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Pranci Tiwari
Department of Plant Molecular Biology and Genetic Engineering, N. D. University of Agriculture and Technology, Kumarganj Ayodhya, Uttar Pradesh, India

Reeshu Singh
Department of Plant Molecular Biology and Genetic Engineering, N. D. University of Agriculture and Technology, Kumarganj Ayodhya, Uttar Pradesh, India

NA Khan
Department of Plant Molecular Biology and Genetic Engineering, N. D. University of Agriculture and Technology, Kumarganj Ayodhya, Uttar Pradesh, India

Praveen Tiwari
Department of Plant Molecular Biology and Genetic Engineering, N. D. University of Agriculture and Technology, Kumarganj Ayodhya, Uttar Pradesh, India

Corresponding Author:
Reeshu Singh
Department of Plant Molecular Biology and Genetic Engineering, N. D. University of Agriculture and Technology, Kumarganj Ayodhya, Uttar Pradesh, India

Sequence characterization of amplifying region by using RAPD marker for bottle gourd (*Lagenaria siceraria*)

Pranci Tiwari, Reeshu Singh, NA Khan and Praveen Tiwari

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Abstract
Bottle gourd [*Lagenaria siceraria* (Mol.) Standl.] (2n = 2x = 22), also known as opo squash or long melon, is diploid belonging to the genus *Lagenaria* of the *Cucurbitaceae* family with a genome sizea of ~334 Mb. Bottle gourd comes under in different sizes some are long, some are round and some are dumbbell shaped. DNA was isolated from 4 varieties of *Lagenaria* (2 long Narendra Shivani and Narendra Rashmi & 2 Round Narendra Madhuri and Narendra Prabha) and was done with 32 primers. RAPD was done in 4 varieties of *Lagenaria* (2 long Narendra Shivani and Narendra Rashmi & 2 Round Narendra Madhuri and Narendra Prabha) and was done with 32 primers. Out of 32 primers, 8 primers shown good amplification and dendrogram was generated using NTSYS analysis. Relationship of *Lagenaria* was established as shown by dendrogram. Narendra Rashmi and Narendra Shivani (both long) falls in one group as shown in dendrogram. 1200 bp long variety specific DNA was identified with OPK 17 primer. 1200 bp band in Narendra Shivani was more intense in Narendra Shivani than Narendra Rashmi. Longest nature of Narendra Shivani may be due to repeat copy of this DNA as amplican was more intense in Narendra Shivani then Narendra Rashmi.

Keywords: *Lagenaria siceraria*, RAPD marker, diversity

Introduction
Bottle gourd [*Lagenaria siceraria* (Mol.) Standl.] (2n = 2x = 22), also known as opo squash or long melon, is diploid belonging to the genus *Lagenaria* of the *Cucurbitaceae* family with a genome size of ~334 Mb (Achigan-Dako *et al.*, 2008) [1]. Bottle gourd is annual herbs and the leaves of bottle gourd are alternate and variable. The flowers are unisexual and white; they are present on the same plant. It has white pulp, often is an indehiscent gourd. Bottle gourd requires a minimum temperature of 18^0C during early growth, but optimal temperature are in the range of 24-27^0C. (Edwin Wosu and Ndukwu, 2008)

The fruits are edible and considered as a good source of vitamin C, β-carotene, vitamin B-complex, pectin and also contain highest choline level. The seed kernels contain 45% oil and about 35% protein. Modern phytochemical screening methods have shown the presence of triterpenoid cucurbitacins B, D, G, H, fucosterol, campesterol and flavone C-glycosides in it. Bottle gourd seeds are a good source of edible protein and oil. They are an excellent source of essential fatty acids (such as omega 3 and omega 6), antioxidands, vitamins and sterols. They contain the high level of vitamin E, A and C (EL-Dengawy *et al.*, 2001; Hassan *et al.*, 2008; Hegazy and EL Kinawy, 2011) [5, 6]. They contain the pharmaceutically active compounds used to treat acne, hyper – seborrhea, BHP, hirsutism and alopecia (Piccirilli *et al.*, 2007; Prashar *et al.*, 2014) [9, 10].

The bottle gourd (*Lagenaria siceraria*) is probably one of Humankinds first domesticated vegetable species, providing food, medicine and a lot more. It probably originated in Africa, from where it got distributed, perhaps by floating in sea to India, China and as far as New Zealand. The gourd is now widely cultivated throughout the tropics, especially India, Sri Lanka, Indonesia, Malaysia, the Philippines, China, tropical Africa and South America. Uttar Pradesh rank second in bottle gourd cultivation within the country. Its production is of about 422.74 of the Countries total production and share about 17.20%.

