Development of two elite water chestnut genotypes (Trapa spp.) and their molecular characterization using RAPD and ISSR markers

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Abstract

Water chestnut (2n = 48) is an aquatic plant of Trapaceae family, the fruits of which are nutritious and have medicinal properties. By selecting from Green Spineless and Red Spineless Biotypes for high TSS and yield from natural population, we developed Improved Red Spineless (IRS) and Improved Green Spineless (IGS) genotypes and registered at N.B.P.G.R as IC 642169 and IC 642170, respectively. These two improved varieties were analyzed with the well-known local varieties using 10 RAPDs and 10 ISSR primers. A total of 78.0 polymorphic fragments with an average PIC of 0.32 and 0.16 and an average MI of 1.48 and 1.09 were produced by the RAPD and ISSR markers, respectively. The dendrogram analysis based on individual and combined RAPD and ISSR markers has demonstrated that the new improved varieties are molecularly distinct from the locally popular varieties. Among four genotypes, the unique gene sequences in 550 bp and 800 bp DNA ladders identified by AP-4 primer and 600 bp DNA ladders identified by AP-32 primer are only found in IRS genotype as dimorphic bands and single band, respectively. Similarly, in IGS water chestnut, DNA fragments AP30-1250, and ISSR23-1500 & ISSR23-1350 are only found as single bands and dimorphic bands, respectively.

Introduction

Water chestnut is a submersed floating herbaceous aquatic plant of the Trapaceae family that grows worldwide in tropical, subtropical and temperate climatic conditions. In most water chestnut growing countries, especially in China, India and Southeast Asia, it is cultivated for edible fruit. Four different species of water chestnut namely *Trapa natan* L, *Trapa bispinosa*, *Trapa quadrispinosa* and *Trapa bicornis* are prevalent in India. Flowers are bisexual and born in axil of the leaves from floating rosettes and are pollinated by insects and sometimes self-pollinated (Hummel and Erik, 2004). The fruit is highly nutritious with about 80% starch, 5.0% protein and significant amounts of vitamins such as thiamine, riboflavin, nicotinic acid, vitamin C, vitamin A and phosphorylases (Crow and Hellquist, 2000). The fruit can be consumed as raw, boiled, canned, roasted, and dried to make flour (Chandana et al. 2013). Furthermore, the fruits have been used as anti-inflammatory, anti-diarrheal, intestinal astringent, antiepileptic agent and in treating urinary discharges, fractures, bronchitis and anemia (Mahato et al. 2018).

Molecular markers are used for genetic diversity analysis, cultivar identification, purity testing and crop improvement (Mandal et al. 2018). Molecular markers likes RFLP, RAPD, SCAR, AFLP, SSR, CpSSR, ISSR, RAMP, SAMPL, SRAP, SSCP, CAPS, SNP, DArT, EST, STS, IRAP, REMAP, RBIP, and IPBS are available that can be used for various purposes. (Amiteye, 2021). When there is no difference in the morphological traits between crop varieties molecular markers help in the differentiation of the genotypes. However, in crops like water chestnut where genome sequence and SSR markers are not available, markers like RAPDS and ISSRs are the option for molecular studies. RAPD markers have been used extensively for genetic diversity studies in many crop plants including water chestnut (Hoque et al. 2005: Kachare et al. 2013; Mahto et al. 2018).

Improved Red Spineless (IRS) and Improved Green Spineless (IGS) were developed from the plant population of Red Spineless Biotype and Green Spineless Biotype, respectively with improvement in yield and TSS and these two varieties are compared with two popular cultivars [Red Spine Large Local (RSLL) and Red Spine Small Local (RSSL)] grown in wetland ecosystem of north Bihar that look morphologically similar with new varieties using RAPDs and ISSR markers and the results of this are discussed in this paper.

Materials And Methods

Water chestnut samples were collected from the Research Centre for Makhana (RCM), Darbhanga, Bihar, India. The varieties used in this study are 1. Improved Green Spineless (IGS) 2. Red Spine Large local (RSLL) 3. Improved Red spineless (IRS) and 4. Red Spine Small Local (RSSL). The RAPD and ISSR markers used are listed in Table 2 and Table 3.

