International Journal of Biodiversity and Conservation Vol. 2(12), pp. 405-413, December 2010
Available online http://www.academicjournals.org/ijbc
ISSN 2141-243X ©2010 Academic Journals

Full Length Research Paper

The use of ISSR and RAPD markers for detecting DNA polymorphism, genotype identification and genetic diversity among *Trichosanthes dioica* Roxb. cultivars

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Accepted 29 July, 2010

Trichosanthes dioica **Roxb. belongs to family Cucurbitaceae and commonly known as pointed gourd. It is perennial, dioecious and cross pollinated vegetable crop, widely cultivated in eastern Uttar Pradesh of India. 22 cultivars of male and female of *T. dioica* from various agro-climatic regions of India have been fingerprinted by RAPD and ISSR markers utilizing 37, 15 primers respectively. To understand genetic relationships among these cultivars, Jaccard's similarity coefficient and UPGMA clustering algorithm were applied to the two marker data sets. The percentage of polymorphism range for RAPD is from 89 to 45% while for ISSR is from 88 to 100%, the UPGMA dendogram obtained from the cluster analysis of RAPD and ISSR data gave similar clustering pattern, with Jaccards similarity coefficient ranging from 0.23 to 0.93. This study showed that RAPD and ISSR markers could provide a practical and efficient tool in quality control of the *T. dioica*. The present report is, therefore, a step to protect the plant breeders rights by making use of reliable and modern DNA technologies.**

Key words: *Trichosanthes dioica* Roxb., RAPD, ISSR, genetic diversity.

INTRODUCTION

Trichosanthes dioica Roxb. commonly known as pointed gourd, is the member of order cucurbitales; family cucurbitaceae. It is perennial, dioecious and cross pollinated vegetable found in wild form throughout the north India. It is known by different names such as Parwar, Padval, Patol, Parwal, Potala and Kommupotla etc. *T. dioica* is one of the most important vegetable crops of eastern Uttar Pradesh, Bihar and west Bengal in India from where it is transported to distant markets (Singh, 1989). The total cultivated area under pointed gourd in India is approximately 15,000 ha (Yadav, 1989).

Pointed gourd is not only consumed as a fresh vegetable but it is also possesses proven medicinal value in diuretic, febrifuge and laxative, lowering total cholesterol, lowering blood sugar, immunomodulatory, antitumor and antihuman immunodeficiency (Chandra-Sekar et al., 1988, Sharma and pant, 1988; Sharma et al., 1988). The fruits are reported to have some protected in the control of cancer like conditions. Parwal leaves with bark of neem are used for treatment of leprosy. The germplasm resources of pointed gourd have so far been characterized entirely on the basis of morphological traits, biochemical characters, agronomic characters, and pathogenecity (Sardar, 1994; Basu 1999; Singh, 1999; Mahapatra, 1999). The improvement of crop plants has been one of the important goals of plant breeders and geneticists of yesteryears, and molecular geneticists and molecular breeders in the recent years. These approaches have, however, not been able to accomplish the desired goals. The precise cataloguing of germplasm resources including cultivars by molecular DNA markers has lately gained lot of attention (Birmeta et al., 2002; Al-Khalifah et al., 2003; Guena et al., 2003) for molecular breeding. DNA molecular marker technology, which are based on sequence variation of specific genomic regions, provide powerful tools for cultivar identification and genetic diversity in various crops with the advantages of time-saving, less labor-consumption and more efficiency (Hu and Quiros, 1991; Mongkolporn et al., 2004; Dongre and Parkhi, 2005; Liu et al., 2007; Garg et al., 2006). Unlike RFLP and AFLP, RAPD, ISSR and SSR, which are

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based on polymerase chain reactions (PCR), are technically simple, do not require radioactive materials and have good throughput with relatively low cost. Hence, these marker systems could be effectively used for seed genetic diversity and cultivar identification, PCR-based DNA markers evolve rapidly enough to be variable within a population and thus they are suited for detecting genotypic diversity (Esselman et al., 1999). Few studies like; hypoglycemic effect, antioxidant potential, glycemic properties, pharmacognostic study, wound healing potential and molecular characterization carried out in *T. dioica* Roxb. (Adoga et al., 2010; Shivhare et al., 2010, Prashant et al., 2008; Singh et al., 2010; Shivhare et al., 2009; Goswami et al., 2009).

