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RESEARCH ARTICLE

SSR and RAPD analysis of genetic diversity in walnut (*Juglans regia* L.) genotypes from Jammu and Kashmir, India

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Abstract In this study, the genetic relatedness of 82 walnut genotypes adapted to the North Western Himalayan region of Jammu and Kashmir, India was analyzed by combination of 13 SSR and 20 RAPD primers. A high level of genetic diversity was observed within populations with the number of alleles per locus ranging from one to five in case of SSR primers and two to six in case of RAPD primers, the proportion of polymorphic loci was 100 %, and similarity ranged from 12 % to 79 % with an average of 49 %. Dendrogram showed that all the accessions formed four main clusters with various degree of subclustering within the clusters. These results have implications for walnut breeding and conservation.

Keywords Genetic diversity · *Juglans regia* · Microsatellites · RAPD

Introduction

Walnut (*Juglans regia* L.) belongs to the family Juglandaceae. In local language it is called 'akhrot'. The genus *Juglans* consists of approximately 20 species having 32 chromosomes (Robert 1930). Different species of the genus are taxonomically grouped into four sections; three of these are *Rhysocaryon* (black walnuts of America), *Cardiocaryon* (Japanese, Manchurian and Chinese walnuts) and *Trachycaryon* (butter nut of Eastern North America). The fourth section, *Juglans*, is comprised of single species *Juglans regia*, which is widely grown in temperate zone (Manning 1978). Cultivated varieties of walnut generally adapt well to the climatic conditions of the different production areas. *Juglans regia* L., has an exceptionally wide natural distribution, it occurs from the Carpathian Mountains of Eastern Europe, all through Western Asia, the Himalayan regions of Pakistan, India, Nepal, Bhutan and east into China. Accurate and rapid cultivar identification is especially important in vegetatively propagated plant species such as most fruit trees both for practical breeding purposes and for proprietary rights protection. Unfortunately, the traditional methods for characterization and assessment of genetic variability in perennial fruit crop species, based on morphological, physiological and biochemical studies are both time consuming and affected by the environment. The introduction of molecular biology techniques, such as DNA based markers, provides an opportunity for genetic characterization that allows direct comparison of different genetic material independent of environmental influences (Weising et al. 1995). Previously, in addition to morphological identification (Zenelli et al. 2005), various biochemical and molecular markers have been used for genetic characterization of walnut genotypes. These included isozymes (Ninot and Aleta 2003; Vyas et al. 2003; Fornari et al. 2001; Busov et al. 2002), restriction fragment length polymorphisms (RFLPs) (Fjellstrom and Parfitt 1995), randomly amplified polymorphic DNAs (RAPDs) (Nicese et al.1998; Yan-Min et al. 2000; Zhang Li et al. 2007), intersimple sequence repeats (ISSRs) (Potter et al. 2002), simple sequence repeats (SSRs) (Woeste et al. 2002; Dangl et al. 2005; Foroni et al. 2005; Victory et al. 2006; Robichaud et al 2006; Karimi et al. 2010), amplified fragment length polymorphisms (Kafkas et al. 2005; Bayazit et al. 2007) and SNPs (Ciarmiello et al. 2011). SSR

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Table 1 Detail of 82 walnut genotypes used in this study

Name of the genotype	Collection site.	Accession No.	Latitude	Longitude	Altitude
CITH-W-1	Ganderbal	IC561050	34.23°	74.78°	5,312 ft
CITH-W-11	Anantnag	IC 587310	33.73°	75.15°	5,253 ft
CITH-W-23	Anantnag	IC 587091	33.73°	75.15°	5,253 ft
GGS-1	Ganderbal	IC 587072	34.23°	74.78°	5,312 ft
CITH-W-13	Budgam	IC 587115	34.63°	76.04°	10,479 ft
PBS-1	Pulwama	IC 587083	33.88°	74.92°	5,350 ft
LG-10	Ganderbal	IC 561052	34.23°	74.78°	5,312 ft
BYS-1	Budgam	IC 587232	34.63°	76.04°	10,479 ft
BSS-4	Budgam	IC 587298	34.63°	76.04°	10,479 ft
CITH-W-17	Baramulla	IC 587071	34.1980°	74.3636°	5,226 ft
GWS-6	Ganderbal	IC 587160	34.23°	74.78°	5,312 ft
CITH-W-6	Pulwama	IC 587141	33.88°	74.92°	5,350 ft
CITH-W-9	Baramulla	IC 587073	34.1980°	74.3636°	5,226 ft
CITH-W-18	Ganderbal	IC 587163	34.23°	74.78°	5,312 ft
GLS-6	Ganderbal	IC 587154	34.23°	74.78°	5,312 ft
APS-12	Anantnag	IC 587113	33.73°	75.15°	5,253 ft
BRTS-3	Baramulla	IC 587192	34.1980°	74.3636°	5,226 ft
CITH-W-2	Ganderbal	IC 561049	34.23°	74.78°	5,312 ft
HS-4	Baramulla	IC 561062	34.1980°	74.3636°	5,226 ft
GG-7	Ganderbal	IC 561055	34.23°	74.78°	5,312 ft
CITH-W-27	Ganderbal	IC 561056	34.23°	74.78°	5,312 ft
CITH-W-16	Budgam	IC 587140	34.63°	76.04°	10,479 ft
BRMS-2	Baramulla	IC 587184	34.1980°	74.3636°	5,226 ft
CITH-W-10	Budgam	IC 561061	34.63°	76.04°	10,479 ft
PTS-11	Pulwama	IC 587287	33.88°	74.92°	5,350 ft
GSS-18	Ganderbal	IC 587139	34.23°	74.78°	5,312 ft
AMC-1	Anantnag	IC 587088	33.73°	75.15°	5,253 ft
CITH-W-5	Pulwama	IC 587086	33.88°	74.92°	5,350 ft
CITH-W-4	Anantnag	IC 587272	33.73°	75.15°	5,253 ft
GKS-1	Ganderbal	IC 587142	34.23°	74.78°	5,312 ft
CWS-4	Budgam	IC 587231	34.63°	76.04°	10,479 ft
AMC-5	Anantnag	IC 587092	33.73°	75.15°	5,253 ft
CITH-W-8	Baramulla	IC 587208	34.1980°	74.3636°	5,226 ft
SHS-11	Shopian	IC 587331	33.90	74.99	5,600 ft
PBS-2	Pulwama	IC 587084	33.88°	74.92°	5,350 ft
GK-2	Ganderbal	IC 561059	34.23°	74.78°	5,312 ft
CITH-W-29	Budgam	IC 587270	34.63°	76.04°	10,479 ft
CWS-7	Budgam	IC 587268	34.63°	76.04°	10,479 ft
BRTS-4	Baramulla	IC 587193	34.1980°	74.3636°	5,226 ft
AMC-4	Anantnag	IC 587091	33.73°	75.15°	5,253 ft
APS-16	Anantnag	IC 587305	33.73°	75.15°	5,253 ft
CITH-W-24	Budgam	IC 587230	34.63°	76.04°	10,479 ft
CITH-W-7	Anantnag	IC 561057	33.73°	75.15°	5,253 ft
KAS-2	Kulgam	IC 587352	33.65°	75.02°	5,705 ft
CITH-W-22	Budgam	IC 587354	34.63°	76.04°	10,479 ft
GLS-1	Ganderbal	IC 587074	34.23°	74.78°	5,312 ft
GLS-2	Ganderbal	IC 5287075	34.23°	74.78°	5,312 ft
GLS-3	Ganderbal	IC 587076	34.23°	74.78°	5,312 ft
GLS-4	Ganderbal	IC 587077	34.23°	74.78°	5,312 ft

