Diversity in Indian and Some Exotic Onion Cultivars as Revealed by Genomic and Mitochondrial DNA

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Abstract

Cytoplasmic genic male sterility in onion has been associated with the mitochondrial genome. Identification of male sterile lines and its maintainers is a major hurdle in exploitation of male sterility in onion hybrid seed production. In this study, we evaluated genomic and mitochondrial genome diversity to identify type of cytoplasm and male sterility among various onion varieties of Indian and exotic origin employing RAPD, SSR and non-radioactive RFLP molecular markers. Among the 68 used random decamer RAPD primers, 39 primers produced 445 total polymorphic bands with an average of 11.40 bands per primer. A total of 16 SSR primers were screened and 11 showed polymorphism and produced 86 polymorphic bands with an average of 8 polymorphic bands per primer. The RFLP studied using non-radioactive gene probes were *cox1*, *cox2*, *atp6*, *atp9* and *nad6*. These studies demonstrate the diversity in the nuclear and mitochondrial genome of onion cultivars.

INTRODUCTION

Onion (*Allium cepa*) and its allied cultivated forms such as garlic, leek, chives and Japanese bunching onion are important crops grown worldwide primarily for culinary purposes. Onion products are available in fresh, frozen, canned, pickled and dehydrated forms. High level of variability is found in onion and is well exploited for cultivation and commercial purposes. For example, high total soluble solids (TSS) and pungent genotypes are used for dehydration purposes while low TSS and mild pungent to sweet varieties are used for fresh consumptions.

Plant breeders in India have released several varieties for table onion and a few for processing. Most of the Indian varieties are open pollinated and possess low TSS (<12%) and high bolting percentage as compared to some of the exotic open pollinated varieties and F_1 hybrids. Being a biennial crop, developing improved open pollinated lines takes several years. The production of F_1 hybrids on commercial scale using hand emasculation is also difficult due to tiny flowers. Exploitation of cytoplasmic male sterility (CMS) for production of F_1 hybrids has been found efficient but identification of CMS lines and their maintainers and restorers is difficult.

Recent developments in biotechnology have provided researchers with possibilities of characterizing the cytoplasms enabling the identification of potential male sterile individuals. Further, DNA polymorphism in the germplasm can be studied using various molecular marker methodologies (Staub et al., 1996). The most common molecular markers currently in use are (i) restriction fragment length polymorphism (RFLP), (ii) random amplified polymorphic DNA (RAPD), (iii) amplified fragment length polymorphism (AFLP), and (iv) simple sequence repeat (SSR). An extensive review by Joshi et al. (1999) on the use of molecular markers in plant genome analysis has provided valuable insight into the understanding of types, properties and usefulness of different molecular markers.

RAPD analysis is a relatively powerful method for identifying inter and intra-specific variations in the DNA. It is a quick and comparatively economical approach for

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the detection of small genetic differences (Friesen and Klass, 1998; Wolfe and Liston, 1998) in the genome. Genetic variability studies in both natural plant populations and germplasms of important crop plants have been reported using RAPD analysis (Orozco-Castillo et al., 1994; Lu et al., 1996) and EST-SSR markers (McCallum et al., 2008). Mitochondrial DNA RAPD have been used in crops such as sorghum (Sane et al., 1996, 1997; Jaiswal et al., 1998) and rice (Seth et al., 1996), to assess genetic variability and differentiate sterile cytoplasms.

Several workers have utilized RAPD for cultivar or line identification in different onion breeding programs (*Allium cepa*: Champion et al., 1995; Havey, 1995; *Allium sativum*: Bradley et al., 1996; Al-Zahim et al., 1999; triploid onion: Puizina et al., 1999). RFLP techniques also have been used to characterize onion/*Allium* germplasm (Havey and Bark, 1994; Bark et al., 1994; Havey, 1997, 2000), and to assess diversity among sterile and fertile cytoplasms (Havey, 2000). Hybrid onion seed production is principally effected through male sterility system (Pike and Yoo, 1990). The CMS trait is characterized by the absence of functional pollen which is a result of an incompatible nuclear-mitochondrial interaction (Hanson, 1991). Two types of CMS have been genetically characterized in onion namely S type and T type. The former type is widely used by seed producers because of the stable male sterility over many environments, no reduction in female fertility and relatively common occurrence of the recessive allele at the nuclear male fertility restoration locus (MS) allowing seed propagation of male sterile lines (Jones and Clarke, 1943). The latter type was identified by Berninger (1965) and was genetically characterized by Schweisguth (1973) and is used to produce hybrid onion seed in Europe. The N cytoplasm depicts a non-sterile (i.e. fertile) nature of the cytoplasm as is found to occur in open-pollinated types (Havey, 1997). It is believed that additional CMS types may exist in onion though no sufficient evidence has been reported in literature.

