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RAPD and ISSR markers for molecular characterization of *Grewia optiva*: an important fodder tree of north western Himalayas

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Abstract

RAPD (random amplified polymorphic DNA) and ISSR (inter simple sequence repeats) markers were used to study DNA polymorphism of thirty *Grewia optiva* genotypes raised from seeds collected from various districts of Himachal Pradesh (India) and selected on the basis of morphological parameters. In general, the genotypes exhibited a very high level of molecular diversity and DNA polymorphism. Using 25 RAPD and 18 ISSR primers molecular diversity based on Jaccard's similarity coefficients among 30 genotypes ranged from 0.17-0.83 in RAPD assay and 0.30-0.84 in ISSR assay. RAPD primers amplified more loci than ISSR primers. RAPD and ISSR primers amplified 96.31 and 91.72% polymorphic loci, respectively. The dendrogram derived using the unweighted pair-group method with arithmetic averaging (UPGMA) clustered the genotypes into different groups reflecting geographical sub-structuring of the genetic diversity. Genotype SO-12 (Kasauli) was found to be the most divergent genotype which could be used for number of combinations to be established in the seed orchards and as a parent in hybridization programme.

Keywords: Fodder tree, Genetic diversity, *Grewia optiva*, ISSR, RAPD

Introduction

Fodder production in hills is very essential for their ecological security and the economy of the people. In absolute terms by 2020, the country is facing an estimated shortage of 728 million tons of green fodder and 157 million tones of dry fodder (Anonymous, 2011). The available fodder is not only insufficient but also poor in nutritive value and season specific (Hazra, 2014). The availability of green fodder is the most important single factor responsible for the success of animal husbandry. Leaf fodder is a very useful resource of green forage, especially during winter months when all other fodder sources have been exhausted. The major sources of tree leaf fodder are the trees growing on forests and common lands. However, with high timber value plantations coming up on scrub forests, this resource is getting depleted from near habitations. Another source of tree leaf fodder is the trees growing on farm lands. One of the important fodder species in improving the nutrition of livestock in the hills is *Grewia optiva* Drummond.

Grewia optiva Drummond, locally called as Beul or Bhimal, belongs to family Tiliaceae. It is a very important agroforestry tree species of the low and mid-hills regions in the western and central Himalaya, which constitutes 50 genera and 450 species (Watson and Dallwitz, 1992). It is an important fodder tree during the winters for villagers when no other fodder is available. Apart from widely used fodder, this species is highly valued for its fibre, fuel and small timber for making agricultural implements. Study of genetic variation is the first step for any breeding programme. High genetic variation within and among populations was demonstrated and this distribution of variation formed the basis of breeding programmes (Namkoong, 1984). Molecular markers successfully developed during the last two decades had largely overcome the problems that were associated with phenotype-based classification. PCR based techniques developed in recent years such as random amplified polymorphic DNA (RAPDs) (Williams *et al*., 1990) and inter simple sequence repeats (ISSR) (Zietkiewicz *et al*., 1994) provided DNA markers that are dispersed throughout plant genomes and are easier to reproduce and analyze. As *G. optiva* is an important agroforestry species and there was paucity of information, so the study was carried out to find the genetic diversity among different genotypes of *G. optiva*.

Table 1. Location and morphological details of *Grewia optiva* genotypes used for study

