The Journal of Animal & Plant Sciences, 29(2): 2019, Page: 500-509
ISSN: 1018-7081

GENETIC RELATIONSHIP ANALYSIS OF WALNUT (*JUGLANS REGIA* L.) GROWN IN WESTERN HIMALAYIAN IN AZAD KASHMIR, PAKISTAN

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ABSTRACT

Walnut (*Juglans regia* L.) is exceptionally widespread nut tree in the world and grow best in Azad Jammu and Kashmir and northern areas of Pakistan is expected to have considerable inter and intra-species genetic variability due to adaptation to the various environmental conditions. We focused to evaluate the genetic characterization of sixty four walnut cultivars including their genetic relationship and distance collected from sixteen selected sites of Neelum valley Azad Jammu and Kashmir in Himalayan region of Pakistan. 35 decamer primers were used which based on discernable bands yielded markable amplification patterns. Cluster analysis and principal component analysis (PCA) separated the sixty four cultivars into six main groups whose alterations were related to their pedigree from each other. In our study we found 44% cultivars had marker alleles associated with the presence of WGA gene. 30% had at VAM gene. 18% had at JM gene. Six markers did not show polymorphism (were not validated). Frequencies of JM and VAM genes need to be improved in future walnut cultivars. Advanced walnut cultivars may routinely be investigated for the presence or absence of their special variety genes by the use of DNA markers. Kagazi akhrot need to be pyramided through MAS to extend the genetic base of future varieties. Moreover, fieldwork study revealed that local walnut germplasm are being eroded from some regions due to urbanization, agriculture, grazing, wood harvesting and natural calamities (earth quake and land sliding) that depicted genetic drift. The insightful difference among walnut accessions confirmed that they did not develop locally but were introduced from abroad germplasm whereas, close relationship showed that they are probably from same population with common ancestry.

Key words: *Juglans regia*; genetic characterization; SSRs; walnut breeding; germplasm conservation.

INTRODUCTION

Walnut (*Juglans regia* L.) popularly known as “akhrot”, is a vital medicinal plant with a potency to cure various diseases in traditional medicines. Its different parts have valuable dietary applications and it is cultivated in diverse regions of the world (You *et al*., 2012). Genus *Juglans* is placed in family Juglandaceae and it includes about 7 to 45 species (Aradhya *et al*., 2004), all having 32 chromosomes and located in temperate zone (Robert, 1930). The phytochemical profile of the plant makes it worth using in medication all over the world and in Azad Jammu and Kashmir. Various physiologically important phytochemicals (oils, quinines, tannins, basic unsaturated fats including cis-linoleic etc.) and compounds like mineral (potassium, phosphorus, magnesium and iron), water, cellulose and kernel having different functionalities have been reported from it.

In Neelum valley Azad Kashmir, Pakistan generally three varieties of walnuts are cultivated. Locally these varieties are known as Kagazi, Wonth and Burzul. *Juglans regia* is well-known plantation in Azad Jammu and Kashmir and different factors like various geological properties, roughness and interspecific cross fertilization incorporate hereditary inconstancy in its germplasm, wild variety of walnut at elevation range from 1550 m to 3000 m (Khan *et al*., 2010).

The term genetic diversity means variations in inherited character amongst alleles in various members of varieties which plays a vital part in evolution of species that allow to accept diverse environments. It is a consequence of mutation, reshuffling and natural selection of individuals that modified the population to adapt to extreme conditions. Genetic relationship among species is fundamental to all streams of biology. All kind of biological studies must start with the recognition of the identities of the subject organisms. The fittest individuals of the species will continue to produce next generations (NBII, 2011). New varieties a hybrids can developed from genetic variability in plants through molecular characterization and morphological traits (Vetriventhan and Srinivasan, 2015).