Keeping in view the above, it was felt necessary to characterize the potentially useful varieties of white onion using molecular approaches. In the past decade or so, genetic variability studies in some Indian lines/varieties were included in previous studies but extensive work on Indian germplasm has not been done to the best of our knowledge. Hence an attempt has been made to undertake detailed studies on aspects involving RAPD patterns of both genomic and mitochondrial DNA, field evaluation, characterization of male sterility using mitochondrial RFLP analysis of some Indian and exotic cultivars suitable for processing.

MATERIALS AND METHODS

Onion Bulb Samples

Five fresh onion bulbs of each of the different parental lines, varieties and hybrids used in the present study were collected from the Research and Demonstration Farm of Jain Irrigation Systems Limited, Agripark, Jain Hills, Jalgaon, India. Two outer layers of the bulbs were peeled off, the core was removed carefully and the remaining part of the bulb was washed thoroughly in sterile distilled water and rinsed with 70% ethanol, air dried and used for DNA extractions (Genomic & Mitochondrial). A brief description of onion germplasm used in present study is given in Table 1.

Isolation of Genomic DNA

High molecular weight genomic DNA was extracted from onion using the Cetyl Trimethyl Ammonium Bromide (CTAB) method (Vorh Bi et al., 1996) with slight modifications. The pellet obtained was dissolved in TE buffer (pH 8.0) and quantified by subjecting the DNA to a 0.7% agarose gel electrophoresis stained with ethidium bromide, against a known standard DNA using the alpha imager software.

Isolation of Mitochondrial DNA

Mitochondrial DNA was isolated from 15 g onion bulb tissue using the method described by Nath et al. (1993) with minor modifications. It essentially consisted of obtaining the mitochondrial pellet through differential centrifugation followed by removal of nuclear DNA with the DNAse treatment. The mitochondrial pellet was then suspended in lysis buffer containing proteinase K and lysed by incubating it at 55°C for 60 min. Mitochondrial DNA was then extracted, purified and dissolved in 50 µl TE buffer and treated with RNase (20 µg/ml) at 37°C, 60 min. The resulting mitochondrial DNA was quantified using a nanodrop spectrophotometer and quality checked using a 0.8% agarose gel electrophoresis.

Primers

Some of the primers used for amplification were purchased from Operon Biotechnologies, GmbH, Nattermannallee, Cologne, Germany and the others were synthesized commercially using primers from published research articles. For RAPD of genomic DNA, 55 random primers were screened and for mitochondrial RAPD, 68 random primers and 16 SSR primers were screened.

Non-Radioactive Restriction Fragment Length Polymorphism (RFLP)

In this study, mitochondrial DNA from cytoplasmic male-sterile (CMS) hybrids, parental lines and some Indian and exotic varieties, were hybridized with five probes of mitochondrial genes: *cox1*, *cox2*, *atp6*, *atp9* and *nad6*. Non-radioactive RFLP analysis was adapted from Fermentas Life Sciences (Lithuania, EU) manufacturer's protocol. Restriction of genomic DNA was performed according to manufacturer's specifications and blotting onto nylon membrane was done according to Sambrook et al. (1989). Probes were labeled and detected using Biotin DecaLabel™ DNA labeling kit from Fermentas Life Sciences (Lithuania, EU). Twenty µg mitochondrial DNA was used for the restriction digestion and subsequent steps for experiment were followed as per manufacturer's instruction.

RAPD-PCR Using Genomic DNA

Hot-start PCR for the amplification of DNA was carried out with 25 µl volume of reaction consisting of 100 ng template DNA, 0.5 unit *Taq* DNA polymerase (recombinant, from Fermentas Life Sciences) 10 mM dNTP, 1x *Taq* DNA Polymerase buffer with KCl, 2.5 mM $MgCl_2$ and 20 ρmole decamer primer. Amplifications were done employing the following conditions: Hot-start at 90.5°C for 2 min; 40 cycles at 90.8°C for 30 s, 41°C for 1 min and 72°C for 2 min, with a final extension at 72°C for 8 min. The PCR products were subjected to 1.8% agarose gel electrophoresis. The DNA was stained by adding ethidium bromide directly into the gel mixture, for better visualization. The DNA bands were photographed under ultra violet light using the gel doc system.