DNA Isolation

DNA was extracted from leaves of all the four varieties Using modified CTAB method. The leaf powder obtained by grinding in liquid nitrogen was suspended in 1 ml extraction buffer (2% CTAB, 2% polyvinylpyrrolidone (PVP), 100mM Tris-HCL (pH 8.0), 1.4M NaCl, 20mM EDTA,and 0.2% of mercaptoethanol). The suspension was incubated at 65^0C for 30 minutes followed by extraction with an equal volume of chloroform. Nucleic acid was precipitated with an equal volume of isopropanol. DNA pellet was washed with 70% ethanol, dried and suspended in 100µl of TAE buffer (10 mM Tris,1 mM EDTA, pH-7.6).

Polymerase Chain Reaction For RAPD And ISSR Markers

PCR was performed in 25µl reaction volumes containing 1X PCR buffer (10mM Tris-HCl (pH 8.3), 50mM KCL, and 1.5mM MgCl2), 200µM dNTPs, 50 ng of template DNA, 5pmol of primer, and 2 units of Taq polymerase (Thermo Scientific).

PCR conditions for RAPD markers included an initial denaturation step at 94°C for 5 min, followed by 45 cycles of 94°C for 1min, 36°C for 1 min and 72°C for 1min and a final extension of 72°C for 7 min. PCR conditions followed for ISSR markers included an initial denaturation step at 94°C for 5 min, followed by 40 cycles of 94°C for 30 sec, 50°C (ISSR-2, ISSR-10, ISSR-22, ISSR-12, ISSR-7, ISSR-13), 55°C (ISSR-23 and ISSR-18), 48°C (ISSR-4 and ISSR-5) for 30 sec and 72°C for 1min and a final extension of 72°C for 7 min. All amplified products were resolved in 1% agarose gels (Lonza Inc., USA) carrying Goodview ^{TM} Nucleic acid stain. Band images were obtained using the Gel documentation system (E-Box, VILBER) and size of the amplified fragments were estimated with 1Kb molecular marker (Thermo Scientific).

Data analysis

Consistent reproducible and well resolved bands, ranging from 180 to 2300 bp in size were manually scored for the presence of band as 1 and absence of the band as 0. Polymorphic information content and Marker index were calculated as described earlier (Roldan-Ruiz et al. 2000, Kayis et al. 2010). DARwin software version 6.0.000 was used to construct a dendrogram which is based on unweighted pair group method of arithmetic mean (UPGMA) using Jaccard's coefficient (Perrier and Jacquemoud-Collet, 2006). Factorial analysis to study the genetic relationships was also performed using DARwin software.

Results And Discussion

Development of two improved water chestnut varieties

Crop improvement for food, fiber and feed is a continuous process to sustain human needs. It is achieved either through selection of plants with desirable traits from the population or through breeding approaches. Two spineless improved water chestnut varieties/genotypes were developed which were superior to the popular spine varieties prevalent in North Bihar. From the Red spine biotype and Green spineless biotype, superior plants of merit in terms of yield and TSS were selected during 2016–2017 at RCM, Darbhanga, Bihar, India and were under subsequent multi-location trial till 2021. Improved variety/genotype from Red spineless biotype is registered by N.B.P.G.R as **IC 642169**, which is mentioned as Improved Red Spineless (IRS) in further sections and the improved variety from Green spineless biotypes is also registered at N.B.P.G.R. as **IC 642169**, which is mentioned as Improved Green Spineless (IGS) (Flow chart a & b). The fruits of IGS are large, dark green and oval shaped with a TSS of 8.5^0B and this variety yields 12.76 t/ha (Plate-2) while the fruit of the original green spineless biotype is very tiny, triangular in shape with a TSS of 7.7^0b and a yield of 4.2 t/ha. (Plate- 1). Similarly, IRS has large oval bright red fruits with a TSS of 9.0^0B and a yield of 9.45 t/ha (Plate-4) compared to that of Red spineless biotype which has oval shaped dull red colored fruits with a TSS of 7.5^0B and a yield of 5.92 t/ha. Both the IRS and IGS varieties are superior to Red Spine Large Local (RSLL) and Red Spine Small Local (RSSL), two popular local varieties of North Bihar. RSLL is cultivated for table purposes and RSSL is generally used for dry nut and atta making.