District	Genotypes	Code	Latitude	Longitude	Height (m)	Diameter (cm)	Leaf area (cm^2)
Bilaspur	Kuthera	BI-4	31° 56ð N	76°48ð E	6.03	8.33	55.04
Bilaspur	Talvano	BI-5	31° 62ð N	76°22ð E	5.90	7.77	52.54
Chamba	Chanaur	CH-1	32°15ð N	75°75ð E	5.77	6.23	72.25
Chamba	Shahu	CH-2	32° 56ð N	76°10ð E	4.52	6.43	52.58
Chamba	Saru	CH-6	32º 56ð N	76º10ð E	6.12	7.63	43.34
Hamirpur	Bharari	HA-1	31°61ð N	76°55ð E	4.53	6.45	55.55
Hamirpur	Patta Balakhar	HA-2	31°61ð N	76°55ð E	7.58	9.25	73.88
Hamirpur	Hamirpur Kanal	HA-4	31°67ð N	76°53ð E	7.23	8.27	41.29
Hamirpur	Ghahar	HA-5	31°73ð N	76°65ð E	5.67	7.53	43.10
Kangra	Dharamshala	KA-1	32°21ð N	76°31ð E	6.50	7.27	58.97
Kangra	Bhalun	KA-2	32°45ð N	76°56ð E	6.07	9.33	44.31
Mandi	Samaila	MA-1	31°53ð N	76°77ð E	6.00	7.40	46.00
Mandi	Bachhwan	MA-2	31°53ð N	76°77ð E	6.40	7.30	59.26
Mandi	Tatahar	MA-4	31°90ð N	76°81ð E	5.40	7.03	40.76
Mandi	Sarkaghat	MA-5	31°70ð N	76°73ð E	5.47	6.64	52.09
Sirmour	Seenaghat	SI-10	30°83ð N	77°46ð E	6.57	9.33	57.19
Sirmour	Adgu	SI-11	30°59ð N	77°67ð E	6.10	9.97	72.58
Sirmour	Mehandobag	SI-15	30°71ð N	77°21ð E	6.50	8.17	69.47
Sirmour	Nainatikker	SI-16	30°89ð N	77°17ð E	6.36	8.60	43.42
Solan	Gaura	SO-1	30°90ð N	77°09ð E	6.77	8.67	40.10
Solan	Nauni	SO-2	30°86ð N	77°16ð E	6.53	8.23	48.79
Solan	Dharja	SO-3	30°91ð N	77°03ð E	6.60	8.57	50.92
Solan	Deog	SO-4	31°10ð N	77°67ð E	5.39	8.10	49.04
Solan	Amberkothi	SO-6	30°87ð N	77°27ð E	6.37	7.60	65.37
Solan	Oyali	SO-7	30°97ð N	76°96ð E	7.10	8.47	48.70
Solan	Kailar	SO-8	31°19ð N	76°71ð E	6.07	8.03	63.10
Solan	Deothi	SO-9	30°92ð N	77°07ð E	6.53	8.67	60.07
Solan	Jaunaji	SO-10	30°90ð N	77°09ð E	5.17	6.67	50.09
Solan	Mishuar	SO-11	31°10ð N	77°67ð E	6.07	8.40	51.56
Solan	Kasauli	SO-12	30°89ð N	76°94ð E	7.07	9.87	51.09

Materials and Methods

Plant material: A set of sixty genotypes of *Grewia optiva* were procured from different districts of Himachal Pradesh (India). Thirty best performing genotypes (Table 1) were selected on the basis of previous study in the department. Fresh and disease free leaves were collected from the trees selected for molecular variability studies and carried to the laboratory in brown paper bags within 2-3 hours of collection. Use of polybags was avoided as it led to degradation of leaf samples and kept in deep freezer (-20°C) for future DNA extraction. Fresh leaf tissue (~ 0.5 g) was crushed in 7 ml extraction buffer (10 per cent (w/v) SDS (sodium dodecylsulfate), 0.5M EDTA (pH 8.0), 5M NaCl, 1M Tris pH 8.0 and 0.75 g PVP (polyvinylpyrrolidone) was added to remove polyphenols. The powder was either stored at -40^{0}C or used for DNA isolation immediately. Total genomic DNA was isolated using the Dellaporta *et al*. (1983) method with slight modification made in buffer concentrations. The nucleic acids were precipitated by addition of 2/3rd parts by volume of the chilled (20°C) ethanol and 1/10th parts by volume of 3 M sodium acetate. The pellet was collected by centrifugation and washed with cold (-20°C) 70 per cent ethanol. The quality of DNA was tested on 0.8% agarose gel (Genei Banglore, Banglore India) and quantification was done using Perkin Elmer UV/VIS spectrophotometer and diluted to 5ng/µl for further PCR (Polymerase chain reaction) amplification using CR Corbett thermocycler.

RAPD amplification: Twenty five decamer primers were used for the current study (Table 2). DNA was amplified by PCR amplification reaction. The 25µl of reaction mixture contained 20ng of DNA, 0.75 units of Taq DNA

polymerase 2.5µl of 10X Taq buffer (50mM $MgCl_2$, 10mM Tris-Cl), 1.25µl of pooled dNTPs (2.5mM each) and 10ng of primer. PCR conditions used for RAPD amplification included initial denaturation for 3 min at 94°C followed by 45 cycles of amplification (denaturation at 92°C for 45 seconds, annealing of primer at 36°C for 1min and primer amplification at 72°C for 2 min) and final extension at 72°C for 10 min.

