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].

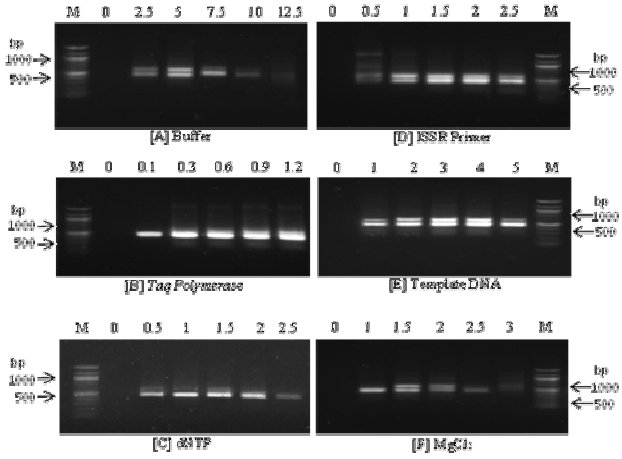

Fig. 2. PCR parameters optimization. Different volumes (µl) of reagent (1.5% agarose).

*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