A great variability is encountered in fruit shape. They may be long, cylindrical, curved, necked, oblong, round, flat round, conical, pear-shaped club shaped etc. But bottle gourd are

broadly classified as long and round types. Variability in fruit characteristics exhibited by bottle gourd has been described by Sirohi and Sivakami (1991) [12]. Most fascinating variability is encountered in its fruit shape and size. Although the crop was neglected from crop improvement point of view about three decades ago but moderately intensive conventional breeding has resulted in the development of several open pollinated and hybrid varieties in this crop in India.

India is endowed with a rich variability of bottle gourd, especially with regard to fruit characteristics (Sivaraj and Pandravada, 2005) [13]. Morphological characters are useful for characterizing genotypes against highly heritable and stable traits. Further, association of any morphological character with desirable traits or yield components serves as a phenotypic marker in the selection process.

Narendra Shivani is a variety of Narendra Deva University of agriculture and technology is a selection of local germplasm. This variety has a speciality of having a log slender fruit, length of 2.0 meters. Yield potential of 1300 q/ha. With proper plant care and nutrient management single plant produces more than 200 fruits on bower. Narendra Madhuri, Narendra Rashmi, NDGB 619 are also the varirties released from NDAU&T through the selection from local germplasm. Fruit of Narendra Madhuri is round shaped which get mature at the time of mid-July to mid-august. The plant will remain in fruiting for 6 month when trained on bowers and having a yield potential of more than 1000 q/ha. The fruit of Narendra Rashmi is long bottle shaped. Its fruit is mainly cultivated in summer/rainy season. It require about 60 days for first fruit picking and yield about 300-400q/ha. Narendra Prabha (NDBG-619) have a Cylindrical fruits, and its seeds are peculiar small conical shaped. It is an early variety, require about 55 days for first picking in spring/ summer crop and average yield of about 300 q/ ha.

We are investigating this study to find out the physiological properties of four bottle gourd varieties *viz* Narendra Shivani is long & while the other three varieties sach as Narendra Madhuri is round shaped, Narendra Rashmi is long and bottle shaped and the rest one variety Narendra Prabha (NDGB-619) is cylindrical and due to the elongation of Narendra Shivani we are also trying to find out the gene responsible for its elongation.

Materials and Methods

A total of four bottle gourd genotypes were Narendra Shivani, Narendra Madhuri Narendra Rashmi, Narendra Prabha selected for DNA amplification using RAPD markers and making the SCAR marker for long specific variety. These genotypes were obtained from net house in Department of PMB & GE and Main Experiment Station, Department of Vegetable Science, of N.D. University of Agriculture and Technology Kumarganj Ayodhya and there description has been provided in Table-1.

Table 1: List of bottle gourd genotypes used in the DNA isolation

S. No.	Name of Variety	Pedigree	Special Features	Year of Release
01.	Narendra Shivani	Selection from local germplasm	Prolific bearer very long slender fruits exceed the length of 2.0 meters. Yield potential of 1300 q/ha. With proper plant care and nutrient management single plant produces more than 200 fruits on bower.	2007
02.	Narendra Madhuri	Selection from local germplasm	Rounded fruits, appropriate time of planting is mid-July to mid-August. With proper plant care and nutrient management, the plants trained on bowers remain in fruiting for about 6 months. Yield potential of more than 1000 q/ha.	2007
03.	Narendra Rashmi	Selection from local germplasm	Fruits are long bottle shaped. Summer type bottle gourd, suitable for cultivation in summer and rainy seasons. Requires 60 days for first fruit Picking. Fruits yield 300-400q/ha.	2001
04.	Narendra Prabha (NDBG-619)	Selection from local germplasm	Cylindrical fruits, peculiar small conical seeds, an early variety, 55 days for first picking in spring/ summer crop an average yield 300 q/ ha.	2009

Collection of round and long varieties of bottle gourd including Shivani variety

Fig 2 shows the seed of bottle gourd varieties Narendra Shivani, Narendra Rashmi, Narendra Madhuri and Narendra Prabha (NDGB 619). The seed of Narendra Shivani and Narendra Rashmi was light colour, long in size while Narendra Madhuri and Narendra Prabha seed were dark in colour and long in size.