Table 1 (continued)

Name of the genotype	Collection site.	Accession No.	Latitude	Longitude	Altitude
Hamdan	Ganderbal	Passport data send to NBPGR, New Delhi for IC number allotment	34.23°	74.78°	5,312 ft
Sulaiman	Ganderbal		34.23°	74.78°	5,312 ft
VL-2	Ganderbal		34.23°	74.78°	5,312 ft
Wussan-3	Ganderbal		34.23°	74.78°	5,312 ft
W-2	Ganderbal		34.23°	74.78°	5,312 ft
S-10	Ganderbal		34.23°	74.78°	5,312 ft
SKAU-003	Ganderbal		34.23°	74.78°	5,312 ft
SKAU-004	Ganderbal		34.23°	74.78°	5,312 ft
SKAU-0020	Ganderbal		34.23°	74.78°	5,312 ft
SKAU-0022	Ganderbal		34.23°	74.78°	5,312 ft
SKAU-008	Ganderbal		34.23°	74.78°	5,312 ft
SKAU-0023	Ganderbal		34.23°	74.78°	5,312 ft
LBPTR-1	Baramulla		34.1980°	74.36°	5,226 ft
BC-3	Budgam		34.63°	76.04°	10,479 ft
Shalimar-2	Ganderbal		34.23°	74.78°	5,312 ft
Wussan-8	Ganderbal		34.23°	74.78°	5,312 ft
Wussan-2	Ganderbal		34.23°	74.78°	5,312 ft
Wussan-25	Ganderbal		34.23°	74.78°	5,312 ft
Saloora-1	Ganderbal		34.23°	74.78°	5,312 ft
Wussan-1	Ganderbal		34.23°	74.78°	5,312 ft
Opex Cultury	Exotic collections				
Nugget					
Victoria					
Serr					
Lake-English					
Spannow					
Paynee					
Turtle					
Pratab					
Blackmore					
Chenovo					
Fragutte					
Waterloo					

markers or SSRs (Litt and Luly 1989) are currently becoming the preferred technique for the molecular characterization of different plant species (Gupta and Varshney 2000). SSR markers in combination with RAPD markers have become an important tool to understand the molecular diversity and the genetic relationships within crops (Mir et al. 2008).

Material and methods

Plant material

A total of 82 walnut accessions were collected from different regions of Jammu and Kashmir and are being maintained at research farm of CITH, Srinagar, India (Table 1, Fig. 1). These accessions are being multiplied and evaluated under different locations across the state of Jammu and Kashmir, India. These accessions were assessed for DNA profiling through RAPD and SSR for diversity analysis.

DNA extraction

DNA was extracted from young walnut leaves using the cetyl trimethyl ammonium bromide (CTAB) method and purified by RNase treatment. The DNA was quantified on 0.8 % agarose gel stained with ethidium bromide in the presence of different concentrations of undigested λ-DNA and a final concentration of 25 ng μl^{-1} was used for PCR.