MATERIALS AND METHODS

Plant material

Leaves materials of *T. dioica* were collected from various locations in India; Narendra dev Agriculture University faizabad, Rajendra Agriculture University Bihar and National Botanical Research Institute (NBRI) Lucknow (Table 1). Young leaf tissue was collected from each plants, washed with distilled water and dry by blotting sheets. The leaves were de-ribbed and powdered rapidly in liquid nitrogen and then either the DNA isolation procedures were immediately followed or the powder tissue was stored at -70°C till further use.

Since there was no protocol available for *T. dioica*, several protocols for DNA isolation were tried and good results were obtained with the protocol of Doyle and Doyle (1987) with some slight modification, pure and highly concentrated DNA was extracted. A fluorometer (Hoefer DyNA Quant^{200} pharmacia Biotech, USA) was used to determine the quantity and agarose gel used to check quality of the DNA. The stock DNA samples were diluted with sterile TE buffer to make a working solution of 5 ng μl^{-1} for use in PCR analysis.

RAPD amplification

Total 80 decamer random primers of 20 each kit OP-AB, OP-A, OP-C and OP-E from Operon technologies (Alameda, Calif.) were used for amplification of the DNA (Table 2). A standard 20 µl reaction contained 50 ng template DNA, 1.5 U Taq DNA polymerase (Bangalore genei, India), 2 X PCR reaction buffer containing 1.5 mM $MgCl_2$, 10 picomoles primer and 100 µmoles of each dNTPs (Amersham Pharmacia Biotech). DNA amplification was performed in Perkin Elmer DNA thermal cycler 9700 according to Williams et al. (1990). The following thermal cycling protocol was used: (1) cycle for 2 min at 94°C (2) 44 cycles of 94°C for 1 min, 36°C for 1.30 min and 72°C for 1.30 min (3) One cycle for 5 min at 72°C, followed by a soaking at 4°C. The RAPD products were separated by electrophoresis according to their molecular weight on 1.4% (w/w) agarose gels submerged in 0.5 X TBE buffer and then stained with ethidium bromide (100 µg ml^{-1}) solution 10 min. The DNAs were visualized on a UV-transilluminator and documented by using the gel documentation system of AlphaImager (System and Control, India). The kilo base DNA marker (Banlalore genei, India) loaded in the gel as standard size marker.

ISSR amplification

60 randomly selected ISSR primers from UBC kit (GCC, India), were used for PCR amplification with male and female cultivars of *T. dioica* genomes (Table 3). The 25 µl reaction volume contained 2.5 µl 10 × assay buffer (Banglore Genei), 2.5 mM $Mgcl_2$ (Promega), 0.24 mM dNTP(Amersham Pharmacia Biotech), 5 µM primer , 50 ng genomic DNA and 1 unit of Taq DNA polymerase(Banglore Genei). Amplification was performed in Perkin Elmer DNA thermal cycler 9700 for 35 cycles. Each cycle, consisted of the following steps: 4 min denaturation at 94°C, then 35 cycles of 94°C for 1 min denaturation, 1.30 min at the annealing temperature specified for each primer and 1 min at 72°C for the extension and a final extension cycle for 5 min at 72°C. The amplification products were separated by standard horizontal electrophoresis in 1.4% (w/w) agarose gels and stained with ethidium bromide. The reproducibility of DNA profiles were tested by repeating the PCR amplifications twice with each primers analyzed. The robust bands were found to be repeatable, and were the products considered in the study.

Data analysis

Amplification product were scored as (1) for its presence or (0) for its absence. A fragment was considered polymorphic if both the presence and absence of that fragment were observed in the same species and monomorphic, it was present among all individual within a species. To reduce the possibility of comparing non-homologous bands, a positive control (an individual possessing the band to be scored) was included on each agarose. On the basis of data a binary matrices were assembled for the two markers. The binary matrices were subjected to statistical analyses using NTSY-S-pc version 2.02 (Rohlf, 2000). Jaccard's similarity coefficient was employed to compute pair-wise genetic similarity. The similarity matrices were constructed for each markers type. The corresponding dendograms were constructed by applying un-weighted pair group method with arithmetic average (UPGMA).