From the literature of walnut research in Azad Jammu and Kashmir Pakistan the systematic information is hardly adequate for the full development of walnut as a future plant. Proper systematic and diversity studies on

walnut are an immediate necessity for three important reasons: i) it is necessary to understand the genetic relationships of various species of walnut in order to conduct planned breeding of new cultivars, ii) identification of new species or assignment of correct taxonomic status to some doubtful species is greatly beneficial for making decisions about the use of new germplasm, iii) Knowledge of the genetic diversity in walnut is useful in conducting an efficient collection and conservation of germplasm resources. This is especially important now as biological diversity of medicinally and economically important plants is shrinking all over the world (Karim *et al*., 2012). It would be a great loss if there is no effort made to protect and understand the available germplasm resources of walnut as it is one of most important economical plant species of Western Himalaya, Azad Jammu and Kashmir (Simon and Potter, 2001). Molecular approaches to assess genetic diversity are becoming more popular because discrete and accurate information can be obtained easily (Vetriventhan and Srinivasan, 2015). In this study, the genetic variations among walnut were evaluated by using SSR markers which is an efficient and authentic method to quantify the genetic relationships of various plant species (Huang *et al.*, 2008).

MATERIALS AND METHODS

Study Area and Sampling Sites: Neelum valley is situated at the high altitude in the Himalayan range of North Pakistan, between 73º.463204 North latitude and 34º.371527S East longitude (Fig. 2.1). The topography of the study site is uneven, characterized by steep mountain slopes varying from 3,200 ft. to 20,750 ft. The climate of the study area is temperate to sub alpine with four distinct seasons with relatively long winters from October to May (Dar, 2003).

Collection of Plant Material: Sixteen sites of Neelum valley Azad Jammu and Kashmir (Table 2.1) were selected for the collection of *Juglans regia* L., seeds commonly known as walnut and each selected site then grown in botanical nursery of the University of Azad Jammu and Kashmir Muzaffarabad at Chehla campus. From the healthy and developed branches of the tree cover grown in the nursery leaf samples were collected and packed in re-sealable plastic bags that contained silica gel crystals, tagged properly and taken to Laboratory of National Agricultural Research Center (NARC) Islamabad for more analysis. These samples were kept at 4 °C in refrigerator until required.

DNA isolation and amplification: Thirty five previously reported amplified SSR microsatellite markers were used for the quantification of genetic diversity among sixty four walnut cultivars (Table 2.2). DNA extraction and polymerase chain reaction were carried out by the protocol which is used in Doyle and Doyle (1987) with few modifications. Amplified PCR segments were determined on agarose gel 1.5 and 2 % and were then stained through ethidium bromide. At NIGAB the bands were then visualized under UV light in the gel documentation system and the presence or absence of bands associated with marker genes were scored by comparing with the DNA ladder with known band sizes.

Table 2.2: List of SSR Primer Pairs and their PCR profiles used for genetic characterization of *Juglans regia* L.

Figure 2.1: Distribution Map of *J. regia* L. Germplasm Collection Sites of Neelum Valley Azad Jammu and Kashmir.

Table 2.1. *Juglans regia* L. Germplasm Collection Sites of Neelum Valley Azad Jammu and Kashmir.

#	Localities	Altitude (Ft)	Latitude	Longitude
1	Chehliana	3710	34º25.172N	73º48.077E
2	Barian	4129	34º26.221N	73º48.544E
3	Jura	4113	34º29.751N	73º50.181E
4	Sandok	4316	34º31.234N	73º50.310E
5	Shahkot	4428	34º34.016N	73º53.126E
6	Danger	4726	34º36.125N	73º55.387E
7	Keran	5142	34º39.535N	73º55.387E
8	Neelum village	6182	34º39.572N	73º56.173E
9	Lawat	5545	34º43.333N	74º00.086E
10	Dowarian	6114	34º43.086N	74º03.495E
11	Changan	6162	34º42.491N	74º05.126E
12	Dudhnial	6264	34º42.544N	74º07.517E
13	Khawja seri	6145	34º46.333N	74º08.542E
14	Sharda	6310	34º47.319N	74º11.315E
15	Kel	7153	34º49.307N	74º21.002E
16	Arangkel	7768	34º48.277N	74º20.445E