Fig 1: shows the leaves of bottle gourd. They were approximately similar in colour, shape and size

A. Narendra Madhuri

B. Narendra Rashmi

C Narendra Prabha

D. Narendra Shivani

A. Narendra Rashmi

B. Narendra Shivani

C. Narendra Prabha

D. Narendra Madhuri

Table 2: Primers used for PCR amplification

S No.	Primer	Sequence
1	OPB 13	5'TTCCCCCGCT3'
2	OPK 11	5'AATGCCCCAG3'
3	OPA 19	5'CAAACGTCGG3'
4	OPA 13	5'CAGCACCCAC3'
5	OPO 12	5'CAGTGCTGTG3'
6	OPB 10	5'CTGCTGGGAC3'
7	OPB 18	5'CCACAGCAGT3'
8	OPO 11	5'GACAGGAGGT3'
9	OPC 15	5'GACGGATCAG3'
10	OPO 10	5'TCAGAGGCGC3'
11	OPK 17	5'CCCAGCTGTG3'
12	OPB 08	5'GTCCACACGG3'
13	OPB 11	5'GTAGACCCGT3'
14	OPN 07	5'CAGCCCAGAG3'
15	OPA 17	5'GACCGCTTGT3'
16	OPC 07	5'GTCCCGACGA3'
17	OPN 01	5'CTCACGTTGG3'
18	OPY 19	5'TGAGGGTCCC3'
19	OPY 18	5'GTGGAGTCAG3'
20	OPF 14	5'TGCTGCAGGT3'
21	OPF 13	5'GGCTGCAGAA3'
22	OPF 06	5'GGGAATTCGG3'
23	OPF 17	5'AATTTGGGAA3'
24	OPY 15	5'AGTCGCCCTT3'
25	OPJ 08	5'CATACCGTGG3'
26	OPZ 03	5'CAGCACCGCA3'
27	OPD 08	5'GTGTGCCCCA3'
28	OPY 02	5'CATCGCCGCA3'
29	OPY 14	5'GGTCGATCTG3'
30	OPJ 13	5'CCACACTACC3'
31	OPY 01	5'GTGGCATCTC3'
32	OPY 09	5'GTGACCGAGT3'

Plasmid

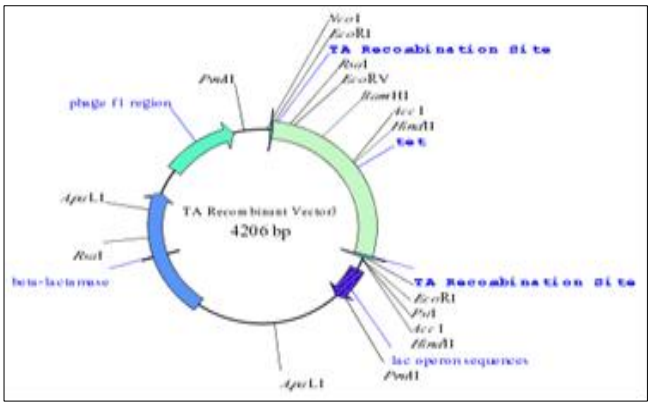

Fig 2: shows the TA cloning vector

E. coli Host

For transformation in E. coli to cloned vector, DH5α was used

DNA extraction, purification and quantification

DNA isolation and quantification: Taken leaves samples ground in liquid nitrogen using sterilized mortar pestle. The powdered mycelium was transferred to a 2 ml Eppendorf tube containing 880 µl of extraction buffer (2% CTAB buffer, 4M NaCl, 0.5M EDTA, 1M Tris-Cl, 0.02% β-Mercaptoethanol). After incubation at 65°C for 1-hour, equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added and centrifuged @ 12000 rpm for 10 minutes. After proper mixing, transfer the clear supernatant to an Eppendorf tube, an equal volume of chloroform: isoamylalcohol (24:1) was added followed by mixing and centrifuged @ 12000 rpm for 10 minutes. Then, add chilled absolute alcohol to the supernatant, mix well and keep at -20°C for 2 hrs. After centrifugation, DNA pellet was washed with 70% ethanol, air dried and resuspended in 100µl TE buffer. The gel electrophoresis and Nanodrop Spectrophotometer were used to determine the quality and quantity of fungal DNA. After quantification, the DNA samples were diluted to a concentration of 30-50 ng/µl for use in PCR reaction