The polymorphic information content (PIC) was calculated by applying the formula given by Powell et al. (1996) and Smith et al. (1997):

$$PIC = 1 - \sum_{i=1}^{n} f_i^2$$

where f i is the frequency of the i^{th} alleles and the summation extends over n alleles.

RESULT

Of the 80 primers used for the initial screening for polymorphism with 22 *T. dioica* male and female cultivars, 33 primers gave no amplification at all while 37 primers amplified polymorphic products and 10 primers generated non scorable band pattern (Figure 1A). These 37 primers were then used for RAPD analysis of vegetative propagated plants of *T. dioica*. Amplification of 22 cultivars with these primers yielded a total of 200 scorable bands, of which 150 were polymorphic. The highest number of bands (19) was obtained with primer OPAB-7 and OPAB-10 while the lowest number, 5 were obtained with primer OPE-10. An average of 6 bands was obtained per primer and the amplification products ranged in size from 200 to 3500 bp. The polymorphism obtained in the 22 cultivars of *T. dioica* with the 37 informative primers

Table 1. The accessions of the *T. dioica* from which the leaf tissue was collected for the present studies.

S.no	Sample name	Variety Name	Accession No.	Tissue Collected From
1.	MI	Male I	TD001	NBRI, Lucknow
2.	MII	Male II	TD002	NBRI, Lucknow
3.	MIII	Male III	TD003	NDUV, Faizabad
4.	MIV	Male IV	TD004	NDUV, Faizabad
5.	NP602	Narendra Parval 602	TD005	NDUV, Faizabad
6.	NP305	Narendra Parval 305	TD006	NDUV, Faizabad
7.	NP3	Narendra Parval 3	TD007	NDUV, Faizabad
8.	NP801	Narendra Parval 801	TD008	NDUV, Faizabad
9.	NP751	Narendra Parval 751	TD009	NDUV, Faizabad
10.	NP260	Narendra Parval 260	TD010	NDUV, Faizabad
11.	NP310	Narendra Parval 310	TD011	NBRI, Lucknow
12.	NP504	Narendra Parval 504	TD012	NDUV, Faizabad
13.	NP605	Narendra Parval 605	TD013	NBRI, Lucknow
14.	NP601	Narendra Parval 601	TD014	NDUV, Faizabad
15.	PG01	IIVR PG01	TD015	NDUV, Faizabad
16.	RPI	Rajendra Parval I	TD016	RAUV, Bihar
17.	RPII	Rajendra Parval II	TD017	RAUV, Bihar
18.	NP604	Narendra Parval 604	TD018	NDUV, Faizabad
19.	NP311	Narendra Parval 311	TD019	NBRI, Lucknow
20.	NP701	Narendra Parval 701	TD020	NDUV, Faizabad
21.	NP307	Narendra Parval 307	TD021	NDUV, Faizabad
22.	NP207	Narendra Parval 207	TD022	NDUV, Faizabad

NDUV, Faizabad: Narendra dev university faizabad, India. NBRI, Lucknow: National Botanical Research Institute, Lucknow, India. RAUV, Bihar: Rajendra Agriculture University Bihar, India.

Table 2. Different series of Operon primers used for RAPD analysis of *T. dioica* the primers respond different genomic DNA and generate variable number and size of amplicons.

Primer No.	Total No. of Amplicon	Total No. of bands	Poly-morphic bands	Mono-morphic bands	PIC value	Average	Average no of bands	Size range of amplified product (bp)
AB01	129	16	15	1	0.224	0.08 - 0.49	5.8	500 - 2200
AB03	217	15	11	4	0.177	0 - 0.49	9.8	400 - 2200
AB04	122	12	9	3	0.16	0 - 0.49	5.5	400 - 2100
AB05	89	5	3	2	0.054	0 - 0.5	4.04	400 - 2200
AB06	144	9	8	1	0.141	0 - 0.49	6.5	700 - 1904
AB09	172	12	10	2	0.146	0 - 0.49	7.8	500 - 2100
AB11	187	14	10	4	0.156	0 - 0.49	8.5	400 - 2200
AB14	198	15	12	3	0.174	0 - 0.48	9	200- 2300
AB16	236	16	15	1	0.242	0 - 0.49	10.7	400 - 2000
AB17	116	10	8	2	0.139	0 - 0.48	5.2	600 - 2500
AB18	131	14	12	2	0.160	0 - 0.48	5.9	550 - 2100
A1	210	21	15	16	0.15	0 - 0.51	9.5	300 - 2100
A2	104	12	12	0	0.183	0 - 0.5	4.7	400 - 1900
A3	91	9	6	3	0.076	0 - 0.48	4.1	600 - 1500
A5	219	8	7	1	0.2	0 - 0.15	9.9	125 - 2500
A7	64	5	3	2	0.046	0 - 0.51	2.9	200 - 2300
A9	196	14	10	4	0.131	0 - 0.28	8.9	200- 2100
A10	155	6	3	3	0.11	0 - 0.15	7.0	300 - 1600
A11	207	15	11	4	0.094	0 - 0.45	9.4	300 - 1600

