf tissue of MRQ74 variety samples was used.
DNA templates to generate a six temperature fragment utilized were in the range of 60 to 40° to determination the best PCR amplification temperature. ISSR primer with highest amplifications result was selected for ISSR evaluates. Used master mix (25µl reaction) was placed in a thermo cycle machine, for PCR gradient analysis, (Bio Rad, T100^{TM} Thermal Cycler).

Consisted which of 14.4 µl sigma water, 5µl 5X PCR buffer, 1.5µl of 10Mm $MgCl_2$, 1µl of 10mM dNTPs mixed, 1µl of 0.4µM ISSR primers, 2µl of template DNA (50-100 ng/ µl) (Table 2), and 0.1µl *Taq* DNA *polymerase*. PCR protocol was started thru hot start then follows thru specific thermal cycles as followed single step of initial [13].

- *Thermocycler Set-up*

Initial denaturation 94°C (2 mines); [Denaturation 94°C (1 min); Annealing 45-60°C (50s); Elongation 72°C (50s)] for 31 cycles; Final extension at 72°C (5 mines); End (10°C). PCR Cycles: Repeat from step (Denaturation to Elongation) for 31 cycles. The enhancement of amplified was separated using electrophoresis on agarose gel (1.5%) in TAE (1X) for 60 min and the DNA fingerprints images were assessed using an automatic imaging system.

- *DNA Quantification and Gel Electrophoresis*

For the purpose of DNA quantification related to the DNA extraction, Nanodrop Spectrophotometer machine by use was used to calculate the concentration of DNA and the purity, the relative purity with the concentrations of the DNA extract was showed. The last DNA concentrations were diluted by using TE buffer. Right after PCR, desired products were then separated on 1.5% agarose gels run at the condition of 70 Volts and 400 m Amps for 50 minutes by using Thermo Scientific EC 300 XL Power Supply model (EC300XL2), USA. The visualized of DNA bands by using automatic imaging system under UV light (MS major science model UVCI-1100, USA) and photographed using Gel Documentation System, model UVCI-1100, Major Science. The size of the desired genes was estimated by using 100 bp DNA ladder. DNA bands images were select using an automatic imaging system.

E. Polymerase Chain Reaction (PCR)

1) Tore all PCR components on ice
2) Prepare a stripe PCR tube (8 tubes with lids)
3) Prepare Master Mix (MM) (Table 3)

F. Primers Optimization ISSR (Rice)

Annealing temperature: 1- 60 C˚, 2- 58.8 C˚, 3- 56.9 C˚, 4- 54.2 C˚, 5- 50.7 C˚, 6- 47.8 C˚

G. Agarose Gel Electrophoresis

1) Dissolve 1.5g Agarose in 100ml 1X TAE (Tris Acetate EDTA) buffer (40 mM Tris, 1% (v/v) acetic acid, 1 mM EDTA, pH 8.0)
2) Once dissolve. Wait to cool and add 1µl Ethidium Bromide Solutions, 10mg/ml.
3) Mix well and pour to cast (7 wells cast)
4) Add 5 µl Gel Loading (6X) mixed well to each of digest samples
5) Run at 70V for 1 h in TAE buffer
6) Observe under imaging system