PCR amplification and gel elution: The PCR reaction was performed in 25 µl reaction volume with, 0.5 µM of primers, 10 mM dNTP, 1.5 mM MgCl2, 50 ng of template DNA, 1X *Taq* buffer and 1U of DNA Taq polymerase. The PCR was performed with following parameters: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94 °C for 45 sec, annealing at 55 °C for 45 sec, extension at 72 °C for 45 sec followed by a final extension at 72 °C for 10 min. The amplified PCR products were analyzed in gel electrophoresis and documented under UV using gel documentation system

Cloning of the PCR product into T-vector

The purified DNA fragments were ligated to TA cloning vector. The ligation mixture along with linearized vector and amplicon DNA were mixed in 0.5 ml micro-centrifuge tubes and incubated at 16 °C overnight.

Component	Quantity taken
Purified amplified products	5 µl
PCR cloning vector	1µl
2X Instant ligation buffer	5 µl
Instant T4 DNA ligase	1µl
Distilled water	8µl
Total volume	20µl

Preparation of competent cells

The competent cells of *E. coli* ($DH_5\alpha$) were prepared by following the protocol mentioned by Sambrook *et al*., (1989) with minor modifications under aseptic and refrigerated condition. An isolated colony from *E. coli* $DH_5\alpha$ plate was inoculated into 5 ml Luria broth and incubated at 37°Covernight at 200 xg. The next day, the culture was diluted to 1:100 using Luria broth *i.e*., 0.5 ml of culture was added to 50 ml of Luria broth. It was incubated for 2-3 hours till it attained an OD of 0.3 to 0.4 at 600 nm. The culture was chilled in ice for 30 min., and 25 ml of culture was dispensed into two centrifuge tubes of capacity 50 ml. The cells were pelleted at 6000 xg for 5 min. The supernatant was discarded and pellet was suspended in 12.5 ml of ice-cold 0.1 M calcium chloride. The centrifuge tubes were again kept in ice for 45 min. and later centrifuged at 4000 xg for 10 min. The pellet was dispensed in 1 ml of 0.1M $CaCl_2$. About 200 µl of cells were distributed to each chilled 1.5 ml micro centrifuge tubes and immediately used.

Transformation of *E. coli* ($DH_5\alpha$) and blue white selection

About 100 µl of freshly prepared competent cells were taken in a chilled centrifuge tube and 5 µl of ligation mixture was added and mixed gently. The mixture was chilled in ice for 45 min. and heat shock was given by shifting the chilled mixture to preheated 42°C water bath for exactly 2 min., then the tubes were immediately transferred to ice for 5 min. To this, 800 µl of Luria broth was added and incubated at 37°C at 200 xg for 45 min. to allow bacteria to recover and express the antibiotic marker encoded by the plasmid. The culture was centrifuged at 10,000 xg for 1 min. and about 700 µl of supernatant was discarded and the pellet was dissolved in remaining supernatant and spread on the plates having Luria agar with Ampicillin (100 mg/ml) and incubated overnight at 37°C for colonies to appear. The recombinant clones were identified by blue/white assay.

After incubation only white colonies, having recombinant vectors were picked up and streaked on plates having Luria agar with Ampicillin (100 µg/ml), X-gal (50 µg/ml) IPTG (Isopropyl 1-ß, D-1 thiogalactopyranoside) (200 mg/ml) and incubated at 37°C overnight.

Dendrogram Preparation

All the eight primers bands for all varieties were scored. Where the band was present 1 was given, where the band was not present 0 was given and then using NTSYS program dendrogram was generated.

Results and Discussion

Narendra shivani variety is too long (up to 2 to 8 feet). To address this objective approach we took as first do fingerprinting of long and round varieties and established their relationship. Following this differentiate between round and long varieties of bottle gourd and again explore what makes Narendra shivani very long. As per requirement first DNA was isolated from the all varieties. Teethed and no sharing definitely suggest that isolated DNA is intact in all varieties and can be used further molecular work. DNA markers are abundant and essentially independent from environmental conditions (Keim *et al*., 1989) [7], and several research group have been using this tool in breeding programs (Shoemaker *et al*., 1992; Young and Kelly, 1996; Young *et al*., 1998) [11, 15, 16]. Further RAPD profiles appear to be useful in discriminating cultivars and show promise as identification makers.