Table 2. Contd.

A12	119	9	7	2	0.092	0 - 0.46	5.4	400 - 1900
A13	155	10	8	3	0.122	0 - 0.48	7.0	300 - 2100
A15	109	9	7	2	0.095	0 - 0.49	4.9	200 - 2500
A16	133	9	4	5	0.08	0 - 0.39	6.0	200 - 2100
A19	149	9	6	3	0.11	0 - 0.15	6.7	400 - 3000
C10	144	12	9	3	0.16	0 - 0.48	6.5	270 - 2000
C11	148	12	9	3	0.14	0 - 0.50	6.7	300 - 2100
C12	187	14	10	4	0.146	0 - 0.48	8.5	500 - 2300
C13	119	12	12	0	0.41	0-0.46	5.4	200 - 2000
C14	114	9	8	1	0.21	0 - 0.32	5.18	210 - 1900
C15	90	7	6	1	0.083	0 - 0.48	4.09	600 - 2000
E1	100	17	14	3	0.237	0 - 0.48	4.5	380 - 2000
E2	156	16	16	0	0.255	0.16 - 0.49	7.09	700 - 3000
E3	194	14	11	3	0.178	0 - 0.43	8.8	200 - 2000
E4	234	16	10	6	0.09	0 - 0.48	10.6	200 - 1400
E5	161	11	7	4	0.26	0 - 0.48	7.3	250 - 1100
E6	260	20	15	5	0.11	0 - 0.48	11.8	150 - 1500
E8	173	15	13	2	0.20	0 - 0.48	7.8	150 - 1375
Total		449	352	97				

Table 3. Amplified fragments of 22 cultivars of male and female *T. dioica* using ISSR analysis

S/No.	Primer	Sequence	Annealing Temp (℃)	Total no. of band	Total no. of poly-morphic band	Percentage polymorphism (%)	Range of fragment size (bp)
1.	834	$(AGA)_3$ $(GAG)_2$ GYT	48℃	9	8	88%	1600-300bp
2.	835	$(AGA)_3$ $(GAG)_2$GYC	49℃	10	9	90%	2000-200bp
3.	838	$(TAT)_3$ $(ATA)_2$ ARC	50℃	9	8	88.8%	1900-200bp
4.	840	$(GAG)_3$ $(AGA)_2$ AYT	52℃	11	10	90%	1584-200bp
5.	846	$(CAC)_3$ $(ACA)_2$ ART	48℃	11	9	81.8%	1700-1400bp
6.	855	$(ACA)_3$ $(CAC)_2$ CYT	51℃	13	11	84.6%	2700-200bp
7.	856	$(ACA)_3$ $(CAC)_2$ CYA	52℃	15	15	100%	2027-300bp
8.	857	$(ACA)_3$ $(CAC)_2$ CYG	52℃	9	6	66.6%	2000-400bp
9.	861	$(ACC)_6$	55℃	14	12	85.7%	2100-400bp
10.	862	$(AGC)_6$	52℃	8	7	87.5%	2500-300bp
11.	864	$(ATG)_6$	52℃	16	16	100%	2800-200bp
12.	865	$(CCG)_6$	52℃	14	13	92.8%	1800-300bp
14.	868	$(GAA)_6$	50℃	9	8	88.8%	1900-200bp
15.	872	$(GAT)_2$ AGATAGATAA	52℃	12	10	85.7%	2100-400bp

showed a distinct variation. The polymorphic information content (PIC) ranged from 0.05 - 0.25 with average of 0.17. Genetic similarity measured through analysis of RAPD data of 22 cultivars belonging to *T. dioica* species revealed varying degree of genetic relatedness among cultivars. Highest similarity (0.85%) was measured between cultivars MII and MIV and least genetic similarity (0.16%) was assessed between NP601 and MI. Dendogram showing distinguishing relationship and grouped into three major clusters. Cluster I consist of accession of NP601. Cluster II consist of 12 accessions and cluster III consist of 9 accessions. Total 60 UBC ISSR primers (University of British Coloumbia biotechnology laboratory, Canada) were screened with 22 male and female cultivars of *T. dioica* genomes. Total 160 amplified products were obtained with 15 informative