RAPD and ISSR analysis on IRS, IGS and two controls

Though the plant and fruit color of IGS and RSSL are different, that of IRS and RSLL are very similar (Table 2), and therefore it is difficult to distinguish them in the field. Hence molecular marker analysis using 10 RAPD and 10 ISSR markers is undertaken to differentiate them at genomic level.. A representative profile of RAPD and ISSR marker is presented in Fig. 2.

RAPD primers generated a total 52 fragments across all 4 varieties of which 46 fragments were polymorphic. RAPD markers generated 1–6 number of fragments in a range of 230 bp to 1750 bp wherein AP-16 generated least number of fragments and AP-32 and AP-39 generated highest number. PIC of the markers ranged from 0.23 to 0.46 and the MI of the markers was 0.42 to 3.6. Of the 10 ISSR markers tested two markers did not work and the remaining eight ISSR markers generated a total of 49 fragments of which 23 fragments were polymorphic with a PIC of 0.05–0.39 and MI of 0.007 to 0.32. Together RAPD and ISSR markers generated 78 polymorphic fragments with a mean PIC of 0.25 and MI of 1.3.

Cluster Analysis

Marker data on RAPD and ISSR markers separately and together was used to construct a dendrogram based on Jaccard's coefficient. Using RAPD markers two groups are formed where IRS and RSSL formed one group and RSLL and IGS formed another group (Fig. 3A) whereas based on ISSR markers RSLL formed one group while other three formed another group (Fig. 3B). However in the second group, IRS and RSSL formed a separate sub group. Factorial analysis based on RAPD markers and ISSR markers also suggest that IGS and RSLL diverge from IRS and RSSL supporting the cluster analysis (Fig. 4). When both RAPD and ISSR data was combined to construct a dendrogram, again two groups were formed one with RSLL and IGS and another with IRS and RSSL (Fig. 3C) and the factorial analysis has clearly shown that all four varieties are different from one another (Fig. 4).

In this study we found that RAPD markers generated more polymorphic fragments compared ISSR markers and the PIC and MI of RAPD markers is higher when compared to that of ISSR markers which suggests that RAPD maker is efficient in detecting variation among the water chestnut. However, in other crops ISSR markers are found to be more informative than RAPD markers (Elmeer et al. 2017; Phong et al. 2011).

Unique gene sequences identified in genotypes

Unique gene sequence in 550 bp and 800 bp DNA ladders identified from AP-4 (5'CAAACGACGG3') primer and 600bp DNA ladder identified from AP-32 (5'AACATCTCCGGG3') primer which are only present in IRS water chestnut as dimorphic and monomorphic band, respectively. Similarly, in IGS water chestnut, DNA fragments viz; AP30-1250 (5'GGACCTCCATCG3') and ISSR23-1500 & ISSR23-1350 (5'AGTAGTAGTTCTCTCTCTCTCTC3') are only present as single DNA band and dimorphic bands, respectively. According to Sharma et al., 2007 the highly allogamous nature of Trapa species creates heterozygosity in their natural populations. Desai et al. (2015) also stated that the DNA polymorphism in a specified population of *Trapa spp.* is due to the presence of genetic variants governed by the different alleles and their frequency of allocation. Utility of molecular analysis of water chestnut plants has been well documented by many workers (Hoque et al. 2005; Mahto et al. 2018). The genotype specific diagonistic bands using RAPD and ISSR markers afford a prospect to be SCAR markers for the differentiation of four genotypes. Genetic fidelity of clonally selected plants and traditional cultivars has immense practical utility and commercial implications under the wetland ecosystem of north Bihar, India. However, morphologically the fruit color. size number of fruits/plant and spine variation in Trapa spp. were considered in the study but it was unsafe to assume that these small morphological variations have been reflected in the RAPD and ISSR analysis. Accordingly, Houque et al. (2005) randomly amplified polymorphic DNA (RAPD) markers viz; AP-1, AP-4, AP-5, AP-11, AP-16, AP-22, AP-30 have proved to be the most polymorphic markers for water chestnut crop.

Conclusion

Thus, using RAPD markers and ISSR markers we were able to distinguish the improved varieties from the local popular cultivated varieties which are registered by the State National Bureau of Plant Genetic Resources (NBPGR) as elite germplasm. The improved varieties, IRS and IGS are also distinct from each other.and are the first improved varieties of water chestnut developed at the Research Centre of Makhana, ICAR, India. The future aim would be to convert the polymorphic fragments of RAPD and ISSR markers into SCAR markers (Cho et al. 2015) for the efficient utilization of these markers in the identification for the varieties as they are more reproducible than RAPD and ISSR markers.