ISSR amplification: Eighteen primers (Genei Banglore, Banglore India) were used for the current study (Table 3). DNA was amplified by PCR amplification reaction. The 25µl of reaction mixture contained 20ng of DNA, 0.75 units of Taq DNA polymerase (Genei Bangalore, Bangalore India), 2.5µl of 10X Taq buffer (50mM MgCl2, 10mM Tris-Cl), 1.25µl of pooled dNTPs (2.5mM each) and 10ng of primer (Genei Bangalore, Bangalore India). PCR conditions used for ISSR amplification included initial denaturation for 3 min at 94°C followed by 45 cycles of amplification (denaturation at 92°C for 45 seconds, annealing of primer at 55°C for 1min and primer amplification at 72°C for 2 min) and final extension at 72°C for 10 min.

Amplification products stained in ethidium bromide were separated on 2 per cent agarose gel using 1X TBE buffer (Tris HCl pH 8.0, boric Acid, Ethylene diamine-tetra acetic acid) on horizontal gel electrophoresis apparatus and photographed in Alpha Imager gel documentation system. 1kb and 100bp DNA mass ladder (Genei Bangalore, India).

Scoring of bands and data analysis: The marker amplification profile of 30 genotypes was used to estimate genetic diversity/relatedness based on number of shared amplified bands. The presence or absence of a particular amplification product was used as an index of genetic diversity/relatedness. The scored bands were analyzed in the form of binary system to prepare the similarity index. The bands with same molecular weight

Table 2. Total numbers of amplified and polymorphic fragments generated by PCR using RAPD primers

Primer name	Base sequences (5'-3')	Total no. of scorable bands (y)	Total no. of polymorphic bands (x)	Total no. of monomorphic bands	Polymorphism (%) $\frac{x}{y} \times 100$	Size range of amplified products (bp)
OPC-01	TTC GAG CCA G	5	5	0	100	200-1500
OPC-02	GTG AGG CGT C	5	5	0	100	300-1150
OPC-04	CCG CAT CTA C	7	7	0	100	300-2000
OPC-06	GAA CGG ACT C	6	6	0	100	350-2000
OPC-08	TGG ACC GGT G	6	6	0	100	200-1350
OPC-11	AAA GCT GCG G	6	6	0	100	200-950
OPC-12	TGT CAT CCC C	3	3	0	100	350-500
OPC-13	AAG CCT CGT C	9	9	0	100	200-1700
OPF-06	GGG AAT TCG G	8	8	0	100	250-1700
OPF-07	CCG ATA TCC C	6	5	1	83.30	350-2000
OPF-08	GGG ATA TCG G	9	9	0	100	300-2000
OPF-11	TTG GTA CCC C	4	4	0	100	300-1000
OPF-12	ACG GTA CCA G	9	9	0	100	200-1500
OPF-13	GGC TGC AGA A	10	10	0	100	200-2000
OPF-14	TGC TGC AGG T	6	6	0	100	200-1000
OPA-01	CAG GCC CTT C	6	6	0	100	200-1000
OPA-02	TGC CGA GCT G	6	6	0	100	200-1000
OPA-03	AGT CAG CCA C	8	5	3	62.50	250-1200
OPA-04	AAT CGG GCT G	9	9	0	100	200-1150
OPA-05	AGG GGT CTT G	7	7	0	100	500-1700
OPO-17	GGC TTA TGC C	6	6	0	100	150-1400
OPO-18	CTC GTA TCC	4	4	0	100	300-900
OPO-20	ACA CACGCT G	8	7	1	87.50	150-1300
OPO-19	GGT GCA CGT T	4	3	1	75.00	250-850
OPF-15	CCA GTA CTC C	6	6	0	100	150-1000
	TOTAL	163	157	6	96.33	150-2000

and mobility were treated as identical fragments. Data matrices were prepared in which the presence of a band was coded as 1 whereas the absence as 0. The similarity matrix value based on Jaccard's coefficient of similarity (Simqual function) was used to generate dendrogram. Clustering was done by UPGMA using SAHN module of NTSYSpc. Ver. 2.02e (Rohlf, 1998).