RAPD-PCR Using Mitochondrial DNA

The procedure followed was similar to that of genomic DNA with modifications in the polymerase chain reaction conditions. These include hot-start at 93°C for 2 min; 35 cycles at 93°C for 30 s, 42°C for 1 min and 72°C for 2 min, with a final extension at 72°C for 8 min.

PCR Markers for Male Sterility

DNA extracted for RFLP studies was also used for this purpose and PCR was performed as described by Engelke et al. (2003).

Data Processing

RAPD patterns were used for 0, 1 scoring based on absence or presence of band, in each lane. Scored data was used for analysis using Windowstat software for D square matrix and dendogram.

RESULTS AND DISCUSSION

RAPD Analysis of Genomic DNA

As illustrated in Figure 1, 355 scorable polymorphic bands were obtained out of a total of 445 bands produced from 31 primers. The number of bands amplified per primer ranged from 10-20, with an average of 14.8 bands per primer and 11.4 polymorphic bands per primer.

Dendrogram produced from the scored bands (Fig. 2) shows two major clusters: In cluster I, which is completely different from other clusters, cultivars JV-7, JV16A, ARS-1, JV-12 and ARL-2 are accommodated; all of these are of exotic origin and have high TSS. Further ARS-1 and ARL-2 (both of which are from Argentina) within this cluster appear closer to each other. In cluster II, cultivars Arka Pitambar, Phule Safed, Arka Kirtiman and Agrifound White and others were accommodated; all of these are of Indian origin and having low TSS. The cluster II is sub divided in three sub groups in which Punjab White is alone in a separate group. One sub group of cluster II included Pusa White Round, Arka Pitambar, Phule Safed and Arka kirtiman while other sub group included Pusa White Flat, UD 102, Gujarat Local and Agrifound White.

Euclidean distance matrix (Table 2) showed higher distance among the exotic cutltivars, JV-7, JV-12, JV-16A, ARS-1 and ARL-2 with distance coefficient varying from 8-9.75. It may be due to the fact that different exotic cultivars are from different countries/continents. Among Indian cultivars lower distance is observed indicating their close proximity to each other in terms of their genome similarities.

RAPD Analysis of Mitochondrial DNA

A total of 68 random decamer and 16 SSR primers were used of which 39 random and 11 SSR primers produced a total of 531 polymorphic bands with an average of 10.62 bands per primer (Fig. 3). Variance analysis and dendogram generated from zero/one scoring revealed two distinct groups, one included cultivars ARS-1, ARL-2, Pusa White, Pusa White Round, Gujarat Local and JV-7 while other group included remaining 10 cultivars included in the experiment (Fig. 4). Within the first group three sub groups each having two varities; ARS-1 and ARL-2, Pusa White and Pusa White Round, Gujarat Local, and JV-7, were found with equal distance. Cultivars of the second group were grouped in two major sub groups. Significant distance among sub groups was also observed e.g. Agrifound and Phule Safed were close to each other but they were significatly away from JV-12 and Arka Pitambar. Agrifound White, Pusa White Flat and Arka Kirtiman were grouped together with mnimum genetic distance. Among all cultivars Gujarat Local was found most distant with JV-12 and JISL-5 having distance matrix value 290 and 288 respectively (Table 3).

The most closely related cultivars were Agrifound and Phule Safed with a distance of 91, followed by JV-12 and Arka Pitamber with a distance of 102. Both Agrifound and Phule Safed have been developed in the same geographical area of India and one expects that they may have considerable similarities.

Grouping based on Genomic RAPD revealed two major groups exotic and Indian or high TSS and low TSS, however in Mitochondrial RAPD grouping these cultivars were mixed together and the grouping was not related to TSS or Origin. It is tempting to suggest on the basis of this limited data that cytoplasm or mitochondrial genome may not have much role in controlling TSS. TSS is a multi gene character and one expects its regulation/control by the nuclear genome.

Characterisation of Male Sterility in Onion Germplasm

1. Field Evaluation of Sterility. All mentioned germplasm bulbs were planted in Rabi (October), 2004 and flowered in January, 2005 at Jain R&D Farm, Jalgaon, India. Umbels of these cultivars were selfed physically to test male sterility. Acetocarmine test for assessing male sterility was also performed. Results of these field screening revealed that JV-7 and Indam were male sterile while JISL-5 had both sterile as well as fertile plants in

3:7 ratio. Other than these three cultivars all others (Agrifound White, Phule Safed, Punjab White, UD-106, Arka Pitambar, Arka Kirtiman, Pusa White Flat, Pusa White Round, V-16, V-12, ARS-1, ARL-2 and Gujarat Local) were found male fertile.