Declarations

Funding

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Tables

Table-1: Morphological characteristics of four genotype of *Trapa*

SL.No.	Genotypes	Cultivar	Fruit Colour	No. of Spine	Fruit Weight (g)	Fruit Shape
1.	*Trapa spp.*	Improved Green Spineless (IGS)	Green	0	30.2	Oval
2.	*Trapa bispinosa*	Red Spine Small Local (RSSL)	Red	2	12.5	Triangular
3.	*Trapa spp.*	Improved Red Spineless (IRS)	Red	0	26.7	Oval
4	*Trapa bispinosa*	Red Spine Large Local (RSLL)	Red	2	19.5	Triangular

Table 2: RAPD markers used on the four water chestnut genotypes and their diversity indices

S.No	Primer Name	Primer Sequence 5'-3'	Size range (bp)	No. of Fragments	No. of Polymorphic Fragments	% of Polymorphism	PIC	RP	MI
1	AP-1	AGCCAGCGAA	280-1100	4	4	100	0.38	2	1.52
2	AP-4	CAAACGTCGG	350-1700	9	9	100	0.4	5.5	3.6
3	AP-5	GTTGCGATCC	230-1200	5	3	60	0.23	1.5	0.42
4	AP-11	ATGCTCCGAG	350-2050	5	5	100	0.5	5	2.5
5	AP-16	GACGTACCCT	850-1400	3	3	100	0.46	2.5	1.38
6	AP-22	AAGGCGCGAACG	1050-1400	2	0	0	0.0	0	0
7	AP-30	GGACCTCCATCG	520-2300	10	7	70	0.29	4.5	1.4
8	AP-32	AACATCTCCGGG	650-1550	9	8	88.9	0.44	8	3.1
9	AP-36	TATCCTACCGGC	600-1750	4	2	50	0.19	1	0.19
10	AP-39	CTTGAGGGATGG	700-1750	6	4	66.7	0.33	4	0.87
Total				**57**	**45**	**735.6**	**3.22**	**34**	**14.98**
Average				**5.7**	**4.5**	**73.5**	**0.32**	**3.4**	**1.48**

Table 3: ISSR markers used on the four water chestnut genotypes and their diversity indices

S.No	Primer Name	Primer Sequence 5'-3'	Size range (bp)	No. of Fragments	No. of Polymorphic Fragments	% of Polymorphism	PIC	RP	MI
1	ISSR-23	AGTAGTAGTTCTCTCTCTCTCTC	300-1500	5	2	40	0.18	1.5	0.14
2	ISSR-18	CACACACACACACACAAGC	480-2000	17	17	100	0.4	10.5	6.8
3	ISSR-4	ACACACACACACACACGA	400-2200	9	6	66.7	0.25	3	1.0
4	ISSR-5	GAGAGAGAGAGAGAGAC	600-1600	6	3	50	0.21	2	0.32
5	ISSR-11	AGAGAGAGAGAGAGAGG	250-1100	9	4	44.4	0.2	2.5	0.35
6	ISSR-10	GAGAGAGAGAGAGAGAT	550-1800	7	1	14.3	0.05	0.5	0.007
7	ISSR-12	CTCTCTCTCTCTCTCTA	180-1300	4	0	0	0	0	0
8	ISSR-6	ACACACACACACACACC	450	1	0	0	0	0	0
Total				**58**	**33**	**315.4**	**1.29**	**20**	**8.62**
Average				**7.25**	**4.1**	**39.4**	**0.16**	**2.5**	**1.09**

Table 4: Banding patterns of RAPD and ISSR markers used on four water chestnut genotype

Components	RAPD	ISSR
Total number of primers	10	8
Total number of bands	57	58
Total number of polymorphic bands	45	33
Percentage of polymorphism	73.5	39.4
Average number of bands /primer	5.7	7.25
Average number of polymorphic bands/primer	4.5	4.1