#	Locus	Primer Sequences ((5'-3')	PCR profile
1	WGA005	F: CAGTTTGTCCCACACCTCCT R: AACCCATGGTGAGAGTGAGC	94°C: 3min 1 cycle, 94°C: 30s, 50 °C: 30s, 72°C: 1min 35 cycles, 72°C 10min 1 cycle
2	WGA032	F: CTCGGTAAGCCACACCAATT R: ACGGGCAGTGTATGCATGTA	94°C : 3min 1 cycle, 94°C: 30s, 57°C: 30s, 72°C: 1min 35 cycles, 72°C: 7min 1 cycle
3	WGA069	F: TTAGTTAGCAAACCCACCCG R: AGATGCACAGACCAACCCTC	94°C: 4min 1 cycle, 94°C:40s, 57°C: 40s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
4	WGA118	F: TGTGCTCTGATCTGCCTCC R: GGGTGGGTGAAAAGTAGCAA	94°C: 3min 1 cycle, 94°C:30s, 57°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
5	WGA202	F: CCCATCTACCGTTGCACTTT R: GCTGGTGGTTCTATCATGGG	94°C : 3min 1 cycle, 94°C: 30s, 52°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
6	WGA276	F: CTCACTTTCTCGGCTCTTCC R: GGTCTTATGTGGGCAGTCGT	94°C : 3min 1 cycle, 94°C: 30s, 52°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
7	WGA332	F: ACGTCGTTCTGCACTCCTCT R: GCCACAGGAACGAGTGCT	94°C : 3min 1 cycle, 94°C: 30s, 57°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
8	WGA349	F:GTGGCGAAAGTTTATTTTTGC R: ACAAATGCACAGCAGCAAAC	94°C : 3min 1 cycle, 94°C: 30s, 57°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
9	WGA027	F: AACCCTACAACGCCTTGATG R: TGCTCAGGCTCCACTTCC	94°C : 3min 1 cycle, 94°C: 30s, 57°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
10	WGA071	F: ACCCGAGAGATTTCTGGGAT R: GGACCCAGCTCCTCTTCTCT	94°C : 3min 1 cycle, 94°C: 30s, 57°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
11	WGA089	F: ACCCATCTTTCACGTGTGTG R: TGCCTAATTAGCAATTTCCA	94°C : 3min 1 cycle, 94°C: 30s, 60°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
12	WGA376	F: GCCCTCAAAGTGATGAACGT R: TCATCCATATTTACCCCTTTCG	94°C : 3min 1 cycle, 94°C: 30s, 60°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
13	WGA331	F: TCCCCCTGAAATCTTCTCCT R: CGGTGGTGTAAGGCAAATG	94°C : 3min 1 cycle, 94°C: 30s, 65°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
14	WGA321	F: TCCAATCGAAACTCCAAAGG R: GTCCAAAGACGATGATGGA	94°C: 3min 1 cycle, 94°C:30s, 60°C: 30s, 72°C: 60s 35 cycles, 72°C: 10min 1 cycle
15	WGA225	F: AATCCCTCTCCTGGGCAG R: TGTTCCACTGACCACTTCCA	94°C : 3min 1 cycle, 94°C: 30s, 51°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
16	VAM-8	F: CGTTTGAATTAGTGTGA R: CTTGACCTTTCACAGTC	94°C : 3min 1 cycle, 94°C: 30s, 50°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
17	VAM-1	F: TCTCTGGCTCTTCACGG R: TCGTCTTCCTTCTCGCA	94°C : 3min 1 cycle, 94°C: 30s, 65°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle