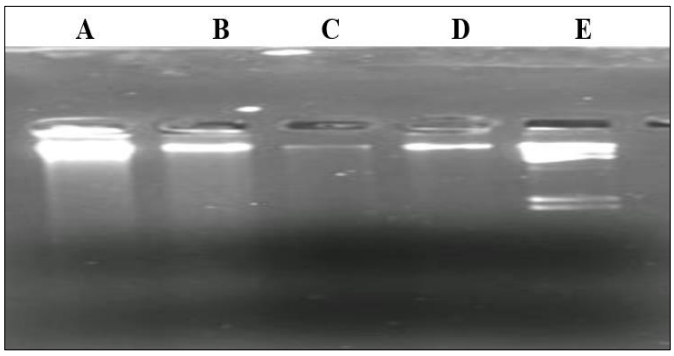

Fig 3: DNA isolation from leaves of bottle gourd

PCR amplification using RADP marker

For primer OPA 19 (Lane 2 to 5), many DNA bands were seen. Maximum bands were present in Narendra Rashmi and bands were of 1500 bp, 1050 bp, 510 bp, 310 bp and 200 bp. In Narendra Shivani bands were of 1050 bp and 310 bp. In Narendra Prabha bands were of 1050 bp and 310 bp. Only one bands was present in Narendra Madhuri band was of 1050 bp. This band was specific as present in Narendra Madhuri and Narendra Rashmi had specific band of 1500 bp and 510 bp. Narendra Shivani had specific band of 310 bp.

For primer OPA 13 (Lane 6 to 9), many DNA bands were seen. Maximum bands were present in Narendra Rashmi which were of 200 bp, 350 bp, 550 bp, and 750 bp, In Narendra Shivani bands were 200 bp, 350 bp, 550 bp and 750 bp In Narendra Prabha bands were 200 bp, 350 bp, 550 bp and 750 bp. Two bands only were present in Narendra Madhuri bands were of 200 bp and 350 bp. For this primer there was only one band present in Narendra Madhuri which was of 350 bp.

For primer OPO 12 (Lane 10 to 13), also many DNA bands were seen. Maximum bands were present in Narendra Madhuri bands of 200 bp, 250 bp, 750 bp and 1000 bp. In Narendra Rashmi bands were 200 bp, 250 bp, 750 bp and 1000 bp. In Narendra Shivani bands were 200 bp, 250 bp, 750 bp and 1000 bp. Two bands were present in Narendra Prabha bands were of 200 bp and 1050 bp. Narendra Madhuri had a band that was 750 bp. Narendra Rashmi had a band that was 750 bp. Narendra Shivani had a band that was 750 bp.

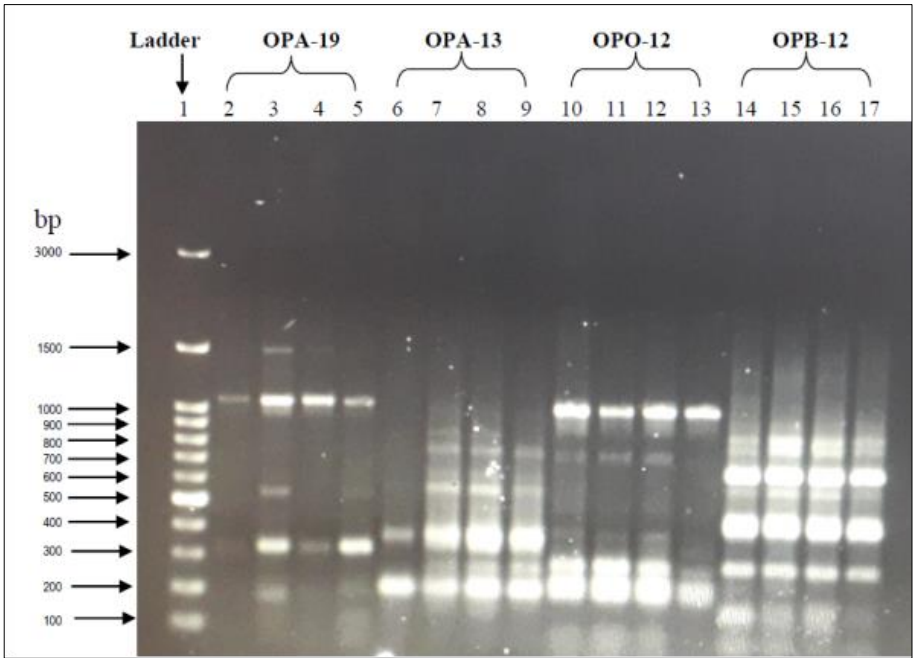

Fig 4: Shows RAPD profile of four varieties using various primers (OPA 19, OPA 13, OPO 12, OPB 12)