Results and Discussion

The 25 RAPD and 18 ISSR primers differentiated among the thirty genotypes of *G. optiva*. The generated band sizes had diverse molecular mass, with the RAPD primers ranging from 150 bp to 2000 bp. The largest number of polymorphic RAPD bands was detected by primer OPF-13 (ten bands), while the least was for primers OPC-12 (three bands) (Table 2). All twenty five RAPD primers produced distinct banding pattern for all the thirty genotypes. A total of 163 amplified products were detected out of which 96.31 per cent were found to be polymorphic. Such a high level of polymorphism reflects the outcrossing nature of the species. These findings are in agreement with Qi *et al*. (2003) who reported screening of twenty-five primers from 119 random primers in jute, reporting 87.78% polymorphism. Similar results were also revealed by Vaishali *et al*. (2008) (86% polymorphism in *Butea monosperma*), whereas Hossain *et al*. (2002) reported only 26% were polymorphism in 9 jute varieties and 12 accessions using 29 RAPD primers. Thus the number of polymorphic primers and fragments generated were not of similar range for tree species. They can vary significantly in different plant species. This is understandable as product amplification depends upon the sequence of random primers and their compatibility within genomic DNA. Jaccard's similarly correlation coefficient value ranged from 0.17 (among the genotypes BI-4 and SO-12) to 0.83 (among the genotypes SO-7 and KA-2). This suggested a fair range of variability in the similarity coefficient values indicating a broad genetic base of *Grewia optiva* genotypes. Weiguo and Yile (2004) reported similar results in *Morus* using RAPD markers revealed the highest similarity (0.9912) between T11 and T12 (Thailand). The result indicated that they almost have the same genetic constituents.

The amplification products for ISSR primers ranged from 150 bp for primer UBC-841 to 4500 bp for primer 825. The largest number of polymorphic ISSR bands was amplified by primer 835 (seventeen bands), while the least was amplified by primer 881 (two bands) (Table 3). A summary of amplified polymorphic and monomorphic fragments obtained from ISSR study revealed that twelve out of eighteen primers were polymorphic and amplified the genomic DNA of different genotypes of *Grewia optiva* successfully. One hundred and forty five amplified products were detected out of which 91.72 per cent were found to be polymorphic. Results from the current study indicate that the genotypes obtained from different districts are of wide genetic base. In similar study by Srivastava *et al*. (2004) and Awasthi *et al*. (2004) in *Morus alba* reported 74.13% and 66.67% polymorphism respectively. Jaccard's similarity correlation coefficient value ranged from 0.29 to 0.84. The highest value (0.84) was found between SO-1 (Gaura) and KA-2 (Bhalun), the genetic similarity coefficients revealed substantial amount of genetic similarity among the genotypes, though the genotypes were collected from different districts varied in climatic conditions. Narayanan *et al*. (2007) reported in teak that molecular diversity based on Jaccard's similarity coefficients among 48 plus trees ranged from 0.27-0.88 in ISSR assay. The ranges indicated very diverse nature of plus trees, which was expected due to rigorous selections made from various teak forest types thriving on different geo-climates of the country. These results are in the line of present results.

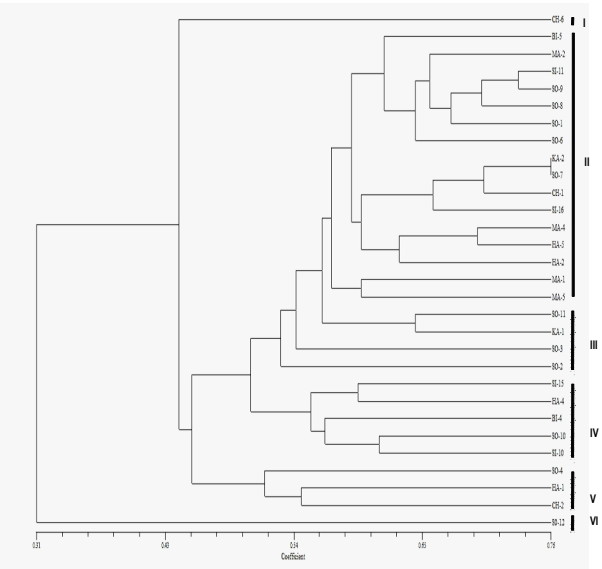

Fig 1. Dendrogram based on UPGMA analysis among 30 genotypes of *Grewia optiva* using RAPD and ISSR markers