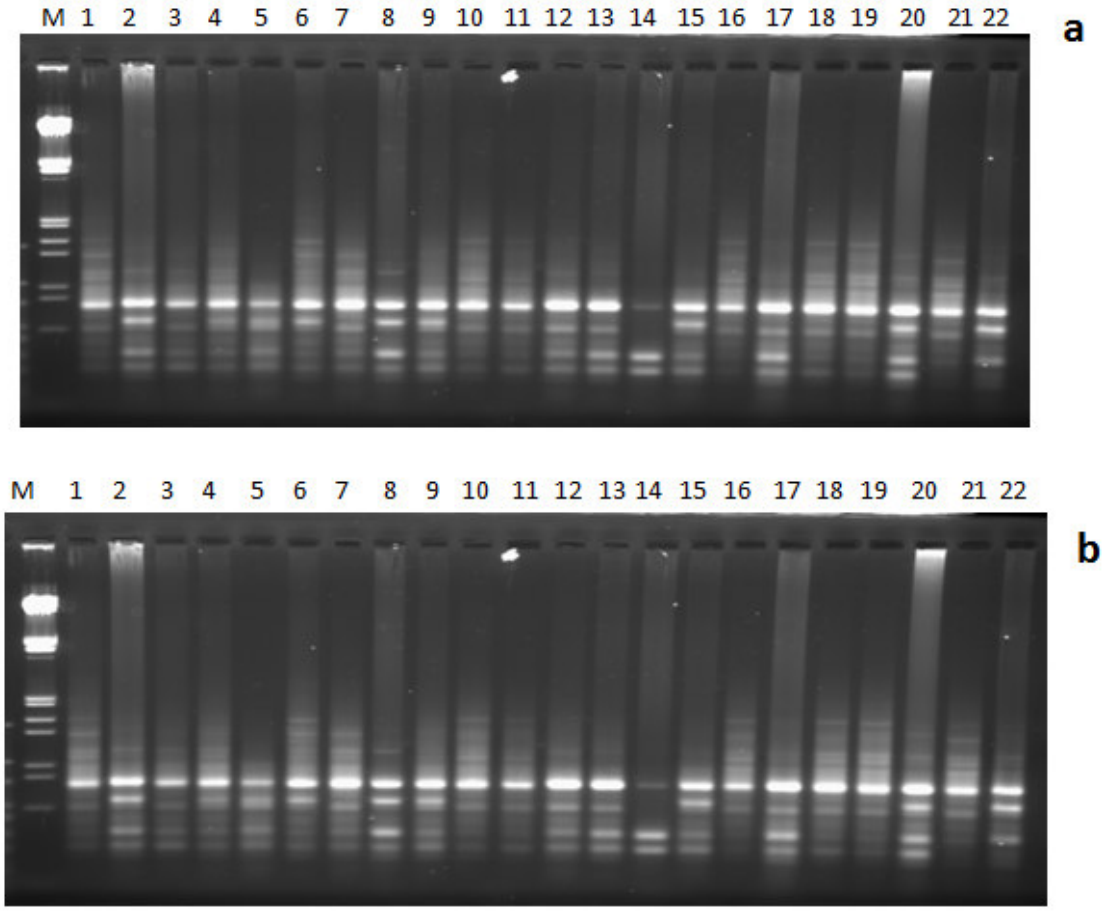

Figure 1. PCR amplification of total genomic DNA of *Trichosanthes dioica* Roxb. using are shown Lane M-EcoRI and Hind III digested λ DNA. Image A Lane 1-22 Different cultivars of *T. dioica* with RAPD Primer OPAB-3. Image B- 1-22 Different cultivars of *T. dioica* with UBC Primer 866.