18	JM6880	F: TCCTTCTGTGTGAGTGCGTG R: GGTCAGGTGAGTGGAGCAAA	94°C: 3min 1 cycle, 94°C:30s, 60°C: 30s, 72°C: 60s 35 cycles, 72°C: 10min 1 cycle
19	JM61666	F: AACTGTTGCCGGAGCTTTCT R: TGGGATAACACCACATGCAGT	94°C : 3min 1 cycle, 94°C: 30s, 57°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
20	JM78331	F: GCAGTGCGCTCCTTTTTCAA R: TTCTCGGGTTGAAGCCACAA	94°C : 3min 1 cycle, 94°C: 30s, 60°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
21	JM77590	F:TGCATGTAAGGGAGCATATGT R: TGCATGAGAAGGTTGTTGGACT	94°C : 3min 1 cycle, 94°C: 30s, 60°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
22	JM59276	F: GAAGCATGCCAACCAAAGCA R: CAGAGGCATTACAGGCAGCT	94°C : 3min 1 cycle, 94°C: 30s, 52°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
23	JM7515	F: ACTGCTTGCGAGATTGCTTG R: CAAATAGGGCAGCCTCGTCT	94°C : 3min 1 cycle, 94°C: 30s, 55°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
24	JM7090	F: TGCATGTAAGGGAGCATATGT R: TGCATGAGAAGGTTGTTGGACT	94°C : 3min 1 cycle, 94°C: 30s, 56°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
25	JM8463	F: GTACCTCCCGCATCCAACAA R: CATTCACGATGCAGACCCTT	94°C : 3min 1 cycle, 94°C: 30s, 55°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
26	JM9158	F: TCAGACAAAGTTGAGGGCTGA R:TGTGAAAAGTCAGGCAGCCA	94°C : 3min 1 cycle, 94°C: 30s, 60°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
27	JM5446	F: ATGCATGCAGCTCCTACCTC R: GGACGTGTCCTGGGTTTTCA	94°C : 3min 1 cycle, 94°C: 30s, 57°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
28	JM2039	F: GAAGGACCTGGATGGAACCG R: GACACCTCCCCATCAAGTGT	94°C : 3min 1 cycle, 94°C: 30s, 60°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
29	JM5969	F: ACAATAGTCTCTGCACCGCC R: AGCTTGTACTTACCGCCGAC	94°C : 3min 1 cycle, 94°C: 30s, 50°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
30	JM1672	F: TGCCAGGGGAGCAAGAAAAA R: CTCCCATTGCGAGTCTCCAT	94°C : 3min 1 cycle, 94°C: 30s, 58°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
31	JM8882	F: CCTGTGGGCGTAACTTTCCA R: AATCCCCAATCCCCCATTTT	94°C : 3min 1 cycle, 94°C: 30s, 60°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
32	JM9040	F: CGGATGGCTTATGCGGGTAT R: TCCTGGCTGAGAGAGGAGAC	94°C : 3min 1 cycle, 94°C: 30s, 57°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
33	JM3976	F: GATCAATCGCTCCTACCCCG R:TCAATGAAACCCCAACACCCA	94°C : 3min 1 cycle, 94°C: 30s, 60°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
34	JM7109	F: TCAATGAAACCCCAACACCC R: GATCAATCGCTCCTACCCCG	94°C : 3min 1 cycle, 94°C: 30s, 57°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle
35	JM5272	F: GAGCGAGGGAGTTTGGAACT R: TGACGGGTTTTTGCGAGTTG	94°C : 3min 1 cycle, 94°C: 30s, 60°C: 30s, 72°C: 1min 35 cycles, 72°C: 10min 1 cycle

Statistical Analysis: All SSR bands were scored as bi-variant 1-0 data matrix (Appendix 36). Loci / alleles (bands) were recorded as absent (0) or present (1). For statistical exploration of the microsatellites, all the scorable bands were taken as a single allele / locus.

PIC (polymorphism information content) was used to determine the efficacy of marker. It was measured by using the following formula given by Smith *et al*., 2000.

$$PIC = 1-\sum fi^2$$

Fi = the i^{th} allele frequency

Multivariate ordination examination of data was carried out by PCA (Principal component analysis) and CA (Cluster analysis) by using past software version 4.0. Procedure which was used (Nei, 1973) is following:

$$GD = 1 - dxy/dx + dy-dxy$$

Where GD = The Genetic Distance between two genotypes

dxy = The common number of bands in two genotypes

dy = the totality of bands

dx = The total bands in 1st genotype

By using bar graph technique the data on the bases of Mean, Standard error, Variance and Standard deviation of each marker was analyzed (Williams *et al*., 1990).

RESULTS AND DISCUSSION

The seed samples of 64 cultivars of walnut (*Juglans regia* L.), collected from the 16 sites of Neelum Valley Azad Jammu and Kashmir were grown in botanical nursery of the University of Azad Jammu and Kashmir Muzaffarabad at Chehla campus and characterized by using SSRs techniques as describe by Fatemeh *et al*., 2014. The sampling sites were carefully chosen on the basis of variations in micro-climatic conditions and presence of different walnut ancestries. From the selected areas the genetic

characterization of walnut has not been carried out before and its cultivation is restricted to certain areas only, by making possible the use of selected and well-established local varieties. Naturally millions of wild walnut trees are growing in Azad Jammu and Kashmir, so for the cultivation of walnut this region have been selected as the representative of the most promising environments Ali *et al*., 2010. It would be a great loss if there is no effort made to protect and understand the available germplasm resources of walnut as it is one of most important economical plant species of Western Himalaya, Azad Jammu and Kashmir (Simon and Potter, 2001).