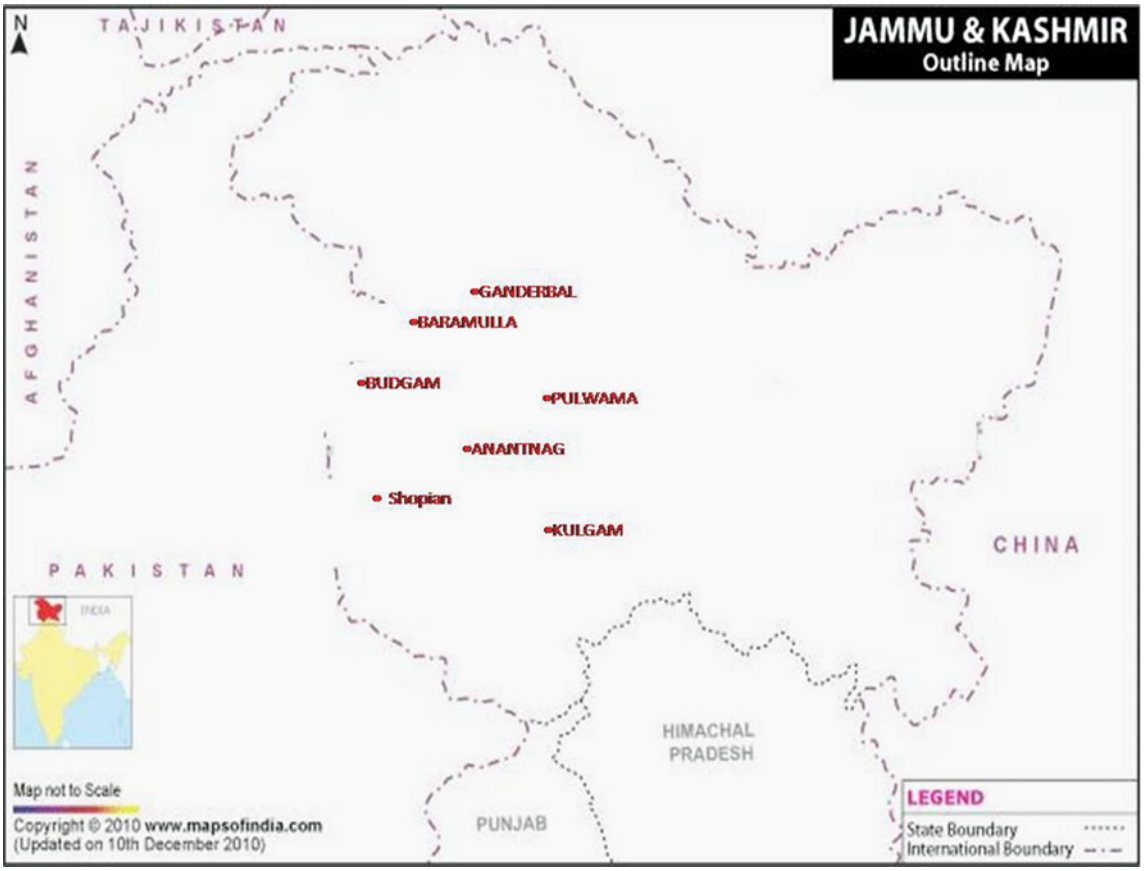

Fig. 1 Map of Jammu and Kashmir showing different collection sites of the walnut genotypes used in this study

RAPD and SSR analyses

DNA from an individual plant of each walnut accession was screened with 13 pairs of SSR primers (Pollegioni et al 2009) were used (Table 3). The PCR reaction (25 μl) contained the following: 1x reaction buffer (20 mM Tris- Cl pH 8.4, 50 mM KCl), 0.2 mM dNTPs, 2 mM $MgCl_2$, 10 pM primer, 1.0 Unit of *Taq* DNA polymerase, and 25–50 ng genomic DNA. For standardization of annealing temperatures of SSR primers, gradient PCR was carried out in a gradient thermal cycler. Initial denaturation at 94 °C for 5 min was followed by 35 cycles at 94 °C for 1 min, 58–63 °C for 1 min and 72 °C for 2 min. The final extension was carried out at 72 °C for 7 min. For RAPD the PCR reaction (25 μl) contained the following: 1x reaction buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 0.2 mM dNTPs, 2 mM $MgCl_2$, 10 pM primer, 1.0 unit of *Taq* DNA polymerase, and 25–50 ng genomic DNA. The DNA was amplified in a thermal cycler (Thermo-Mastercycler) that was programmed as follows: preheating for 5 min at 94 °C; 45 cycles of 60 s at 94 °C (denaturation), 60 s at 37 °C (annealing), and 120 s at 72 °C (extension); and a final extension at 72 °C for 7 min. The RAPD amplified-DNA was analyzed by electrophoresis on 2 % agarose gel in a 0.5X TBE buffer. The SSR amplified fragments were resolved in 3 % metaphore agarose gel in a 0.5X TBE buffer. The gels were stained with ethidium bromide (0.5 μg/ml) and visualized under UV light. The prominent DNA bands that were amplified by a given primer were scored as present (1) or absent (0) for all of the samples that were studied. In order to determine the utility of these markers, number of amplicons per primers, percent polymorphism, Polymorphic information content (PIC), effective multiplex ratio (EMR)/Resolving power (Rp) and marker index (MI) was calculated. Percent polymorphism was calculated as percentage of polymorphic loci from total loci obtained per primer. The Polymorphism Information Content (PIC) value of individual primers were calculated based on the formula $PIC=2\times F$ (1- F) (Anderson et al 1993). Marker index, a product of information content, as measured by PIC, and effective multiplex ratio (E), was calculated following Powell et al (1996) and Resolving power (RP) of each primer combination was calculated according to Prevost and Wilkinson (1999). The Jaccard's similarity index was calculated using NTSYS-pc version 2.02e (Applied Biostatistics, Inc., Setauket, NY, USA) package to compute pairwise Jaccard's similarity coefficients (Jaccard 1908) and this similarity matrix was used in cluster analysis using an

Table 2 Polymorphic profile of 20 RAPD primers across 82 genotypes of walnut (*Juglans regia* L.)

Primer	PIC	Ib	MI	Number of amplicon	Percent polymorphism
OPA 1	0.226413	5.121951	1.132064	5	100
OPA 2	0.238846	5.390244	1.19423	5	100
OPA 3	0.243704	4.00	0.731112	3	100
OPA 5	0.363697	4.02439	1.454789	4	100
OPA 6	0.172814	0.804878	0.518441	3	100
OPA 7	0.364366	2.609756	1.093099	3	100
OPA11	0.287626	3.926829	1.438132	5	100
OPA 12	0.113325	1.902439	0.226651	2	100
OPA 13	0.275679	6.95122	1.654075	6	100
OPA 14	0.345181	4.390244	1.380726	4	100
OPA 15	0.342653	8.512195	1.370613	1	100
OPA 16	0.182183	13.65854	0.728733	2	100
OPA 17	0.271713	3.853659	1.086853	4	100
OPA 20	0.237061	1.512195	0.474123	2	100
OPJ3	0.314099	1.609756	0.314099	1	100
OPO19	0.225758	0.58	0.225758	2	100
OPO 18	0.169096	0.41	0.169096	1	100
OPR1	0.347591	2.56	0.347591	1	100
OPR2	0.392623	1.46	0.392623	1	100
OPV5	0.070196	0.15	0.070196	1	100

Table 3 Polymorphic profile and characteristics of 13 SSR primers used across 82 genotypes of walnut (*Juglans regia* L.)