Figures

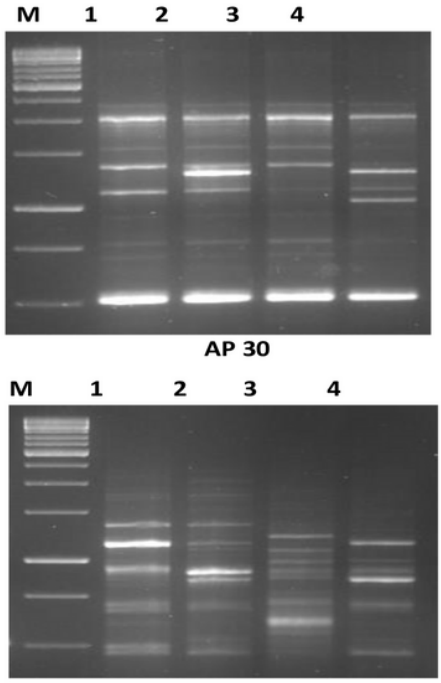


Fig 2 Profile of four water chestnut varieties with A RAPD (AP30) and ISSR (ISSR 18) marker. Lane 1: Red Small Spine Local (RSSL), Lane 2: Improved Green Spineless (IGS), Lane 3: Red Spin Large Local (RSLL), Lane 4: Improved Red Spineless (IRS). M is the 1Kb Molecular weight size standard.

Figure 1

See image above for figure 2 legend.

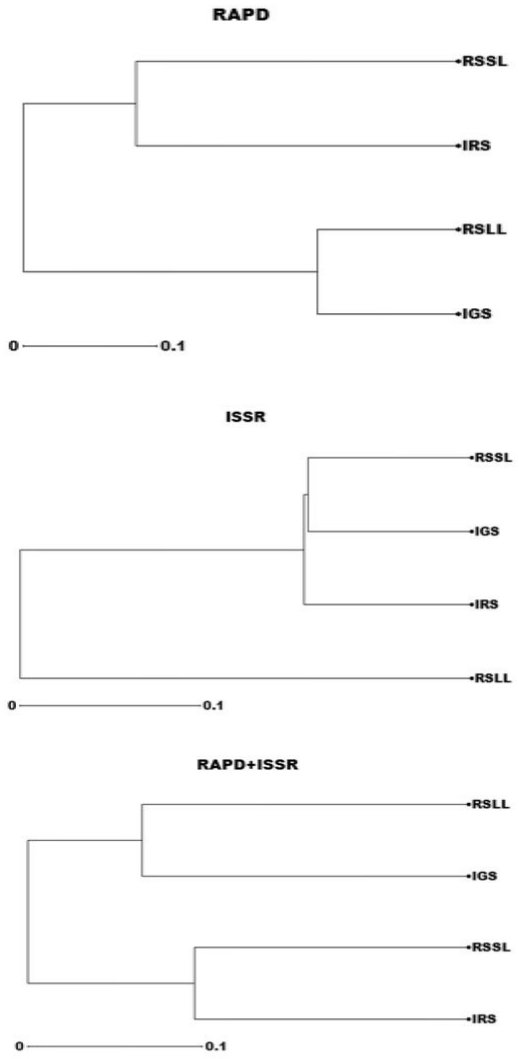


Fig 3: Dendrograms derived from UPGMA analysis using Jaccards coefficient for A) RAPD markers, B) ISSR markers C) combined RAPD and ISSR markers.

Figure 2

See image above for figure 3 legend.