TABLE II
OPTIMIZATION OF PCR BY USING DIFFERENT VOLUMES OF REAGENTS BY USING PRIMER IS 32 AT 54.2 ^{O}C

Reagent	**5X PCR buffer**						**Reagent**	**Primer (µl)**					
Σ H_2O	18.4	15.9	13.4	10.9	8.4	5.9	Σ H_2O	15.4	14.9	14.4	13.9	13.4	12.9
$MgCl_2$	1.5	1.5	1.5	1.5	1.5	1.5	$MgCl_2$	1.5	1.5	1.5	1.5	1.5	1.5
PCR Buffer	0	2.5	5	7.5	10	12.5	PCR Buffer	5	5	5	5	5	5
dNTP	1	1	1	1	1	1	dNTP	1	1	1	1	1	1
Primer	2	2	2	2	2	2	Primer	0	0.5	1	1.5	2	2.5
Template DNA	2	2	2	2	2	2	Template DNA	2	2	2	2	2	2
Taq Polymerase	0.1	0.1	0.1	0.1	0.1	0.1	*Taq Polymerase*	0.1	0.1	0.1	0.1	0.1	0.1
Reagent	**$MgCl_2$ (µl)**						**Reagent**	**Taq polymerase (µl)**					
Σ H_2O	14.9	13.9	13.4	12.9	12.4	11.9	Σ H_2O	13.5	13.4	13.2	12.9	12.6	12.3
$MgCl_2$	0	1	1.5	2	2.5	3	$MgCl_2$	1.5	1.5	1.5	1.5	1.5	1.5
PCR Buffer	5	5	5	5	5	5	PCR Buffer	5	5	5	5	5	5
dNTP	1	1	1	1	1	1	dNTP	1	1	1	1	1	1
Primer	2	2	2	2	2	2	Primer	2	2	2	2	2	2
Template DNA	2	2	2	2	2	2	Template DNA	2	2	2	2	2	2
Taq Polymerase	0.1	0.1	0.1	0.1	0.1	0.1	*Taq Polymerase*	0	0.1	0.3	0.6	0.9	1.2
Reagent	**dNTP (µl)**						**Reagent**	**DNA (µl)**					
Σ H_2O	14.4	13.9	13.4	12.9	12.4	11.9	Σ H_2O	15.4	14.4	13.4	12.4	11.4	10.4
$MgCl_2$	1.5	1.5	1.5	1.5	1.5	1.5	$MgCl_2$	1.5	1.5	1.5	1.5	1.5	1.5
PCR Buffer	5	5	5	5	5	5	PCR Buffer	5	5	5	5	5	5
dNTP	0	0.5	1	1.5	2	2.5	dNTP	1	1	1	1	1	1
Primer	2	2	2	2	2	2	Primer	2	2	2	2	2	2
Template DNA	2	2	2	2	2	2	Template DNA	0	1	2	3	4	5
Taq Polymerase	0.1	0.1	0.1	0.1	0.1	0.1	Taq *Polymerase*	0.1	0.1	0.1	0.1	0.1	0.1

III. RESULT AND DISCUSSION

For DNA purity, all samples had a robust absorbance ratio according to the range of the absorbance, and our results are in agreement with Ferdous et al. [14] the range of absorbance A260/A280 ratio ~ 1.8-2 for DNA purity and quality was more than sufficient. In the present investigation, PCR reactions were chosen for better reproducibility, banding patterns and amplification.

To obtain PCR amplification, 5X PCR buffer, 25 mM $MgCl_2$, 10 mM dNTP, ISSR primer, Template DNA and 5 units/ml *Taq polymerase* are required in specific concentrations for selection of relatively polymorphic bands that are reproducible and produced clear bands. Buffers at (0, 2.5, 5, 7.5, 10, and 12.5 µl) buffer concentrations were optimum in these experimental. However, 5 µl buffers showed clear amplification of PCR products bands (Figure 2; A).

The optimization of *Taq polymerase* using different concentrations includes (0, 0.1, 0.3, 0.6, 0.9 and 1.2 µl). The result of gel electrophoresis image showed all concentrations reproduced the amplification of PCR products but the clarity

bands were observed with 0.1 µl of *Taq polymerase* (Figure 2; B). The dNTP concentrations (0, 0.5, 1, 1.5, 2, and 2.5 µl), were optimum in this study, clear bands were observed in 1 µl of dNTP (Figure 2; C). The ISSR primer concentrations (0, 0.5, 1, 1.5, 2, and 2.5 µl) were optimumal, in the present study the concentration 1 µl of ISSR primer showed band clarity of PCR amplicons (Figure 2; D).

TABLE III
AOUNT OF PCR MASTER MIX

Reagent	**Final Concentration**	**Volume (µl)**	**Volume (µl) (8 tubes)**
Sigma water	-	14.4	115.2
5X buffer	1X	5	40
25mM $MgCl_2$	1.5 mM	1.5	12
10 mM dNTP	0.2 mM	1	8
10 µM ISSR Primer	0.4 µM	1	8
Template DNA	50-100 ng/ µl	2	16
Taq DNA Polymerase	0.1 unit/µl	0.1	0.8
Total Volume:	25 µl	200 ul	

DNA template concentrations (0, 1, 2, 3, 4 and 5 µl) were optimum, all concentrations between (1-5 µl) reproduced the amplification of PCR products but the clarity of bands decreased with the 2 µl template DNA (Figure 2; E). Optimum $MgCl_2$ used at different concentrations (0, 1, 1.5, 2, 2.5 and 3 µl) presented at 1.5 µl $MgCl_2$ per PCR Master Mix where amplification showed clear amplification of PCR products that was reproducible and produced clear bands (Figure 2; F).