2. PCR Markers for Male Sterility. The PCR- marker, which anchors in the upstream region to the mitochondrial gene *cob*, is referred to as 5' *cob*-marker (Sato, 1998). These markers are able to distinguish between S and N cytoplasm. Cytoplasm T, which was not studied by Sato (1998) shows the same amplified product as N cytoplasm. Therefore, Engelke et al. (2003) developed a new *orf*A501-marker that amplifies in S and T cytoplasm only, but not in N cytoplasm. The combination of 5' *cob*-marker with *orf*A501-marker allowed distinguishing between all three cytoplasm types in individual plants. 5'-cob primer with 414 bp band indicates S cytoplasm while 180 bp band indicate N or T cytoplasm. Orf 501primer with 473 bp band indicates S or T cytoplasm. A representative pattern for 10 of the 16 lines is shown in Figure 5. This experiment revealed that Arka Kirtiman, Arka Pitambar, JV-7 and Indam have S type cytoplasm and JISL-5 has T cytoplasm (Table 4).

RFLP Analysis of Onion Mitochondrial DNA

The limited RFLP data (Table 5) shows some indications. Of the 12 lines under this study JV-7 and Indam are male sterile under field condition while JISL-5 shows segregation for this character. Apparently the JISL-5 selection is still not homogeneous. JV-7 is characterized by a unique 4 kb band for the *cox1*/*Xba*I combination that is present in only JISL-5. However JISL-5 has an additional band of 5 kb size which is present in all the other lines that have shown bands for this combination. All others have two bands of 3 and 5 kb size. We are not certain if the presence of two bands is due to a restriction site of *Xba*I within the *cox1* gene in the mitochondria of fertile cytoplasms or if there are two genes of *cox1* in fertile lines. It appears that *cox1* gene in a 4 kb band associated with JV-7 may have sequence differences from the *cox1* gene of fertile lines. This aspect is being looked into.

JV-7 is the only one that has two bands of 10 and 4 kb sizes for the *atp9/Hind*III combination while all those that show bands for this combination possess two bands of 3 and 6 kb sizes except those having no band. JISL-5, Gujarat Local, ARL-2 and Indam did not show any band for this combination. However *cox2/Eco*RV combination produced two bands of identical sizes, 8 and 6 kb, in both the sterile cytoplasms of JV-7 and Indam. All others produced 6 and 4 kb sized bands except few having no band (Table 2).

Interestingly, whenever the bands have been produced in fertile cytoplasms they have been identical irrespective of the gene/restriction enzyme combination. Thus this limited RFLP data correlates well with the field data on male sterile and fertile lines. Extension of these studies will enable us to show what kinds of sequence differences exist in some of the mitochondrial genes between the sterile and fertile cytoplasms. Newly developed markers like SNPs (McCullam et al., 2008) have also proved useful in studying genetic diversity analysis. Our group is currently implementing the use of this new type of molecular markers.

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Tables

Table 1. Origin and type of cultivars used in the present study.

Sr. no	Cultivars	Origin	Day length	Type of cultivar
1	JV-7	North America	Short day	Hybrid
2	JV-12	North America	Short day	Open pollinated
3	JV-16A	North America	Short day	A line
4	Agrifound White	India	Short day	Open pollinated
5	Pusa White Round	India	Short day	Open pollinated
6	Punjab White	India	Short day	Open pollinated
7	Phule Safed	India	Short day	Open pollinated
8	Arka Kirtiman	India	Short day	Open pollinated
9	Arka Pitambar	India	Short day	Open pollinated
10	Pusa White Flat	India	Short day	Open pollinated
11	Gujarat Local	India	Short day	Open pollinated
12	UD-102	India	Short day	Open pollinated
13	ARS-1	Argentina	Short day	Open pollinated
14	ARL-2	Argentina	Long day	Open pollinated
15	Indam	India	Short day	Hybrid

Table 2. Genomic DNA RAPD based Euclidean Distance matrix of onion germplasm.