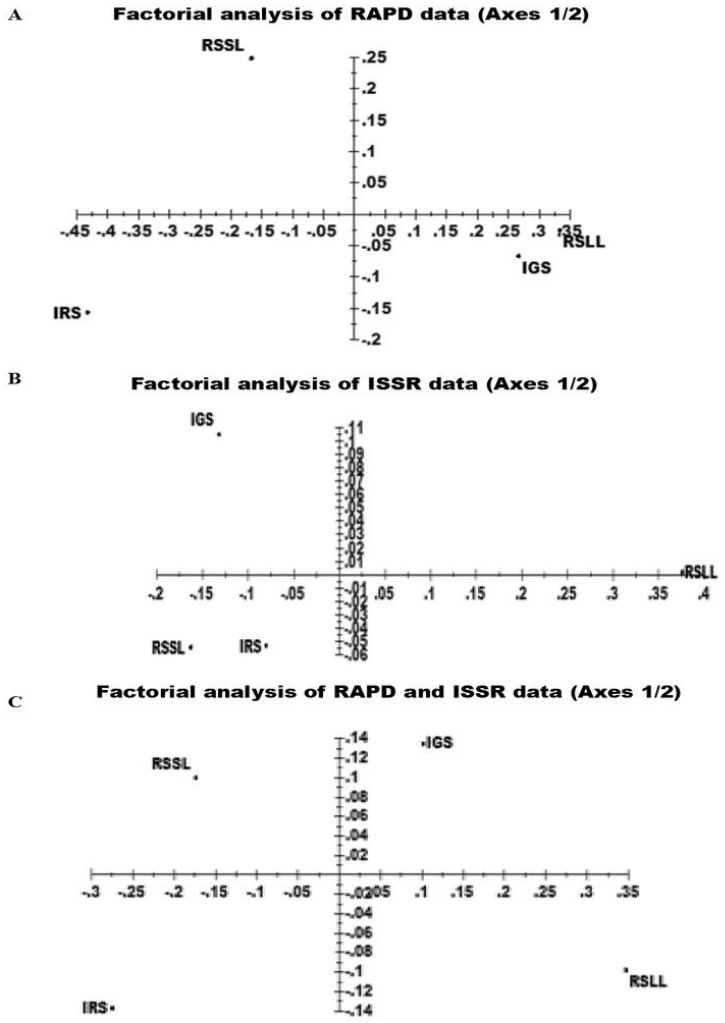


Fig 4: Factorial analysis with A) RAPD B) ISSR and c) combined ISSR and RAPD markers

Figure 3

See image above for figure 4 legend.