The results showed that RAPD primers revealed more DNA polymorphism (96.31%) among the genotypes of *G. optiva* than ISSR primers (91.72%). Whereas, in contrast Srivastava *et al*. (2004) in the study of *Morus alba* observed that ISSR primers revealed 74.13% polymorphism while RAPD generated 60.75 % polymorphism among the 11

Table 3. Total numbers of amplified and polymorphic fragments generated by PCR using ISSR primers of *Grewia optiva*

Primer name	Base sequences (5'-3')	Total no. of scorable bands (y)	Total no. of polymorphic bands (x)	Total no. of monomorphic bands	Polymorphism (%) $\frac{x}{y} \times 100$	Size range of amplified products (bp)
809	AGAGAGAGA GAGAGAGG	14	11	3	78.57	200-2500
810	GAGAGAGA GAGAGAGAT	8	7	1	87.5	200-2000
811	GAGAGAGAG AGAGAGAC	13	12	1	92.30	250-2000
812	GAGAGAGA GAGAGAGAA	8	8	0	100	100-2000
825	CACACACA CACACACC	8	8	0	100	200-4500
830	TGTGTGTG TGTGTGTGG	5	5	0	100	350-2000
834	AGAGAGAGA GAGAGAGYT	7	4	3	57.14	200-1000
835	AGAGAGAGA GAGAGAGYC	17	16	1	94.11	150-4500
850	GTGTGTGTG TGTGTGTYA	5	5	0	100	250-700
861	ACCACCACC ACCACCACC	5	5	0	100	100-1000
862	AGCAGCAGC AGCAGCAGC	5	5	0	100	100-400
864	ATGATGATG ATGATGATG	10	10	0	100	100-2500
881	GGGGTGGG GTGGGGTC	2	2	0	100	700-1700
UBC-807	AGAGAGAG AGAGAGAGT	9	9	0	100	200-2000
UBC-820	GTGTGTGT GTGTGTGTC	6	6	0	100	100-1500
UBC-826	ACACACACA CACACACAC	7	4	3	57.14	250-1500
UBC-827	ACACACACA CACACACAG	7	7	0	100	250-2250
UBC-841	GAGAGAGAG AGAGAGAYC	9	9	0	100	100-1500
	TOTAL	145	133	12	91.72	100-4500

mulberry genotypes which showed that ISSR primers were more efficient in revealing the DNA polymorphism. This can be attributed to the hyper variable nature of the ISSR markers, which are expected to reveal high levels of variation. However, in this study polymorphism was higher using ISSR primers (91.72%) as compared to earlier studies using ISSR primers by Srivastava *et al*. (2004) in *Morus*, Lau *et al*. (2005) in *Bauhinia blakeana* and Okun *et al*. (2007) in *Eucalyptus grandis* who observed 74.13%, 46.7% and 65.9% polymorphism. Because the ISSRs indicated polymorphism among different genotypes it can be anticipated that the results of ISSR based study will play a major role in the management, conservation and improvement of this tree crop. The RAPD and ISSR data were combined for UPGMA cluster analysis. The dendrogram and cluster analysis gave a similar clustering pattern to that of ISSR and RAPD analysis, separately, with Jaccard's similarity coefficient

ranging from 0.25 to 0.76. The dendrogram exhibited six clear clusters (Fig. 1). The dendrograms based on RAPD and ISSR combined markers showed partially different genetic distance levels when used individually. But when used together, RAPD-based cluster is similar to the combined cluster than ISSR-based cluster. Results are in agreement with the studies in *Ficus* species (Hadia *et al*., 2008). However, in old world Lupin (*Lupinus* sp.) it was found that ISSR-based cluster is more similar to the combined cluster than RAPD-based cluster (Yorgancilar *et al*., 2009). *Jatropha curcas* also showed similar result when RAPD and ISSR dendrogram patterns were combined (Gupta *et al*., 2008).

Conclusion

It was concluded that both the marker systems RAPD and ISSR either individually or in combination can be effectively used in determination of genetic relationships among *G. optiva* genotypes. It was found that genotype SO-12 (Kasauli) came as outliner in RAPD, ISSR and combined RAPD+ISSR dendrogram analysis and was considered as the most divergent genotype.

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