The optimization of PCR reagents at several treatments used ISSR primer (IS32). The optimum volumes for PCR reactions chosen were 5 X PCR buffer (5.0 µl), 25 mM $MgCl_2$ (1.5 µl), 10 mM dNTP (1 µl), ISSR primer (1 µl), Template DNA (2 µl) and 5 units/ml *Taq polymerase* (0.1 µl). These conditions resulted in a sharp band and that was clearer when it was tested for all *Oryza sativa* L. regarding the amplification (Figure 2).

PCR buffer: selected to provide a monovalent salt environment and optimal pH for the final reaction volume. PCR buffer was necessary for optimizing *Taq* DNA activity and 5 µl PCR buffers elicited clear amplification of PCR products bands. 1.5 µl $MgCl_2$ per PCR master mix generated clear amplification of PCR products that were reproducible and produced clear bands. The lower concentration of $MgCl_2$ increased the non-specificity and yield of the PCR product [15]. dNTP: clear bands and superb PCR results were observed in 1 µl. Many DNA isolation procedures also yielded large amounts of RNA - large amounts of RNA in the sample can chelate Mg and reduce the yielded DNA isolated. Kumari et al. [16] observed that high concentrations of dNTP reducesd free Mg, interfering with the enzyme. ISSR primer: the concentration of 1 µl of ISSR primer showed band clarity of PCR amplicons, while higher and lower concentrations of ISSR primer resulted in primer dimer formation and the absence of amplification. Kumari et al. [16] observed that high concentrations of ISSR primer may raise the probability of generating a template-independent artifact, termed a primer-dimer, and could promote mispriming and accumulation of non-specific product. DNA template: the clarity of bands decreased with 2 µl of template DNA, and there was the presence of smearing at higher concentrations of the DNA template, which affected the repeatability; there was an absence of amplification with lower concentration [15]. However, the *Taq polymerase* employed exhibited clear bands with 0.1 µl *Taq polymerase*. Kumari et al. [16] found that high concentrations of *Taq polymerase* decreased specificity. Here, *Taq polymerase* remained active over a broad range of temperatures, and primer extension took place at low temperatures, including the annealing step. GC-rich templates were thought to be potentially problematic because of an inefficient separation of the two DNA strands or the tendency for the complementary GC-rich primers to form intermolecular secondary structures, which will compete with primers annealing to the template [17].

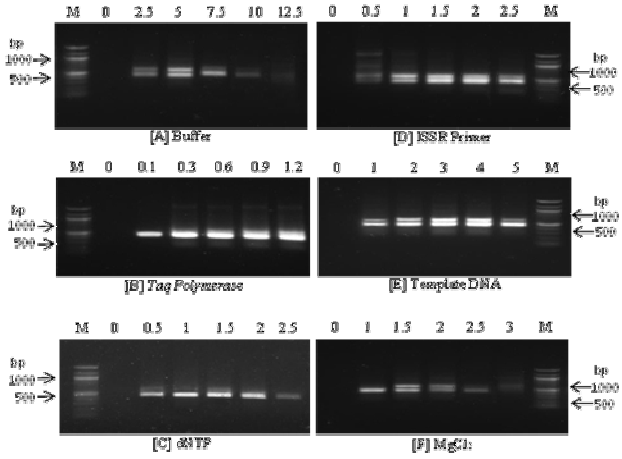

Fig. 2. PCR parameters optimization. Different volumes (µl) of reagent (1.5% agarose).

*M; DNA Marker 100 bp DNA ladder
[A]; Buffer concentrations (0, 2.5, 5, 7.5, 10, and 12.5 µl)
[B]; *Taq Polymerase* concentrations (0, 0.1, 0.3, 0.6, 0.9 and 1.2 µl)
[C]; dNTP concentrations (0, 0.5, 1, 1.5, 2, and 2.5 µl)
[D]; ISSR Primer concentrations (0, 0.5, 1, 1.5, 2, and 2.5 µl)
[E]; Template DNA concentrations (0, 1, 2, 3, 4 and 5 µl)
[F]; $MgCl_2$ concentrations (0, 1, 1.5, 2, 2.5 and 3 µl)

In the present investigation, 100 ISSR primers, 20 degenerate commercial and 80 specific primers Table (6) were screened for amplification of rice for the optimum ISSR annealing temperature. However, for optimizing of primers used DNA one variety MRQ74 rice. The chosen optimizing annealing temperatures for PCR protocol was determined based on the great clear bands created. Seventeen degenerate and 39 specific ISSR primers were able to enhance amplified products at specific annealing temperature and were utilized for genotypical characteristics of rice examples.