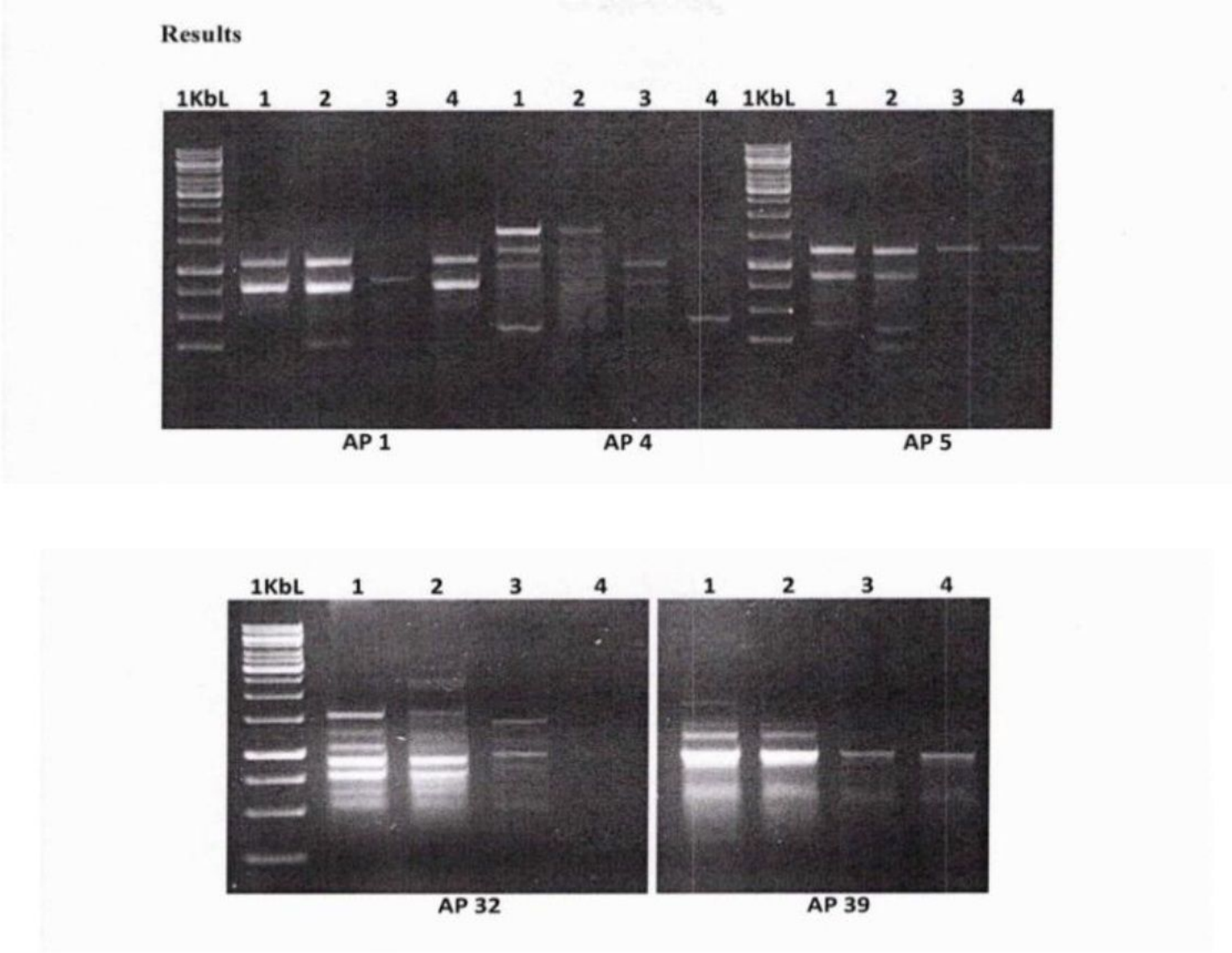
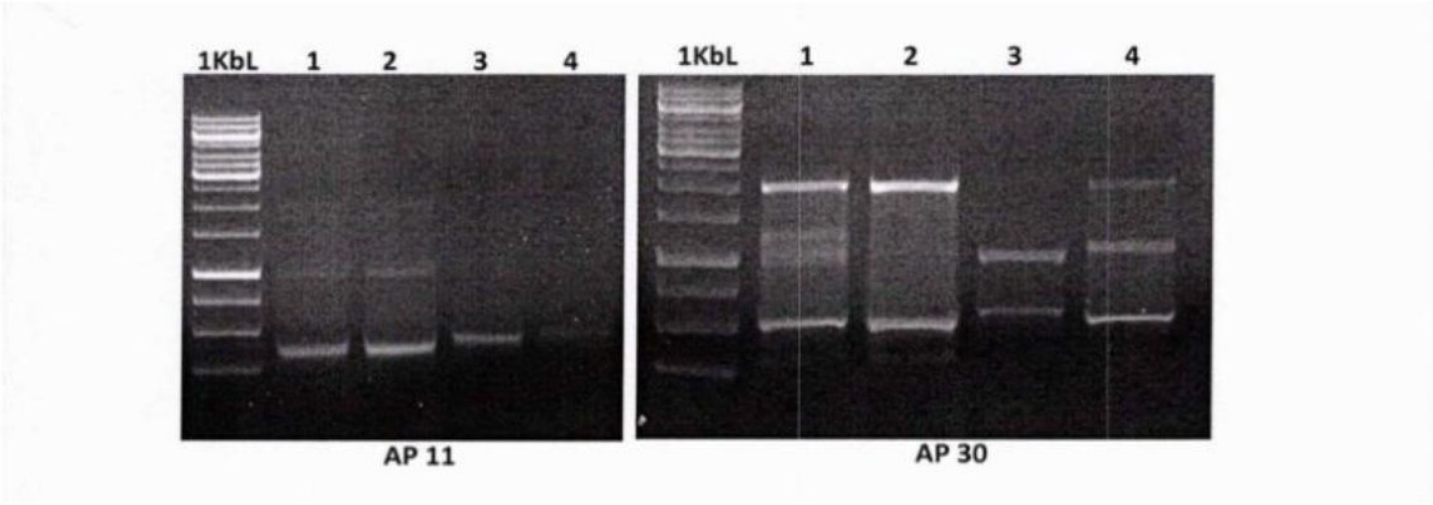


Figure 4

Fig-4-5: The presence of DNA fragment 550 bp DNA and in 800 bp from AP-4 and 600 bp DNA ladder identified from AP-32 primer only present in IRS

[Profile of four water chestnut varieties with 10 RAPD markers. In the figure Lane-1: Improved Green Spineless (IGS) , Lane-2: Red Spine Large local (RSLL), Lane-3: Improved Red Spineless (IRS), Lane-4: Red Small Spine Local, M is the 1Kb Molecular weight size Standard.]