For primer OPB 10 (Lane 14 to 17), many bands were also seen. Maximum bands were present in Narendra Madhuri of size 150 bp, 250 bp, 450 bp and 650 bp. In Narendra Rashmi bands were of 150 bp, 250 bp, 450 bp and 650 bp. In Narendra Shivani bands were of 150 bp, 250 bp, 450 bp and 650 bp. Three bands was present in Narendra Prabha which were of 250 bp, 450 bp and 650 bp. There was no specific band corresponding long variety in above used four primer sets. RAPD profile of four varieties using various primers (OPC 15, OPO 10, OPK 17, OPB 08). For primer OPC 15 (Lane 2 to 5), many bands amplified. Maximum bands were present in Narendra Rashmi and Narendra Shivani. In Narendra Rashmi bands were of 400 bp, 510 bp, 700 bp and 810 bp. In Narendra Shivani it was 400 bp, 510 bp, 700 bp and 810 bp. Two bands were present in Narendra Madhuri and Narendra Prabha bands were of 400 bp and 510 bp. Narendra Rashmi and Narendra Shivani had a specific band of 810 bp. (Fig. 3)

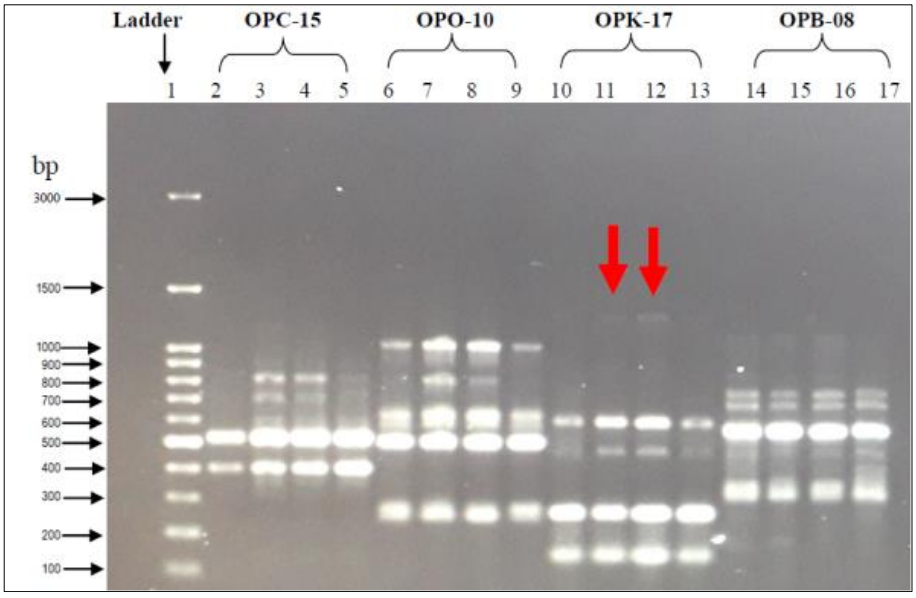

Fig 5: Shows RAPD profile of four varieties using various primers (OPA-15, OPC- 10, OPK-17, OPK-8).

For primer OPO 10 (Lane 6 to 9), many bands were seen. Maximum bands were present in Narendra Rashmi bands of 200 bp, 510 bp, 610 bp, 810 bp and 1010 bp. In Narendra Shivani bands were 200 bp, 510 bp, 610 bp, 810 bp and 1010 bp. Minimum bands were present in Narendra Madhuri of 200 bp, 510 bp, 610 bp of 1010 bp. In Narendra Prabha bands were of 200 bp, 510 bp, 610 bp and 1010 bp. Specific band were present in Narendra Rashmi and Narendra Shivani of 810 bp.

For primer OPK 17 (Lane 10 to 14), many bands amplified in all varieties. Maximum bands were present in Narendra Rashmi and Narendra Shivani. In Narendra Rashmi bands were of 100 bp, 250 bp, 500 bp, 600 bp and 1200 bp. In Narendra Shivani bands were of 100 bp, 250 bp, 500 bp, 600 bp and 1200 bp. Minimum bands were present in Narendra Madhuri bands was 100 bp, 250 bp and 600 bp. In Narendra Prabha bands were of 150 bp, 250 bp and 600 bp. Specific band were present in Narendra Rashmi and Narendra Shivani which was 1200 bp.