The optimal annealing temperature on choose primers are recorded in (Table 6). Result showed that both specific and degenerate primers gave positive amplification temperatures. The specific primers were given highest chance in the annealing process compared to degenerate primers and so were additionally beneficial in generating more polymorphisms to identify variations. Selected the clear and well visible bands were considered for better assessment. Consequently, 56 primers generated strong amplification products that were used for genetic analysis for annealing temperature for ISSR primers (Table 5).

The DNA isolation procedure for seedling was effectively utilised with application of certain modifications for DNA isolation; using fresh *indica* seedling was established as reliable for molecular characterization.

Optimum components and primer choices led to six annealing temperature to choose from (60-45°C). On the other hand, 56 out of 100 ISSR primers (Table 4) were possible for use in the rice examination. The most suitable primers and amplified bands were utilised for *Oryza sativa* L. Saiki [18], which showed optimal primer annealing temperatures for a particular PCR amplification dependent on the concentration, length, nucleotide sequence and base composition of the primers. The higher temperature amplification of large fragments was favored whilst lower temperatures favored short fragment amplification [19].

The primer extension time relied upon the concentration and length of the target sequence as well as the extension temperature [20]. The adjustment of the annealing temperature allowed identifying a higher number of polymorphic loci for every combination [20]. Moraga-Suazo et al. [19] observed primer annealing temperatures and DNA concentration were critical factors for obtaining high banding-pattern quality.

TABLE IV
OPTIMUM ANNEALING TEMPERATURE FOR ISSR PRIMERS

No.	Annealing Temperature (°C)	Total Primers	ISSR Primers
1	60	1	IS 7
2	58.8	3	IS 20, IS 21, IS 60, IS 92
3	56.9	9	IS 29, IS 30, IS 43, IS 48, IS 69, IS 72, IS 89, IS 93, IS 94
4	54.2	13	IS 4, IS 10, IS 15, IS 27, IS 28, IS 31, IS 32, IS 33, IS 37, IS 40, IS 46, IS 51, IS 52
5	50.7	10	IS 19, IS 23, IS 24, IS 26, IS 44, IS 45, IS 49, IS 58, IS 83, IS 96
6	47.8	20	IS 1, IS 8, IS 11, IS 14, IS 16, IS 18, IS 22, IS 25, IS 35, IS 36, IS 38, IS 39, IS 42, IS 59, IS 85, IS 88, IS 90, IS 91, IS 95,

IV. CONCLUSIONS

The conditions described in the present work suggest there was an ability to employ ISSR primers as an efficient determination tool for further molecular studies in particular rice species genetically, and has been documented for all rice varieties. The present optimized protocol for DNA isolation and ISSR technique may serve as a strong starting point for future genetic improvement and molecular characterization works in this promising rice plant.

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APPENDIX

TABLE V

POLYMORPHIC PRIMERS SHOW PRIMER SEQUENCE (5′ -3′) LENGTH, ANNEALING TEMPERATURE, AND PERCENTAGE OF AMPLIFIED BANDS G, A, T, C AND C+G CONTENT FOR ISSR PRIMERS