Sr. no	Cultivar	1	2	3	4	5	6	7	8	9	10	11	12	13	14	**Average D2**
1	JV-7	0.00	9.75	8.00	8.31	8.25	8.83	9.11	9.17	8.37	8.60	8.60	8.06	8.72	8.19	8.61
2	JV-12		0.00	9.54	9.27	9.33	9.22	9.38	9.00	9.00	9.00	9.85	9.27	9.64	9.06	9.33
3	JV-16A			0.00	7.55	7.75	8.72	7.94	7.87	7.87	7.87	8.12	7.42	8.49	8.43	8.12
4	Agrifound White				0.00	6.86	7.81	7.62	7.55	7.28	6.71	7.28	6.48	8.19	8.00	7.61
5	Pusa White Round					0.00	7.35	7.42	6.78	6.63	6.78	7.62	6.56	7.87	8.06	7.48
6	Punjab White						0.00	8.31	7.75	8.00	7.75	8.25	7.68	8.60	8.43	8.21
7	Phule Safed							0.00	6.08	6.40	7.00	7.14	7.35	8.66	8.60	7.77
8	Arka Kirtiman								0.00	6.63	6.16	7.21	7.00	8.60	7.94	7.52
9	Arka Pitambar									0.00	6.16	7.48	6.86	8.60	8.43	7.52
10	Pusa White Flat										0.00	6.78	6.08	8.49	8.19	7.35
11	Gujarat Local											0.00	6.08	8.49	7.68	7.74
12	UD-102												0.00	7.94	7.75	7.27
13	ARS-1													0.00	7.55	8.45
14	ARL-2														0.00	8.18

Table 3. Mitochondrial DNA RAPD and SSR based Euclidean Distance matrix of onion germplasm.

Sr. no	Cultivar	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	**Average D2**
1	ARS-1	0	128	205	200	246	272	218	186	242	237	192	158	168	218	249	240	210.60
2	ARL-2		0	183	184	226	242	180	162	218	225	174	146	158	208	225	224	192.20
3	Agrifound			0	91	177	185	153	179	149	152	181	233	209	257	246	171	184.73
4	Phule Safed				0	160	176	158	164	146	161	180	222	192	254	249	168	180.33
5	UD-102					0	194	194	226	182	191	220	262	218	264	261	210	215.40
6	JISL-5						0	176	208	174	173	238	268	240	288	267	212	220.87
7	Arka Kirtiman							0	154	148	139	178	226	200	256	243	184	187.13
8	Pusa White Flat								0	166	187	176	198	198	252	243	210	193.93
9	JV-12									0	103	198	258	222	290	269	178	196.20
10	Arka Pitambar										0	191	257	219	271	258	185	196.20
11	Agri Founf White											0	172	168	208	217	206	193.27
12	Pusa White												0	122	194	237	252	213.27
13	Pusa White Round													0	200	229	212	197.00
14	Gujarat Local														0	147	294	240.07
15	JV-7															0	259	139.93
16	JV-16T																0	213.67

Table 4. Results of PCR based cytoplasm detection on onion germplasm.

Sr. no.	Cultivar	Male fertile (F)/ sterile (S)	Cytoplasm	Sr. no.	Cultivar	Male fertile (F)/ sterile (S)	Cytoplasm
1	Agrifound White	F	N	9	JV-7	S	S
2	Phule Safed	F	N	10	JV-16T	F	N
3	Punjab White	F	N	11	JV-12	F	N
4	Udaipur-102	F	N	12	ARS-1	F	N
5	Arka Kirtiman	F	S	13	ARL-2	F	N
6	Arka Pitambar	F	S	14	Gujarat Local	F	N
7	Pusa White Flat	F	N	15	JISL-5	7:3 F:S	T
8	Pusa White Round	F	N	16	Indam	S	S

Table 5. Approximate restriction fragment sizes of different mitochondrial gene probe-enzyme combinations in different cultivars.

Sr. no.	Genotype	Band Size kb (approx)				
		*cox1/Xba*I	*atp-9/Hind*III	*cox2/Eco*RV	*nad6/Eco*RI	*atp6/Hind*III
1	JV-7	4	4 & 10	6 & 8	No band	8
2	JV-12	3 & 5	3 & 6	4 & 6	12 & 15	5
3	JISL-5	4 & 5	No band	No band	No band	10
4	Gujarat Local	3 & 5	No band	4 & 6	6 & 10	No band
5	Pusa White Flat	3 & 5	3 & 6	4 & 6	6 & 10	5 & 8
6	Agrifound	3 & 5	3 & 6	4 & 6	No band	No band
7	UD-102	3 & 5	No band	4 & 6	6 & 10	10
8	Phule Safed	3 & 5	No band	No band	No band	No band
9	ARL-2	No Band	No band	4 & 6	No band	No band
10	Indam	No Band	No band	6 & 8	12 & 15	8
11	V-16A	3&5	3 & 6	No band	No band	5
12	Cascade	No Band	3 & 6	No band	No band	8