microsatellite primers, out of which 142 (88%) were polymorphic in the size ranged from 100 bp with primer UBC840 to 2800 bp with primer UBC 864 (Figure 1B). The average number of 10.6 bands obtained per primer. The percentage of polymorphism ranged from 66.6% (ISSR primer UBC-885) to 100% (ISSR primer UBC-858) with an average polymorphism of 88.7% across all cultivars. 37 primers gave unreadable band pattern and 4 primers produced 3 and 4 banding with primer. Genetic similarity measured through analysis of ISSR data of 22 cultivars belonging to *T. dioica* species revealed varying degree of genetic relatedness among cultivars. Highest similarity (0.95%) was measured between cultivars NP602 and NP801 and least genetic similarity (0.12%) was assessed between NP601 and RP1. Multivariable analysis of the genetic similarity data div-ided *T. dioica* cultivars into 3 major clusters. Cluster one consist one cultivars NP601, Cluster II consist 4 Male *T. dioica* and cluster III includes 9 cultivars of female. The distance data were further analyzed by NJ method using the free tree program to describe the relative clustering of the cultivars. The RAPD and ISSR data were combined for UPGMA cluster analysis. 3 clusters were formed similar to ISSR cluster. The UPGMA Dendogram obtained from the cluster analysis of RAPD and ISSR data gave similar clustering pattern, with Jaccards similarity coefficient ranging from 0.23 to 0.93. Dendograms based on RAPD, ISSR as RAPD+ISSR combined discussed (Figure 2). The pattern of clustering of the cultivars remained more or less the same in ISSR and RAPD data, whereas the dendogram based on RAPD showed variation in the clustering of cultivars. The metrics for RAPD and ISSR markers are compared.

Twenty two cultivars of *T. dioica* were grouped in three clusters. ISSR also grouped all cultivars in three clusters, where as a Dendogram based on RAPD data, showed four clusters. In all the 3 dendogram male cultivars shows low value of correlation coefficient with female cultivars.

The dendograms obtained with the two marker systems are a proper representation of their respective similarity matrices. Significant correlation, however, was obtained between RAPD and ISSR markers. Of the total amplification products scored in the RAPD analysis 89% were polymorphic and shown high level of polym-orphisms.