For primer OPB 08 (14 to 17), many bands were also seen. Maximum bands were present in Narendra Madhuri bands of 300 bp, 650 bp, 750 bp and 850 bp. In Narendra Rashmi 300 bp, 650 bp, 750 bp and 850 bp. Minimum bands were present in Narendra Shivani and Narendra Prabha of 300 bp and 650 bp, respectively.

With OPK 17, long specific DNA band of 1200 bp was seen in Narendra Shivani and Narendra Rashmi. Band was more intense in Narendra Shivani then Narendra Rashmi. Previous studies of bottle gourd also suggests fragment size variation of total genomic DNA using RAPD primers (Decker *et al.*, 2001) [3] The DNA fragment corresponding to RAPD maker OPK_{1200} was cloned and partially sequenced for making SCAR marker. The reliability of SCAR makers linked to a unique locus has been reported in some important crops (Naqvi and Chattoo., 1996; Barret *et al.*, 1998) [8, 2] as well as in grapevine (Lahogue *et al.*, 1998).

Dendrogram generation using NTSYS-pc

Dendrogram of all four varieties after NTSYS analysis. Bands of each varieties with each primer were scored giving 1 for DNA band present and 0 for no band of same size. NTSYS analysis showed their genetic coefficient as shown in figure Narendra Rashmi and Narendra Shivani falls in one group while Narendra Madhuri and Narendra Prabha in different group. This suggests that long variety are clustered together. (Fig 6)

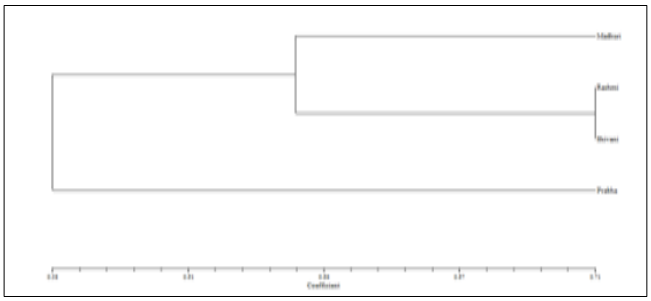

Fig 6: Dendrogram of *Lagenaria*

Cloning in long specific DNA in TA Cloning vector

PCR product was cloned in TA vector. For this 1200 bp PCR product was first eluted from agrose gel. Long specific DNA after elution from gel was transformed as described in method and material and was put in TA cloning vector. From blue and white colony, white colony was selected as recombinant.

Discussion

Broad objective of this work was to find out why Narendra Shivani variety is too long (up to 2 to 8 feet). To address this objective approach we took as first do fingerprinting of long and round varieties and established their relationship. Following this differentiate between round and long varieties of bottle gourd and again explore what makes Shivani very long. As per requirement first DNA was isolated from the all varieties. Teethed and no sharing definitely suggest that isolated DNA is intact in all varieties and can be used further molecular work. DNA markers are abundant and essentially independent from environmental conditions (Keim *et al*., 1989) [7], and several research group have been using this tool in breeding programs (Shoemaker *et al*., 1992; Young and Kelly, 1996; Young *et al*., 1998) [11, 15, 16]. Further RAPD profiles appear to be useful in discriminating cultivars and show promise as identification makers. Out of 32 RAPD primers 8 primers shown good amplification. Unique polymorphic bands produced by these primer were able to identified the genotype. Cluster analysis based on RAPD marker grouped the genotype in three main clusters. Analysis of RAPD variation revealed that the evolutionary history of bottle gourd is complex. Long varieties fall in one group but round Variety Madhuri and Prabha were distant apart. Previous studies of bottle gourd also suggests fragment size variation of total genomic DNA using RAPD primers (Decker *et al.*, 2001) [3]. The DNA fragment corresponding to RAPD marker OPK_{1200} was cloned and partially sequenced for making SCAR marker. The reliability of SCAR makers linked to a unique locus has been reported in some important crops (Naqvi and Chattoo., 1996; Barret *et al.*, 1998) [8, 2] as well as in grapevine (Lahogue *et al.*, 1998).

Further, the DNA fingerprints of *Lagenaria* developed would be of immense use identifying these genotype individually which would be useful in plant variation rights to safe guard the countries genetic resources.

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