Marker	Primer sequence (5′- 3′)	PIC	Ib	MI	Number of amplicon	Number of alleles/locus	Percent polymorphism
WGA9	F: CATCAAAGCAAGCAATGGG R: CCATTGGTCTGTGATTGGG	0.464	1.27	0.46	1	1	100
WGA 202	F: CCCATCTACCGTTGCACTTT R: GCTGGTGGTTCTATCATGGG	0.212	1.27	1.06	5	5	100
WGA 5	F: AGTTTGTCCCACACCTCCT R: ACCCATGGTGAGAGTGAGC	0.452	1.39	0.90	2	2	100
WGA 1	F: ATTGGAAGGGAAGGGAAATG R: CGCGCACATACGTAAATCAC	0.464	1.27	0.46	1	1	100
WGA 27	F: AACCTCACGCCTTGATG R: TGC TCA GGC TCC ACT TCC	0.414	1.41	0.41	1	1	100
WGA 69	F: TTAGATTGCAAACCCACCCG R: AGATGCACAGACCAACCCTC	0.492	1.12	0.49	1	1	100
WGA 71	F: ACCCGAGAGATTTCTGGGAT R:GGACCCAGCTCCTCTTCTCT	0.480	1.2	0.48	1	1	100
WGA 89	F: ACCCATCTTTCACGTGTGTG R: TGCCTAATTAGCAATTTCCA	0.381	0.51	0.38	1	1	100
WGA 118	F: TGTGCTCTGATCTGCCTCC R: GGGTGGGTGAAAAGTAGCAA	0.498	1.05	0.50	1	1	100
WGA 276	F: CTCACTTTCTCGGCTCTTCC R: GGTCTTATGTGGGCAGTCGT	0.285	1.64	0.85	3	3	100
WGA 321	F: TCCAATCGAAACTCCAAAGG R: TGTCCAAAGACGATGATGGA	0.372	1.88	1.11	3	3	100
WGA 4	F: TGTTGCATTGACCCACTTGT R: TAAGCCAACATGGTATGCCA	0.219	1.43	0.43	2	2	100
WGA 32	F: CTCGGTAAGCCACACCAATT R: ACGGGCAGTGTATGCATGTA	0.361	2.07	1.44	4	4	100

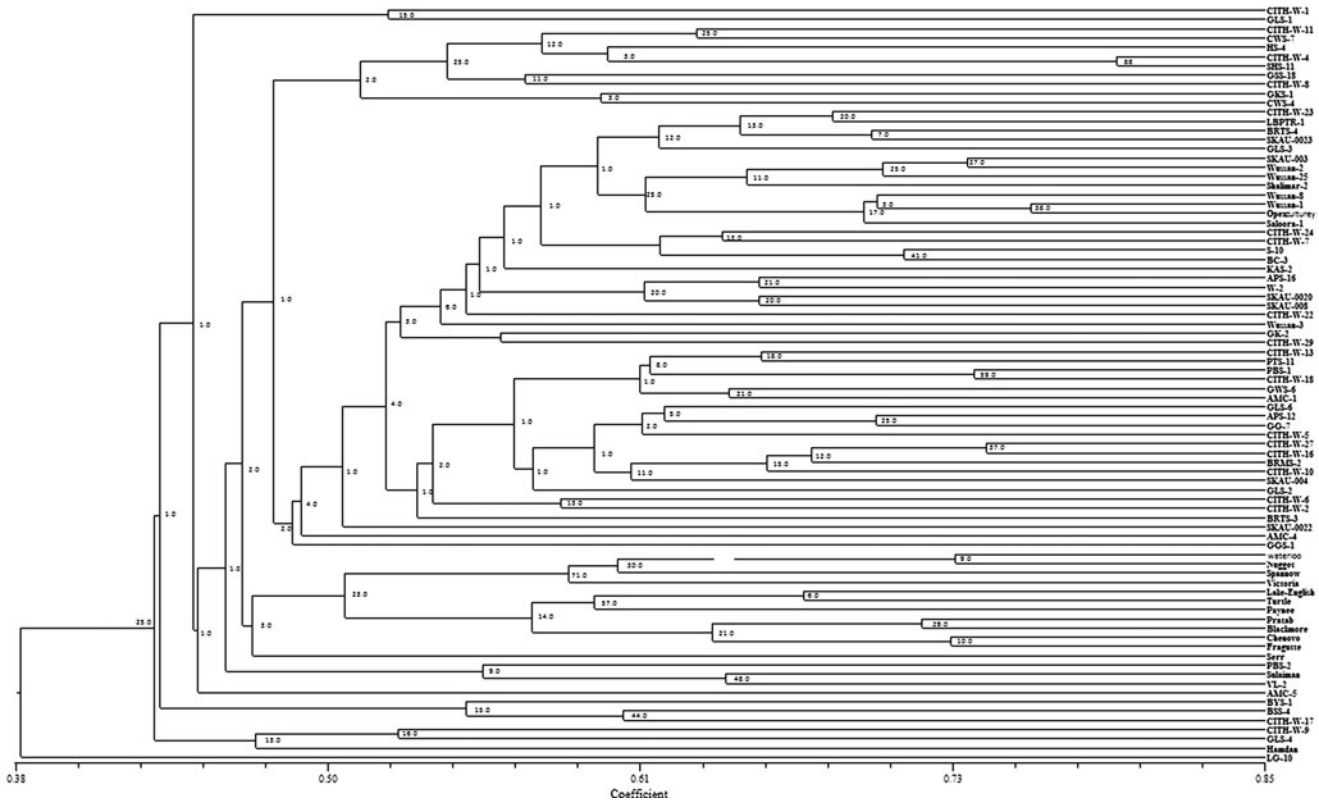

Fig. 2 UPGMA dendrogram showing clustering pattern of walnut accessions. The bootstrap values are given on the nodes

unweighted pair-group method with arithmetic averages (UPGMA) and sequential, agglomerative, hierarchical and nested (SAHN) clustering algorithm to obtain a dendrogram. To judge the confidence of the group revealed in the