No.	Primer name	Primer sequence (5'-3')	Length (mers)	Tm (°C)	Percentage of				
					G	A	T	C	C+G
1	IS 1	$(CAC)_7T$	22	47.8	0.0	31.8	4.5	63.6	63.6
2	IS 4	$(CAC)_7G$	22	54.2	4.5	31.8	0.0	63.6	68.1
3	IS 7	$(CA)_{10}G$	21	60	4.8	47.6	0.0	47.6	52.4
4	IS 8	$(CT)_9G$	19	47.8	5.3	0.0	47.4	47.4	52.7
5	**IS 10**	$BDBT(CCT)_6$	22	54.2	16.7	5.6	55.6	77.8	68.2
6	IS 11	$HVH(TCC)_6$	21	47.8	5.0	15.0	40.0	75.0	71.4
7	IS 14	$(GA)_8T$	17	47.8	47.1	47.1	0.5	0.0	47.1
8	IS 15	$(GA)_8C$	17	54.2	47.1	47.1	0.0	5.9	53.0
9	**IS 16**	$(GA)_8A$	17	47.8	47.1	52.9	0.0	0.0	47.1
10	IS 18	$(CT)_8G$	17	47.8	5.9	0.0	47.1	47.1	53.0
11	IS 19	$(CT)_8T$	17	50.7	0.0	0.0	52.9	47.1	47.1
12	IS 20	$(CA)_8A$	17	58.8	0.0	52.9	0.0	47.1	47.1
13	IS 21	$(CA)_8G$	17	58.8	5.9	47.1	0.0	47.1	53.0
14	IS 22	$(GT)_8A$	17	47.8	47.1	5.9	47.1	0.0	47.1
15	IS 23	$(GT)_8C$	17	50.7	47.1	0.0	47.1	5.9	53.0
16	IS 24	$(GT)_8T$	17	50.7	47.1	0.0	52.9	0.0	47.1
17	IS 25	$(TC)_8A$	17	47.8	0.0	5.9	47.1	47.1	47.1
18	IS 26	$(GT)_9C$	19	50.7	47.1	0.0	47.1	5.3	52.4
19	IS 27	$(GT)_7GGTG$	18	54.2	58.8	0.0	47.1	0.0	58.8
20	IS 28	$(AC)_8T$	17	54.2	0.0	47.1	5.9	47.1	47.1
21	IS 29	$(AC)_8C$	17	56.9	0.0	47.1	0.0	52.9	52.9
22	IS 30	$(AC)_8G$	17	56.9	5.9	47.1	0.0	47.1	47.1
23	IS 31	$(TG)_8A$	17	54.2	47.1	5.9	47.1	0.0	47.1
24	IS 32	$(TG)_8G$	17	54.2	52.9	0.0	47.1	0.0	52.9
25	IS 33	$(AG)_8YT$	18	54.2	44.4	44.4	11.1	5.6	50.0
26	IS 35	$(CT)_8RA$	18	47.8	5.6	11.1	44.4	44.4	50.0
27	IS 36	$(CT)_8RC$	18	47.8	5.6	5.6	44.4	50.0	55.6
28	IS 37	$(CA)_8RT$	18	54.2	5.6	50.0	5.6	44.4	50.0
29	**IS 38**	$(CA)_8RC$	18	47.8	5.6	50.0	0.0	50.0	50.0
30	IS 39	$(GT)_8YA$	18	47.8	44.4	5.6	50.0	5.6	50.0
31	IS 40	$(GT)_8YG$	18	54.2	50.0	0.0	50.0	5.6	55.6
32	IS 42	$(AC)_8YG$	18	47.8	5.6	44.4	5.6	50.0	55.6
33	IS 43	$(AC)_8YA$	18	56.9	0.0	50	5.6	50.0	50.0
34	IS 44	$(AC)_8YT$	18	50.7	0.0	44.4	11.1	50.0	50.0
35	IS 45	$(TG)_8RT$	18	50.7	50.0	50.6	50.0	0.0	50.0
36	IS 46	$(TG)_8RC$	18	54.2	50.0	5.6	44.4	5.6	55.6
37	IS 48	$(ATG)_8$	24	56.9	33.3	33.3	33.3	0.0	33.0
38	IS 49	$(CTC)_6$	18	50.7	0.0	0.0	33.3	66.7	66.7
39	IS 51	$(GACA)_6$	24	54.2	25.0	50.0	0.0	25	50.0
40	IS 52	$(TCC)_5RY$	17	54.2	5.9	5.9	35.3	64.7	55.6
41	IS 58	$(GT)_8YC$	18	50.7	44.4	0.0	50	11.1	55.6
42	IS 59	$(GGAGA)_3$	15	47.8	60.0	40.0	0.0	0.0	60.0
43	IS 60	$BDB(CA)_7$	17	58.8	17.6	47.1	17.6	52.9	58.8
44	IS 69	$(TGG)_5$	15	56.9	66.7	0.0	33.3	0.0	66.7
45	IS 72	$(GTC)_5$	15	56.9	33.3	0.0	33.3	33.3	66.6
46	IS 83	$(AG)_5TTG$	13	50.7	46.2	38.5	15.4	0.0	46.2
47	IS 85	$(CT)_5CACC$	14	47.8	57.1	7.1	35.7	0.0	57.1
48	IS 88	$(AG)_8T$	17	47.8	47.1	47.1	5.9	0.0	47.1
49	IS 89	$(AG)_8C$	17	56.9	47.1	47.1	0.0	5.9	53.0
50	IS 90	$(AG)_8G$	17	47.8	52.9	47.1	0.0	0.0	52.9
51	IS 91	$(CA)_8T$	17	47.8	0.0	47.1	5.9	47.1	47.1
52	IS 92	$(CA)_8C$	17	58.8	0.0	47.1	0.0	52.9	52.9
53	IS 93	$(AGC)_6$	18	56.9	33.3	33.3	0.0	33.3	66.6
54	IS 94	$(ATG)_6$	18	56.9	33.3	33.3	33.3	0.0	33.3
55	IS 95	$(GATA)_4$	16	47.8	25.0	50.0	25.0	0.0	25.0
56	IS 96	$(GACA)_4$	16	50.7	25.0	50.0	0.0	25	50.0
Total			995		1462.5	1523.6	1316	1553.8	2943