Thirty five primer combinations (Table 2.2) used in the present study were chosen based on the feature of producing the most variation. Each pair of SSR primers produced the variable number of noticeable fragments. Depending upon the primer combinations the percentage of polymorphisms ranged from 9 to 95 percent. Out of 64 accessions selective amplification of marker WGA202 yielded 57 fragments (89%), WGA069 depicted 61 fragments (95%), WGA005 produced 52 fragments (81%), WGA032 showed 44 fragments (69%), WGA118 represents 12 fragments (19%), WGA089 exhibited 21 fragments (33%), WGA349 contained 24 fragments (38%), WGA071 amplified 10 fragments (19%), WGA225 held 16 fragments (30%), WGA331 yielded 35 fragments (55%), WGA376 yielded 33 fragments (52%), WGA276 yielded 6 fragments (9%), WGA321 yielded 15 fragments (23%), WGA332 yielded 22 fragments (34%), WGA027 yielded 15 fragments (23%), VAM-1 yielded 15 fragments (23%), VAM-8 yielded 24 fragments (38%), JM61666 yielded 29 fragments (45%), JM59276 yielded 15 fragments (23%), JM5272 yielded 19 fragments (30%), JM78331 yielded 11 fragments (17%), JM68820 yielded 13 fragments (20%), JM3882 yielded 11fragments (17%), JM77590 yielded 17 fragments (27%), JM9158 yielded 6 fragments (9%), JM2039 yielded 7 fragments (11%), JM1672 yielded 29 fragments (45%), JM7515 yielded 19 fragments (30%), JM5446 yielded 15 fragments (23%), JM8463 yielded 7 fragments (11%), JM7109 yielded 13 fragments (20%), JM3979 yielded 16 fragments (30%), JM5969 yielded 21 fragments (33%), JM9040 yielded 15 fragments (23%) and JM7090 yielded 8 variable fragments (13%). This study ran the analysis on 2240 alleles and concatenated the runs, of which 733 (33%) were variable.

SSRs markers technology have been used previously for the relationship and genetic diversity measurements in walnut cultivars. The walnut samples were also investigated by (Fatemeh *et al*., 2014; Foroni *et al*., 2006; Zhuang *et al*., 2011; Ehteshamnia *et al*., 2009) that comprised of several important cultivars from Middle East, western Mediterranean region, Greece and Turkey. The polymorphism level revealed by SSR transferrable markers probably depends on genetic distance and the phylogenetic distance within the cultivars due to their nature of outcrossing. Foroni *et al*., (2005) characterized the germplasm of walnut using SSR transferable markers and detected from 22 walnut genotypes 33 putative alleles, 9 alleles from these were distinctive for one genotype. The expected heterozygosity of 0.72 as reported by Galli *et al*., (2005), the high level of polymorphism with average PIC value, which are basically the same, probably reveals the walnut's outcrossing nature and using combination of RAPD and SSR in other plants similar results have been obtained (Ebrahimi *et al*., 2011; Mir *et al*., 2008). Higher values of expected heterozygosity (0.47-0.75 with an average of 0.63) were observed by (Mahmoodi *et al*., 2013) in walnut due to higher levels of polymorphism between the genotypes they used. In the present study the overall observed polymorphism ratio among 64 samples collected from walnut tree of Azad Jammu and Kashmir is analogous with these examination. Appropriate natural surroundings and little anthropogenic activities may be the principle explanations of the diversity inside these walnut populaces. Different atmosphere and biological conditions in these regions have prompted to a high genetic and morphological characteristics of walnut. The present results are in agreement with the results obtained by (Peng *et al*., 2005). The parameters of genetics measured from the SSRs microsatellite data designated that in all experimented populations there is a significant degree of genetic diversity. Higher heterozygosity values (0.139-0.2715 with an average of 0.227) for 82 walnut cultivars due to the presence of higher polymorphism in the population. The high level of genetic variation in the samples of walnut is due to the propagation through seeds, high heterozygosis and dichogamy (Aslantas, 2006; Germain, 2004). In Turkey Dogan *et al*., (2005) found the high amount of genetic diversity based on fruit characters in walnut.