Figures

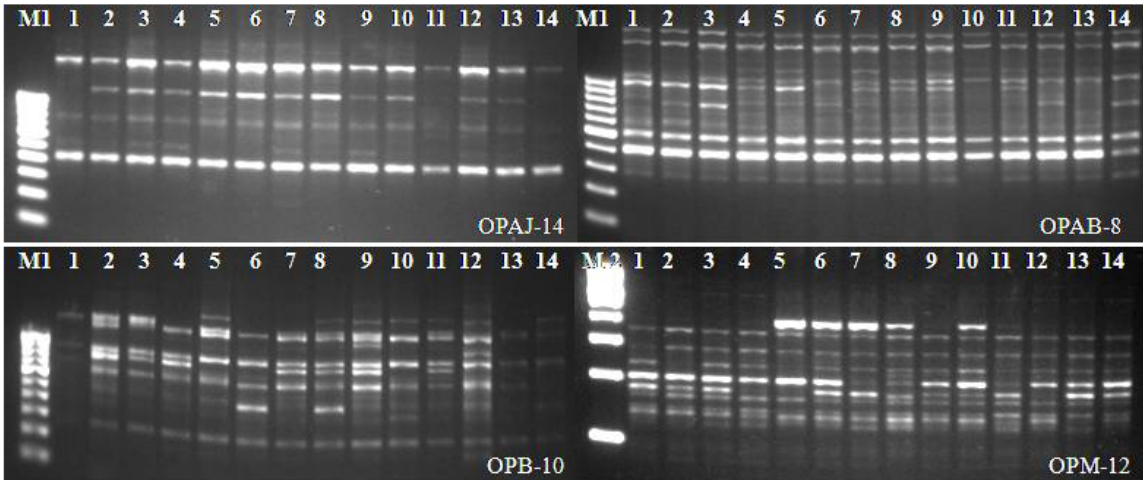

Fig. 1. RAPD patterns of genomic DNA from different onion cultivars. Lane 1-JV7, 2-JV12, 3-JV16, 4-Phule Safed, 5-Arka Kirtiman, 6-Punjab White, 7-Agrifound White, 8-Udaipur 102, 9-Arka Pitambar, 10-Pusa White Flat, 11-Pusa White Round, 12-Gujarat Local, 13-ARS-1, and 14-ARL-2. M1=500 and M2=100 bp DNA ladders.

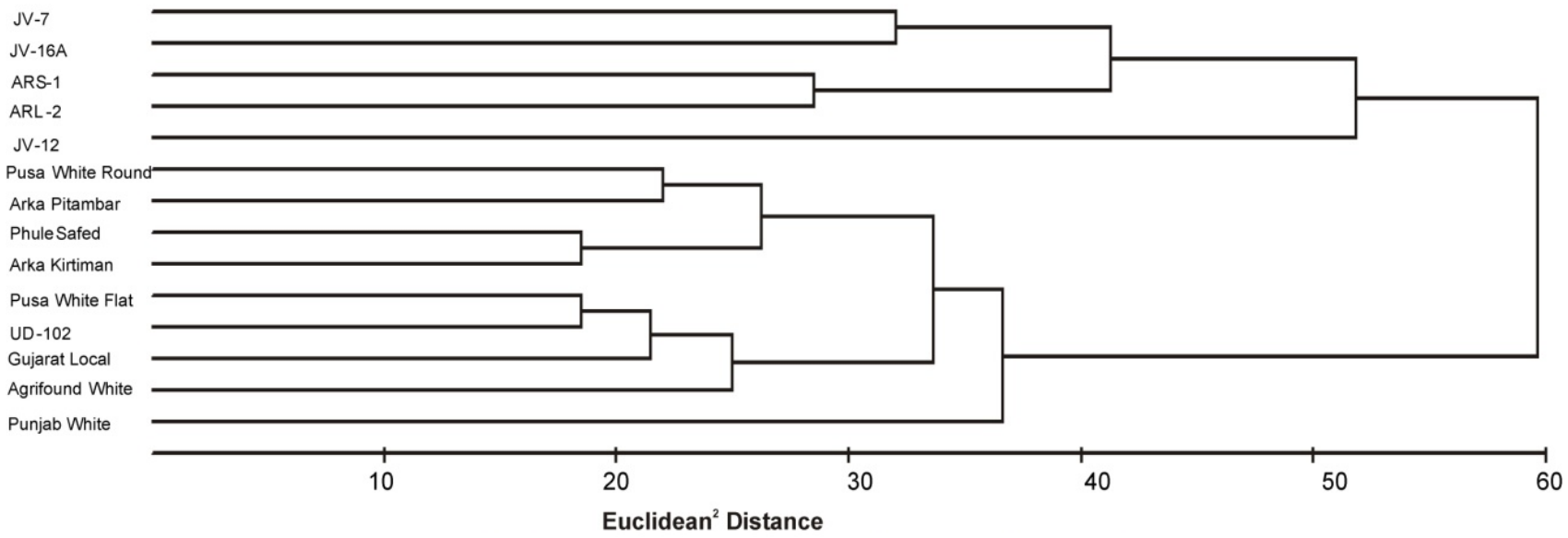

Fig. 2. Dendrogram of 14 onion cultivars derived from genomic DNA RAPD data.