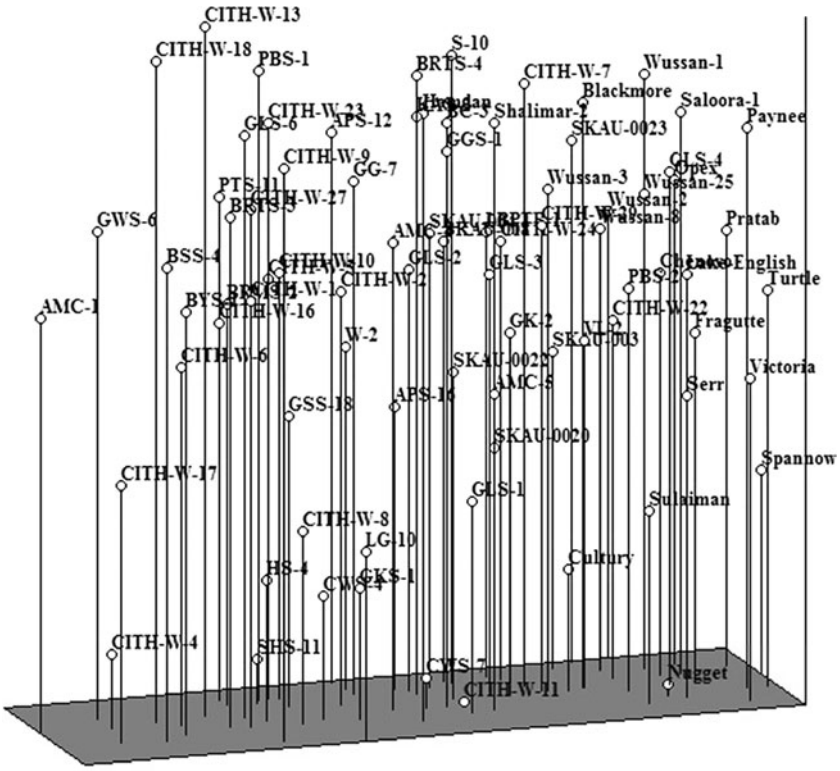

Fig. 3 Three dimensional PCO (principal co-ordinate analysis) scaling of 82 Walnut genotypes using RAPD and SSR markers

dendogram, bootstrap analysis was performed using the WINBOOT program (Yap and Nelson 1996) with 1,000 replications.

The co-relation of matrices obtained from SSR and RAPD profiles was judged by two-way Mantel test (Mantel 1967) using MxComp Module of $NTSYS_{PC}$ version 2.02e (Applied BioStatistics, Inc., Setauket, NY, USA) package.

Model-based cluster analysis was performed to infer genetic structure and to define the number of clusters (gene pools) in the dataset using the software STRUCTURE version 2.2 (Pritchard et al. 2000). The number of presumed populations (K) was set from 1 to 13, and the analysis was repeated 2 times. For each run, burn-in and iterations were set to 100,000 and 200,000 respectively, and a model without admixture and correlated allele frequencies was used. The run with maximum likelihood was used to assign individual genotypes into groups. Within a group, genotypes with affiliation probabilities (inferred ancestry) ≥80 % were assigned to a distinct group, and those with <80 % were treated as "admixture", i.e., these genotypes seem to have a mixed ancestry from parents belonging to different gene pools or geographical origins. The expected heterozygosity (gene diversity) and population differentiation (*Fst*) between individual in a sub-population was also worked out using STRUCTURE programme.

Results and discussion

RAPD and SSR analysis

All the chosen primers amplified fragments, which varied in size from 150–1,500 bp. All the 62 amplified bands were found polymorphic, with an average of 3.1 bands per primer. The respective values for overall genetic variability for PIC, Rp and MI across all the 82 genotypes are given in Table 2. PIC is a feature of a primer and therefore the PIC values were calculated for all primers. Highest PIC value (0.39) was observed for the primer OPR2 and lowest PIC value (0.07) was recorded for the primer OPV5 (Table 2). Average PIC value was 0.25. MI is a feature of a marker and therefore the MI values were calculated for all markers. The MI values ranged from 0.07 to 1.65 with an average of 0.80. Highest value (1.65) was scored with the primer OPA13 and the lowest value (0.07) for the primer OPV5 (Table 2). The RP is a feature of the primer that indicates the discriminatory potential of the primer. RP ranged from 0.15 to 13.65 with an average of 3.67 per primer. Highest value (13.65) was scored with the primer OPA16 and the lowest value (0.15) for the primer OPV5 (Table 2). Allele number per locus varied from two to six (OPA-13). Previously, in addition to morphological identification (Zenelli et al. 2005), various biochemical and molecular markers have been used for genetic characterization of walnut genotypes. These included isozymes (Fornari et al. 2001; Busov et al. 2002; Ninot and Aleta 2003; Vyas et al. 2003,), restriction fragment length polymorphisms (RFLPs) (Fjellstrom et al.1995), randomly amplified polymorphic DNAs (RAPDs) (Nicese et al.1998; Yan-Min et al. 2000; Zhang Li et al. 2007), inter-simple sequence repeats (ISSRs) (Potter et al. 2002), simple sequence repeats (SSRs) (Woeste et al. 2002; Dangl et al. 2005; Foroni et al. 2005; Victory et al. 2006; Robichaud et al. 2006; Karimi et al. 2010), amplified fragment length polymorphisms (Kafkas et al. 2005; Bayazit et al. 2007) and SNPs (Ciarmiello et al. 2011). In our study, the average number of alleles per locus was 2 which is higher than 1.3 detected in *J. regia* with RAPDs (Nicese et al. 1998) but lesser than 3.9 and 5.5 using ISSR (Potter et al. 2002) and SSR (Foroni et al. 2007) markers respectively. Average number of bands generated per primer following RAPD were 3.1 which agrees with earlier studies in other species (Casas et al. 1999; Galderisi et al. 1999; Goulao et al. 2001; Solar et al. 2005) but are lesser than the number of bands obtained by Francesca et al. 2010, due to a superior genetic variability among the genotypes studied by them. Our results agree with earlier studies using RAPD at Juglans species, RAPD markers revealing the genotypic diversity of Juglans (Nicese et al. 1998; Yan-Min et al. 2000; Francesca et al. 2010). Similarly all 13 SSR primers resulted in number of amplified fragments which varied in size from 200–1,200 bp. All the 26 amplified bands were found polymorphic, with an average of 2 bands per primer. The respective values for overall genetic variability for PIC, Rp and MI across all the 82 genotypes are given in Table 3. Highest PIC value (0.49) was observed for the primer WGA69 and lowest PIC value (0.21) was recorded for the primer WGA4 (Table 3). Average PIC value was 0.39.