B = C, G, T; D = A, G, T; H = A, C, T; R = A, G; V = A, C, G; Y = C, T

TABLE VI
SUMMARY INFORMATION FOR EACH 100 ISSR PRIMERS AFTER PCR OPTIMIZATION
* THE BOX INDICATE A SIGNIFICANT AMPLIFICATION PRODUCTS A GOOD BANDS

Primer	Primer Sequence 5' - 3'	Good	Intermediate	Not amplify	Ta (°C)
IS 1	$(CAC)_7T$ (22 mer)	✔			47.8
IS 2	$(GA)_9C$ (19 mer)		✔		-
IS 3	$G(TG)_9$ (19 mer)			✔	-
IS 4	$(CAC)_7G$ (22 mer)	✔			54.2
IS 5	$(CAC)_7GT$ (23 mer)			✔	-
IS 6	$(GTG)_7C$ (22 mer)		✔		-
IS 7	$(CA)_{10}G$ (21 mer)	✔			60
IS 8	$(CT)_9G$ (19 mer)	✔			47.8
IS 9	$(GA)_9AY$ (20 mer)		✔		-
IS 10	$BDBT(CCT)_6$ (18 mer)	✔			54.2
IS 11	$HVH(TCC)_6$ (20 mer)	✔			47.8
IS 12	$(AG)_8T$ (19 mer)			✔	-
IS 13	$(AG)_8G$ (17 mer)		✔		-
IS 14	$(GA)_8T$ (17 mer)	✔			47.8
IS 15	$(GA)_8C$ (17 mer)	✔			54.2
IS 16	$(GA)_8A$ (17 mer)	✔			47.8
IS 17	$(CT)_8A$ (17 mer)			✔	-
IS 18	$(CT)_8G$ (17 mer)	✔			47.8
IS 19	$(CT)_8T$ (17 mer)	✔			50.7
IS 20	$(CA)_8A$ (17 mer)	✔			58.8
IS 21	$(CA)_8G$ (17 mer)	✔			58.8
IS 22	$(GT)_8A$ (17 mer)	✔			47.8
IS 23	$(GT)_8C$ (17 mer)	✔			50.7
IS 24	$(GT)_8T$ (17 mer)	✔			50.7
IS 25	$(TC)_8A$ (17 mer)	✔			47.8
IS 26	$(GT)_9C$ (19 mer)	✔			50.7
IS 27	$(GT)_7GGTG$ (17 mer)	✔			54.2
IS 28	$(AC)_8T$ (17 mer)	✔			54.2
IS 29	$(AC)_8C$ (17 mer)	✔			56.9
IS 30	$(AC)_8G$ (17 mer)	✔			56.9
IS 31	$(TG)_8A$ (17 mer)	✔			54.2
IS 32	$(TG)_8G$ (17 mer)	✔			54.2
IS 33	$(AG)_8YT$ (18 mer)	✔			54.2
IS 34	$(GA)_8YT$ (18 mer)			✔	-
IS 35	$(CT)_8RA$ (18 mer)	✔			47.8
IS 36	$(CT)_8RC$ (18 mer)	✔			47.8
IS 37	$(CA)_8RT$ (18 mer)	✔			54.2
IS 38	$(CA)_8RC$ (18 mer)	✔			47.8
IS 39	$(GT)_8YA$ (18 mer)	✔			47.8
IS 40	$(GT)_8YG$ (18 mer)	✔			54.2
IS 41	$(TC)_8RT$ (18 mer)			✔	-
IS 42	$(AC)_8YG$ (18 mer)	✔			47.8
IS 43	$(AC)_8YA$ (18 mer)	✔			56.9
IS 44	$(AC)_8YT$ (18 mer)	✔			50.7
IS 45	$(TG)_8RT$ (18 mer)	✔			50.7
IS 46	$(TG)_8RC$ (18 mer)	✔			54.2
IS 47	$(ACC)_6$ (18 mer)		✔		-
IS 48	$(ATG)_8$ (24 mer)	✔			56.9
IS 49	$(CTC)_6$ (18 mer)	✔			50.7
IS 50	$(GAA)_6$ (18 mer)			✔	-