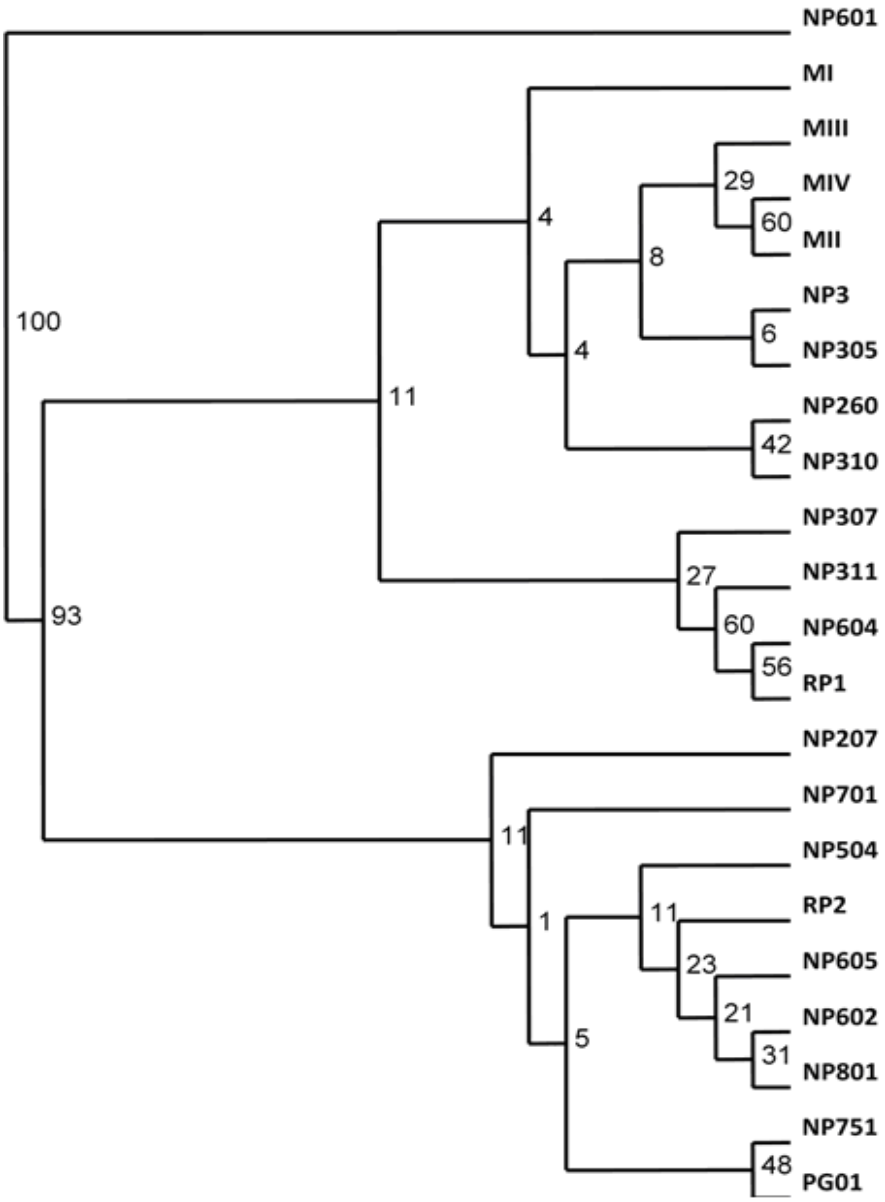

Figure 2. Cluster analysis of cumulative ISSR+ RAPD data for *T. dioica* cultivars. The phenogram was generated by the NJ method and a 500 replicate bootstrap analysis was used to assess the robustness of the tree. The scale represents the distance scale.

Cluster analysis of the RAPD data led to the clear distinction of male and female cultivars of *T. dioica*. The putative similar bands originating for RAPDs in different individuals are not necessarily homologous, although they may share the same size in base pair. This situation may lead to poor reliability of results when calculating genetic relationship (Fernandez et al., 2002).

Dendogram generated two major clusters; one cluster consisted 13 cultivars of male and female cultivars of *T. dioica* and divided into five sub clusters; sub cluster I comprised one male cultivar (MI), sub cluster II included four cultivars (RPI, NP311, NP701 and NP207), sub cluster III included one cultivar (MII), sub cluster IV consisted MIII, NP602, MIV, NP3 and NP801 and sub cluster V included NP310 and NP504. Cluster II divided into 3 sub clusters; sub cluster I included 5 cultivars (NP305, NP751, NP601, NP604 and NP605), sub cluster II comprised two cultivars NP260 and RPI and sub clusters III consisted one cultivar NP307. Cluster II included 8 cultivars and cluster III consisted one accession PG01. All these cultivars do not follow any location perhaps due to the cross pollination. The polymorphic information content (PIC) indicated large diversity among the cultivars with an average 0.17. MI and MII collected from

the same location but they were not grouped together. The distance of both cultivars was 0.6. Another male MIII and MIV collected from the same location but were also not grouped together. Dendogram showed there was no clear grouping between four male cultivars of *T. dioica* and 18 female *T. dioica* and existing large diversity among them.

DISCUSSION

RAPD data was not supporting the clear pattern between male and female cultivars of *T. dioica*. RPI and RPII both cultivars showed large distance but RPII was more close to NP311 and RPI with NP260. Highest similarity (0.91%) was measured between NP602 and PG01 while least (0.15%) between MIII and NP602. Dendogram was not grouped according to location where they were collected. Cultivars with the most distinct DNA profiles are likely to contain the greatest number of novel alleles. These cultivars were likely to uncover the largest number of unique and potentially agronomic useful alleles. Our results indicate the presence of large genetic variation among the cultivars. Different geographical conditions allow some possible genetic changes or DNA errors like translocation, deletion and point mutation etc. Sometimes out breeding population were able to change gene pool of the particular species. *T. dioica* is a vegetatively propagated species. Fruit and leaf size variation in this species show a wide range of variation and selection more characters simultaneously. Therefore, information on genotypic, phenotypic and environmental inter-relationships between various agronomic traits is of interest of the plant breeders.