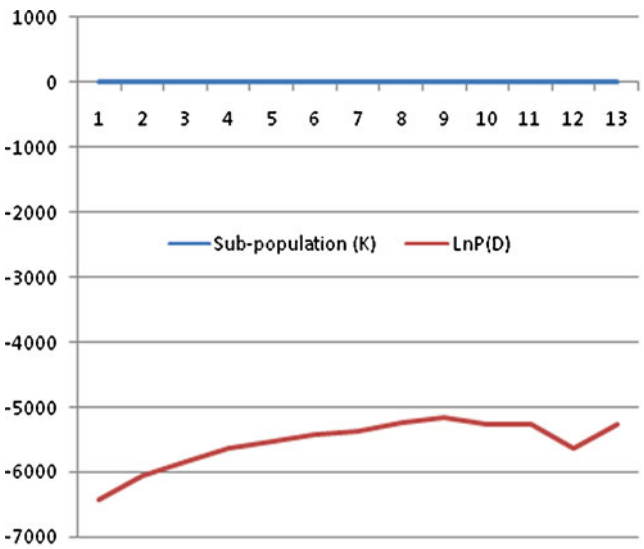

Fig. 4 Probability values of different presumed populations (K)

Table 4 Assignment of sub-populations (K) to the individuals based on probability

Genotype	Code	K-1	K-2	Pop-3	Pop-4	Pop-5	Pop-6	Pop-7	Pop-8	Pop-9	Assignment to sub-population
CITH-W-1	1	0.001	0	0	0	0	0	0.988	0	0.01	7
CITH-W-11	2	0	0	0	1	0	0	0	0	0	4
CITH-W-23	3	0	0.001	0	0	0	0	0.999	0	0	7
GGS-1	4	0.049	0	0	0	0	0	0.933	0	0.019	7
CITH-W-13	5	0	0.994	0	0	0	0	0.006	0	0	2
PBS-1	6	0	0.999	0	0	0	0	0	0.001	0	2
LG-10	7	1	0	0	0	0	0	0	0	0	1
BYS-1	8	0	1	0	0	0	0	0	0	0	2
BSS-4	9	0	1	0	0	0	0	0	0	0	2
CITH-W-17	10	0	1	0	0	0	0	0	0	0	2
GWS-6	11	0	1	0	0	0	0	0	0	0	2
CITH-W-6	12	0	0	0	0	0	0	1	0	0	7
CITH-W-9	13	0	0.019	0	0	0	0	0	0	0.981	9
CITH-W-18	14	0	1	0	0	0	0	0	0	0	2
GLS-6	15	0	0.524	0	0	0	0	0	0.476	0	ADMIXURE OF 2 & 8
APS-12	16	0	0	0	0	0	0	0	1	0	8
BRTS-3	17	0	0.005	0	0	0	0	0	0.995	0	8
CITH-W-2	18	0.008	0	0	0	0	0	0.485	0.507	0	ADMIXURE
HS-4	19	0	0	0	0.999	0	0	0	0.001	0	4
GG-7	20	0	0	0	0	0	0	0	1	0	8
CITH-W-27	21	0	0	0	0	0.001	0	0	0.999	0	8
CITH-W-16	22	0	0	0	0	0	0	0	1	0	8
BRMS-2	23	0	0	0	0	0	0	0	1	0	8
CITH-W-10	24	0	0	0	0	0	0	0	1	0	8
PTS-11	25	0	0	0	0	0	0	1	0	0	7
GSS-18	26	0.996	0	0	0.004	0	0	0	0	0	1
AMC-1	27	0	0.999	0	0	0	0	0	0.001	0	2
CITH-W-5	28	0	0	0	0	0	0	0	1	0	9
CITH-W-4	29	0	0	0	1	0	0	0	0	0	4
GKS-1	30	0.003	0	0	0.997	0	0	0	0	0	4
CWS-4	31	0	0	0	1	0	0	0	0	0	4
AMC-5	32	1	0	0	0	0	0	0	0	0	1
CITH-W-8	33	0.001	0	0	0.999	0	0	0	0	0	4
SHS-11	34	0	0	0	1	0	0	0	0	0	4
PBS-2	35	0.003	0	0	0	0.001	0	0	0	0.996	9
GK-2	36	0.25	0	0	0	0.75	0	0	0	0	ADMIXURE
CITH-W-29	37	1	0	0	0	0	0	0	0	0	1
CWS-7	38	0.006	0	0	0.993	0	0	0	0	0	4
BRTS-4	39	0.002	0	0	0	0.98	0	0	0	0.018	5
AMC-4	40	0.004	0	0	0	0	0	0	0	0.996	9
APS-16	41	0.003	0	0	0	0	0	0	0	0.997	9
CITH-W-24	42	0	0	0	0	1	0	0	0	0	5
CITH-W-7	43	0	0	0	0	1	0	0	0	0	5
KAS-2	44	0	0	0	0	0.999	0	0	0.001	0	5
CITH-W-22	45	0.999	0	0	0	0	0.001	0	0	0	1
GLS-1	46	0.002	0	0	0.22	0	0	0	0	0.778	ADMIXURE
GLS-2	47	0	0	0	0	0	0	0	0	1	9
GLS-3	48	1	0	0	0	0	0	0	0	0	1
GLS-4	49	0	0	0	0	0	0	0	0	1	9