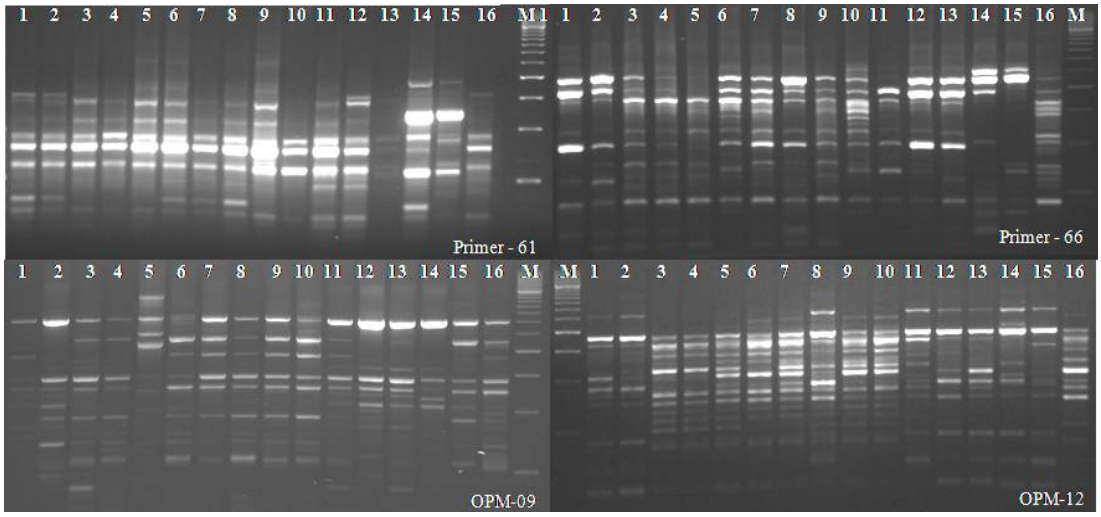

Fig. 3. RAPD patterns of mitochondrial DNA from different onion cultivars. Lane 1-ARS-1, 2-ARL-2, 3-Agrifound, 4-Phule Safed, 5-UD-102, 6-JISL-5, 7-Arka Kirtiman, 8-Pusa White Flat, 9-JV-12, 10-Arka Pitambar, 11-Agrifound White, 12-Pusa White Round, 13-Pusa White Round, 14- Gujarat Local, 15-JV-7, 16-JV16T, and M=500 bp marker.