Several doubts have been raised regarding the suitability of RAPD for diversity studies, the most important one being that co-migrating bands may not be allelic or composed of similar sequence. However, the homology of co-migrating RAPD bands has been demonstrated in some species of *Glycine* and *Allium* (Williams et al., 1990; Wilkie et al., 1993). In addition conformity of phylogenetic groupings based on RAPD data to those based on conventional approaches of morphology, cytology and isozyme analysis, itself indirect but are considered significant evidences in support of the allelism of co-migrating RAPD bands (Virk et al., 1995). Some such specific examples include studies in genome relationships of *Musa* and *Brassica* (Howell et al., 1994; Demeke et al., 1992) and between species of *Stylosanthes* (Kazan et al., 1993). The use of a large number of polymorphic markers would minimize the skewing of results due to non-allelism. Another problem often encountered in RAPD analysis is of reproducibility of band patterns between different PCR reactions. This aspect can be overcome by using a thoroughly optimized PCR protocol and by scoring only reproducible bands. The RAPD method has been employed in the past successfully for the detection of genetic relationship among individuals (Fernandez et al., 2002). Our studies have shown that RAPD is a robust and reliable method to detect genetic diversity and genetic relationship between *T. dioica* cultivars.

The reproducibility of RAPD method and markers has often been questioned and currently the trend is to develop the more reliable SCAR markers from sequenced RAPD markers (data not shown) (Paran and Michel-more, 1993). Longer length (20 - 25 primer) and higher annealing temperature (75°C) of SCAR primers make the reactions more reproducible, reliable and stable. The correlation between RAPD and ISSR Jaccard similarity coefficient value was low in magnitude. A possible explanation for the differences in resolution of RAPD and ISSRs is that the two marker technique target different portions of the genome. The ability to resolve genetic variation among different cultivars may be more directly related to the number of polymorphism detected with each marker technique rather than a function of which technique is employed. Nei (1978) reported that a relatively reliable estimate of average heterozygosity can also be obtained from a small number of individuals if a large number of loci are examined. The number of polymerphisms detected among cultivars influences the standard errors of the genetic diversity estimates.

The clustering pattern of cultivars within groups was not similar when RAPD and ISSR derived dendogram were compared. The dendogram prepared using RAPD and ISSR data showed greater similarity with ISSR pattern but not similar to RAPD clusters. RAPD + ISSR data based Dendogram grouped all 22 cultivars into the three major clusters. Cluster I consisted one female cultivar NP601 and Cluster II consisted of twelve cultivars which was further divided into four sub clusters. Sub cluster I included one male cultivar MI, sub cluster II comprised five cultivars, out of which three were male. Sub cluster III consisted of only two cultivars NP307 and NP310, and sub cluster IV included four cultivars (NP260, NP751, NP3, and RP1). Cluster III consisted of nine cultivars and divided into IV sub cluster. Sub cluster I included one cultivar (NP 701), Sub cluster II included NP207, sub cluster III included five cultivars (NP504, RP2, NP605, NP602 and NP801) and sub cluster IV included only two cultivars (NP311 and PG01).

These differences may be attributed to markers sampling error and/or the level of polymorphism detected reinforcing again the importance of the number of loci and their coverage of the overall genome in obtaining reliable estimates of genetic relationship among cultivars (Loarce et al., 1996). Identification of genes specific markers is yet another important finding in the present study. These markers could be of potential use for detecting mixtures and duplicates in the germplasm. Furthermore, the markers, if tagged to some gene of interest, can serve as an addendum to the existing breeding and selection programmes in *T. dioica.* The

evolution of species in distinct agro-climatic zone demonstrates significant levels of variation in response to the selection pressure in the zones (Singh et al., 1998). It is therefore significant levels of polymorphism among 22 cultivars of *T. dioica* in RAPD (89%) and ISSR (88.7%) markers. Geographically isolated population accumulates genetic differences as they adapt to different environment conditions. RAPD and ISSR analysis of *T. dioica* could be useful to select ancestors to be crossed for generating appropriate populations intended for both genome mapping and breeding purposes. In several crops, ISSR markers closely linked to numerous traits of economic importance have been developed, which will allow indirect selection for desirable traits in early segregating gene-ration at the seeding stage (Caetano-Anolles et al., 1997). The present study concluded that molecular evidence for systematic characterization of *T. dioica* cultivars.

ACKNOWLEDGEMENTS

We thank Dr. Rakesh Tuli, Director NBRI Lucknow India, for providing necessary facilities and Council of Scientific and Industrial Research (CSIR, India) for providing senior research fellowship to SG.

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