Table 4 (continued)

Genotype	Code	K-1	K-2	Pop-3	Pop-4	Pop-5	Pop-6	Pop-7	Pop-8	Pop-9	Assignment to sub-population
Hamdan	50	0	0	0	0	0	0	0	0	1	9
Sulaiman	51	0	0	0.069	0	0	0	0	0	0.931	9
VL-2	52	0	0	0	0	0	0	0	0	1	9
Wussan-3	53	0	0	0	0	0.957	0.003	0	0	0.04	5
W-2	54	0.425	0.039	0	0.001	0.522	0	0	0	0.014	ADMIXURE
S-10	55	0	0	0	0	1	0	0	0	0	5
SKAU-003	56	0.001	0	0	0	0.999	0	0	0	0	5
SKAU-004	57	0.002	0	0	0	0.961	0	0	0.022	0.015	5
SKAU-0020	58	1	0	0	0	0	0	0	0	0	1
SKAU-0022	59	0.989	0	0	0	0.011	0	0	0	0	1
SKAU-008	60	0	0	0	0	1	0	0	0	0	5
SKAU-0023	61	0	0	0	0	0.974	0	0	0	0.026	5
LBPTR-1	62	0.011	0	0	0	0.989	0	0	0	0	5
BC-3	63	0.001	0	0	0	0.999	0	0	0	0	5
Shalimar-2	64	0	0	0	0	1	0	0	0	0	5
Wussan-8	65	0.723	0	0	0	0.193	0.07	0	0	0.014	ADMIXURE
Wussan-2	66	0.003	0	0	0	0.995	0.002	0	0	0	5
Wussan-25	67	0	0	0	0	1	0	0	0	0	5
Saloora-1	68	0.001	0	0	0	0	0.999	0	0	0	6
Wussan-1	69	0	0	0	0	0	1	0	0	0	6
Opex Cultury	70	0.006	0	0	0	0.002	0.993	0	0	0	6
Nugget	71	0	0	1	0	0	0	0	0	0	3
Victoria	72	0	0	1	0	0	0	0	0	0	3
Serr	73	0.001	0	0.999	0	0	0	0	0	0.001	3
Lake-English	74	0.928	0	0	0	0.001	0.025	0	0	0.046	1
Spannow	75	0	0	0	0	0	1	0	0	0	6
Paynee	76	0	0	0.961	0	0	0.039	0	0	0	3
Turtle	77	0	0	0	0	0	1	0	0	0	6
Pratab	78	0	0	0	0	0	1	0	0	0	6
Blackmore	79	0	0	0	0	0	1	0	0	0	6
Chenovo	80	0	0	0	0	0	1	0	0	0	6
Fragutte	81	0	0	0	0	0	1	0	0	0	6
Waterloo	82	0	0	0	0	0	1	0	0	0	6

Highest MI value (0.49) was scored with the primer WGA69 and the lowest value (0.21) for the primer WGA4. Highest Rp value (2.07) was scored with the primer WGA32 and the lowest value (0.51) for the primer WGA89. Allele number per locus varied from one to five (WGA202) with an average of 2.0 alleles per loci, which is lesser than 5.5 alleles per loci obtained in Sorrento walnut using SSR markers (Foroni et al. 2007) but are higher than 1.3 in *J. regia* with RAPDs (Nicese et al.1998). Two way Mantel test (Mantel 1967) was done between the SSR and RAPD data matrices. The correlation coefficient was estimated to be 0.099 between matrices generated by SSR and RAPD markers using the Mantel Test (t=1.69, P=0.95). Microsatellite markers are useful for genetic diversity analysis studies at varietal, species and genus level due to their sequence conservation at flanking regions (Hamza et al. 2004). Although a great number of population genetic studies have been conducted on Juglans species (Fornari et al. 2001; Victory et al. 2006), no genetic research based on the use of molecular markers has been published previously on *J. regia* populations in India. In our work, inter specific transferability of walnut microsatellites, originally selected to study the genetic diversity in *J. nigra* (Woeste et al. 2002), has been tested on *J. regia* varieties. The high level of variability observed at 26 SSR loci we used is consistent with results from studies carried out on different species (Carriero et al. 2002). For all loci, the observed number of alleles was lower than that reported in *J. nigra* (Victory et al. 2006), the species from which the markers were originally

Table 5 Heterozygosity and Fst values calculated for nine subpopulations of walnut

Sub-population (K)	Expected heterozygosity	Fst value
1	0.2615	0.1666
2	0.2596	0.2747
3	0.1396	0.5847
4	0.2021	0.3423
5	0.2314	0.2254
6	0.1941	0.3621
7	0.2341	0.3086
8	0.2442	0.2603
9	0.2715	0.1913
Average	0.226455556	0.301778

developed (Woeste et al. 2002). This may be because of the large number of samples and populations analyzed in previous *J. nigra* studies (Ana et al. 2000). The genetic parameters estimated from the microsatellite data indicated that there are substantial levels of genetic diversity in all sampled populations. This result is in accordance with the common observation of high levels of variation detected in wind pollinated, long-lived tree species (Streiff et al. 1998; Victory et al. 2006; Karimi et al. 2010). In the present study, SSR markers showed interspecific transferability and polymorphism but Gunn et al. 2010 showed that SSR markers lack interspecific differentiation between *J. regia* and *J. sigillata*.