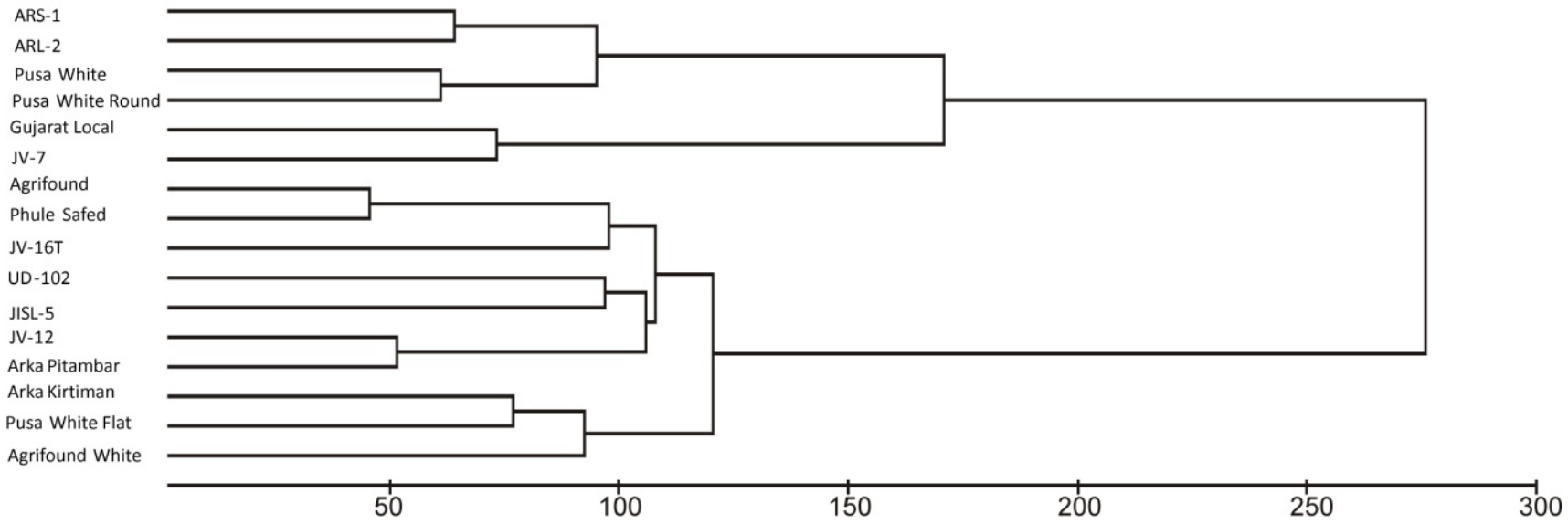

Fig. 4. Dendrogram of 16 onion cultivars derived from Mitochondrial DNA RAPD using random and SSR primers using Euclidean distance.

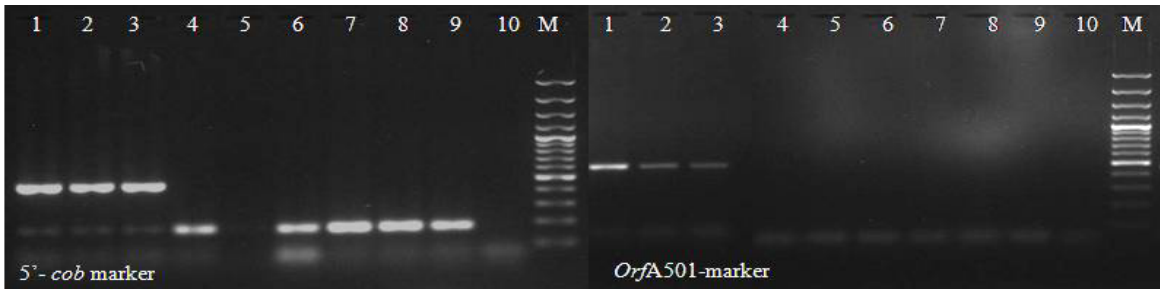

Fig. 5. PCR based analysis of onion cytoplasm. 1=JV-7 (S), 2=Indam (S), 3=Arka Pitambar (S), 4=JV-12 (N), 5=JV-16T (N), 6=ARS-1 (N), 7=ARL-2 (N), 8= Phule Safed (N), 9=Agrifound White (N), 10=Punjab White (N), and M=100 bp DNA ladder.

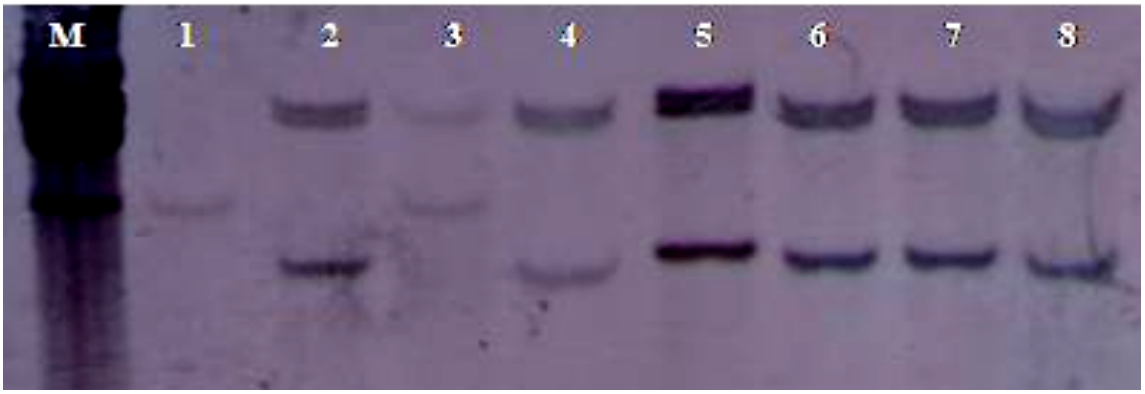

Fig. 6. RFLP patterns of *Xba*1 digested DNA probed with *cox1*. Lane: M=λDNA *Eco*R1 digested marker, 1=JV-7 (S), 2=JV-12 (N), 3=JISL-5 (T), 4=JV16T (N), 5=Arka Kirtiman (S), 6=Agrifound (N), 7=Arka Pitambar (S), and 8=UD-102 (N).