to be continued…

… continuation

Primer	Primer Sequence 5' - 3'	Good	Intermediate	Not amplify	Ta (°C)
IS 51	$(GACA)_6$ (24 mer)	✔			54.2
IS 52	$(TCC)_5$RY (17 mer)	✔			54.2
IS 53	$(CT)_8$C (17 mer)			✔	-
IS 54	$(AG)_8$C (17 mer)			✔	-
IS 55	$(AG)_8$A (17 mer)			✔	-
IS 56	$(TC)_8$C (17 mer)			✔	-
IS 57	$(GA)_8$CT (18 mer)			✔	-
IS 58	$(GT)_8$YC (18 mer)	✔			50.7
IS 59	$(GGAGA)_3$ (15 mer)	✔			47.8
IS 60	BDB$(CA)_7$ (17 mer)	✔			58.8
IS 61	$(GA)_9$T (19 mer)			✔	-
IS 62	$(GA)_9$A (19 mer)			✔	-
IS 63	$(AGAGGC)_4$ (24 mer)			✔	-
IS 64	$(TA)_5$ (10 mer)			✔	-
IS 65	$(TA)_8$ (16 mer)			✔	-
IS 66	$(TA)_6$ (12 mer)			✔	-
IS 67	$(AT)_7$ (14 mer)			✔	-
IS 68	$(AT)_9$ (18 mer)			✔	-
IS 69	$(TGG)_5$ (15 mer)	✔			56.9
IS 70	$(GAA)_6$ (18 mer)			✔	-
IS 71	$(AG)_7$ (14 mer)			✔	-
IS 72	$(GTC)_5$ (15 mer)	✔			56.9
IS 73	$(CT)_8$ (16 mer)			✔	-
IS 74	$(CT)_{14}$ (28 mer)			✔	-
IS 75	$(CTT)_5$ (15 mer)			✔	-
IS 76	$(CCG)_6$ (18 mer)			✔	-
IS 77	$(AT)_5(AAT)_7$ (31 mer)			✔	-
IS 78	$(AGA)_7$ (21 mer)		✔		-
IS 79	$(TC)_{11}$ (22 mer)			✔	-
IS 80	$(AAT)_8$ (24 mer)			✔	-
IS 81	$(AG)_5$T (11 mer)			✔	-
IS 82	$(AG)_5$TT (12 mer)			✔	-
IS 83	$(AG)_5$TTG (13 mer)	✔			50.7
IS 84	C$(AG)_5$ (11 mer)			✔	-
IS 85	$(CT)_5$CACC (14 mer)	✔			47.8
IS 86	$(GT)_5$GA (12 mer)			✔	-
IS 87	$(AATGC)_3$AAT (18 mer)			✔	-
IS 88	$(AG)_8$T (17 mer)	✔			47.8
IS 89	$(AG)_8$C (17 mer)	✔			56.9
IS 90	$(AG)_8$G (17 mer)	✔			47.8
IS 91	$(CA)_8$T (17 mer)	✔			47.8
IS 92	$(CA)_8$C (17 mer)	✔			58.8
IS 93	$(AGC)_6$ (18 mer)	✔			56.9
IS 94	$(ATG)_6$ (18 mer)	✔			56.9
IS 95	$(GATA)_4$ (16 mer)	✔			47.8
IS 96	$(GACA)_4$ (16 mer)	✔			50.7
IS 97	$(GATA)_3$GACA (20 mer)			✔	-
IS 98	$(GAT)_4$A (13 mer)			✔	-
IS 99	$(GAT)_4$T (13 mer)			✔	-
IS 100	$(GAT)_4$G (13 mer)			✔	-

B = C, G, T; D = A, G, T; H = A, C, T; R = A, G; V = A, C, G; Y = C, T