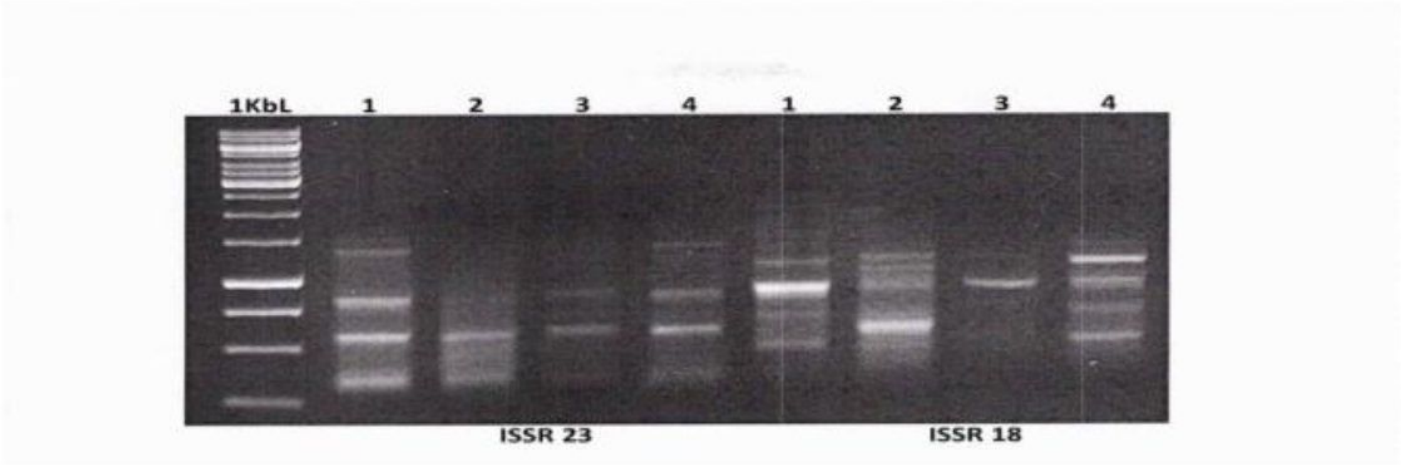


Figure 5

Fig-6-7: Distinguishing Features of IGS: the presence of DNA fragments AP30-1250, and ISSR23-1500 & ISSR23-1350

[Profile of four water chestnut varieties with 10 RAPD and 10 ISSR markers . In the figure Lane-1: Improved Green Spineless (IGS) , Lane-2: Red Spine Large local (RSLL), Lane-3: Improved Red Spineless (IRS), Lane-4: Red Small Spine Local, M is the 1Kb Molecular weight size Standard.]

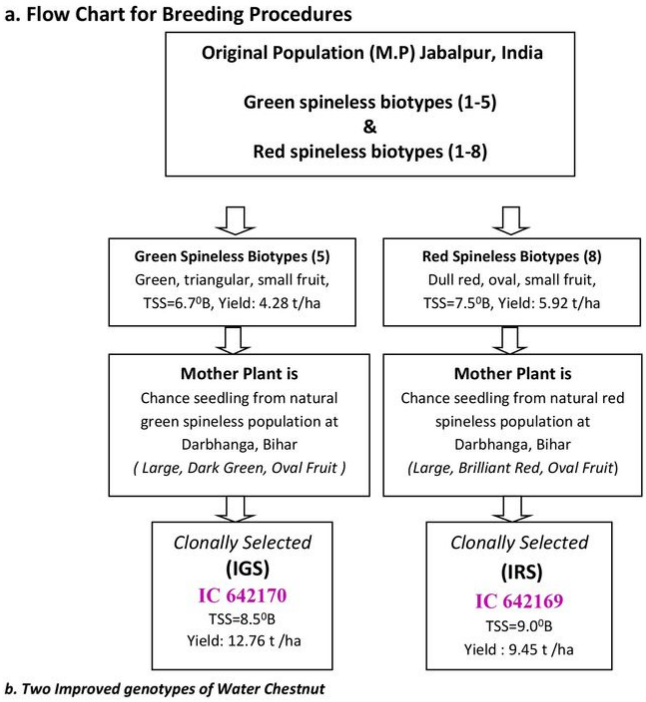


Figure 6

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