Genetic relationship among the walnut accessions

In order to find out the genetic relationship between different walnut accessions, SSR and RAPD data sets were combined together and this combined data was used for analysis using NTSYSPC version 2.02e. The Jaccard's similarity coefficient ranged from 0.12 to 0.79 with an average of 0.49 among all the 82 accessions used. The genetic relationship between the accessions was clearly depicted in the dendrogram which was constructed from the DNA profile. The dendrogram showed that all the accessions formed four main clusters (Fig. 2). Within the first cluster, two subgroups consisting of eleven genotypes were observed. The second and third cluster consisted of 26 and 22 genotypes respectively. Fourth cluster consisted of 23 genotypes with various degree of sub-clustering. There was no relationship between the spatial and genetic proximity of the germplasm apart from few genotypes which were collected from the same location. Genotypes collected from various districts of Jammu and Kashmir showed diverse clustering; this may be due to out-crossing nature of walnut. The principal coordinate analysis (PCO) showed that the first three axes accounted for 57 % (49.58, 3.79 and 2.80 by Ist, 2nd and 3rd co-ordinate respectively) of total variation. The grouping shown in dendrogram was at par with that shown in 3D scatter (Fig. 3). The results obtained, using twenty RAPD and 13 SSR primers that yield a total of 88 polymorphic loci produced a unique fingerprint for each of the 82 walnut genotypes included in this study (Table 2 and Table 3) allowing a unequivocal identification of each genotype. Besides, the fingerprint of each genotype is defined by multiple RAPD and SSR bands presumably at multiple genetic loci. This is important for cultivar characterization since each cultivar is not defined by a single marker but by a set of several markers. This high level of polymorphism probably reflects the outcrossing nature of walnut since similar results have been obtained using combination of SSR and RAPD markers in other crops (Mir et al. 2008: Ebrahimi et al. 2011).

Model-based cluster analysis grouped 82 walnut genotypes into 9 genetically distinct sub-populations {K=9, having maximum natural log probability (−5150.4), which is proportional to the posterior probability (Fig. 4)}. Of the total 82 cultivars, only 6 (7.31 %) showed admixtures (membership probability <0.8) (Table 4). The expected heterozygosity, which measures the probability that two randomly chosen individual will be different (heterozygous) at a given locus ranges from 0.1396 in third sub-population to 0.2715 in 9th sub-population with an average of 0.227.

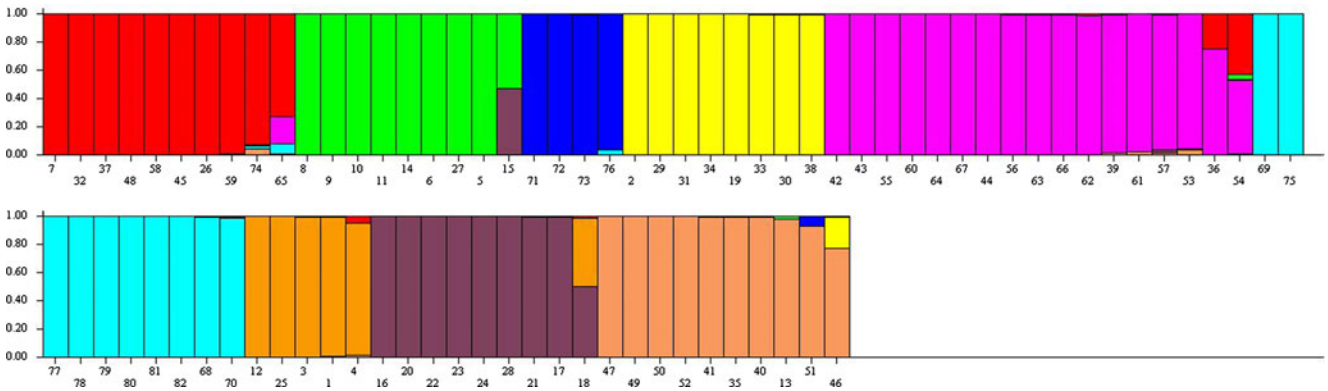

Fig. 5 Assignment of walnut samples to populations using STRUCTURE

Similarly, population differentiation measurements (Fst) range from 0.1666 (in first sub-population) to 0.5847 (in 3rd sub-population), with an average of 0.301 (Table 5). Similar type of results has been observed in Sorrento walnut using SSR primers highlighted the genetic distance between the Sorrento peninsula and Caserta groups, assigning the samples to two different clusters (or populations) corresponding closely, but not perfectly, to each sample's geographic origin (Foroni et al. 2007). The Structure analysis during the present study showed genetic divergence/differentiation in the walnut genotypes selected from different geographical areas (Fig. 5). This spatial divergence may be due to the selection pressure exercised during the breeding/selection process. The information gained through population structure analysis will prove useful in future in conducting association mapping in walnut for a variety of traits.

The results in this study illustrate the combined effectiveness of the SSR and RAPD techniques in discriminating the walnut genotypes analyzed. The SSR and RAPD data has separated the accessions into four major genetic similarity groups. However, the significance of this bifurcation is currently unknown. But the current findings can be exploited in future once these findings will be correlated with traits of interest. Bearing of all these accessions has not been started yet once the bearing will start nut characteristics will be correlated with the data generated and will be used in further crop improvement programmes in walnut. However, this was not unexpected as the genotypes used in this study were selected from the natural walnut populations confined in one geographic region within the North West Himalayan region of Jammu and Kashmir, India. Both cluster analysis and STRUCTURE indicated that the walnut genotypes are genetically different. Whether they will remain distinct will depend on how the forces of selection and gene flow interact. These findings would provide valuable information for decision making in future walnut breeding studies as well as germplasm management activities to maximize genetic diversity in walnut germplasm.

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