On the basis of Wards cluster analysis and SSRs data the dendrogram were created as presented in Figure 3.1. The dendrogram revealed 5 discrete groups that clearly separated walnut accessions from each other and could be seen in one main branch of dendrogram. The dendrogram indicated that group I included 17 walnut samples collected from Sharda-4, Kel-3, Barian-4, Jura-4, Shahkot-1, Danger-1, Shahkot-3, Neelum village-4, Sharda-1, Kel-4, Jura-3, Kel-1, Khawaja seri-2, Khawaja seri-3, Sharda-3, Sharda-2 and Barian-2. In this group all the samples were cultivars except samples Sharda-4, Kel-3. These walnut samples were essentially two landrace genotypes of Neelum Valley and were actually separated as singleton from the sub-clusters. Similarly group II comprises of ten genotypes of walnut which were taken from Shahkot-4, Dowarian-1, Dowarian-2, Dowarian-4, Jura-1, Jura-2, Shahkot-3, Dowarian-3, Danger-2 and Danger-3. In this group also all the samples were cultivar except the samples from Jura-2, Shahkot-3. These walnut

samples were also the 2 landrace genotypes of Neelum Valley and were essentially divided as singleton from the sub-clusters. All these walnut collections were taken from distinct sites which were located at diverse altitude in the study area (Table 2.1). In the groups I and II all the 27 samples of walnut clustered with each other due to genetic relationship with some subgroups. Now a days vegetative propagation of walnut (*J. regia*) has been carried out through grafting or top working on existing walnut trees in Azad Kashmir. They may have a limited area of diffusion and a common ancestry. It is also worth noticing, that some accessions showed less diversity suggested homogeneity that indicated genetic flux or that they had a common origin (Amel *et al*., 2004), in the region from where *J. regia* accessions were collected.

Certain degree of relationship between walnut cultivars is perhaps due to the seed based propagation system of walnut is Neelum valley Azad Jammu and Kashmir, taking into account that walnut trees needs more than twelve year for their maturation. The current result in harmony with previous study of Ebrahimi *et al*., (2010, 2011). Not at all like various tree, the walnut crop has been propagated by seed merely all over its history a high rate of the hereditary change ability of an area is kept up inside walnut genotypes.

The dendrogram revealed that group III comprised of ten walnut accessions collected from Arangkel-3, Arangkel-4, Arangkel-2, Chehliana-2, Changan-1, Changan-3, Changan-4, Sandok-l, Sandok-2, Sandok-1 and Khawaja seri-1. Walnut sample of Sandok-l in main cluster represented as singleton.

Group IV includes ten genotypes sampled from distinct sites of Neelum Valley i.e. Lawat-1, Lawat-2, Dudhnial-4, Neelum village-1, Shahkot-2, Dudhnial-1, Keran-4, Chehliana-2, Chehliana-1 and Neelum village-3 cultivars. The group V comprised sixteen cultivars contained Dudhnial-3, lawat-3, Chehliana-3, Keran-1, Barian-3, Danger-4, Khawaja seri-4, Kel-2, Lawat-4, Arangkel-1, Keran-2, Keran-3, Dudhnial-2, Barian-1, Chehliana-4 and Neelum village-2 cultivars. Similar groups had better homogeneity as compared to those cultivars which are branched with other groups and sub clusters. Thus above clustering explanation indicated that most of the cultivars are clustered based on their selection sites. This may be due to their continuous multiplication at one site for many years and all the selections from that site are more or less similar in genetic constitution. Although the difference in geographical locations is not more the use of germplasm for further multiplication in the region is very much local. Development of new varieties in the region is due to seedling origin. Walnut being heterogeneous leads to development of new genotypes after sowing of seeds.

Electrophoretic markers including SSR markers appear to be independent of physiology and cultivar morphology and have significant benefits over morphological approaches of variety and species identification as they are relatively rapid and cheap, eliminate the need to wait for the maturity of trees and is mainly unaffected by growth conditions. Recently, with the applications of molecular biological techniques, the number of markers existing in plants has intensely amplified. Now it is possible to identify the variations at the DNA level with the help of these techniques, which may not express into proteins to differentiate phenotypic diversity. But these techniques are not accessible in developing countries for the most of researchers because they need enough capital outlay (Thanh and Hirata, 2002).

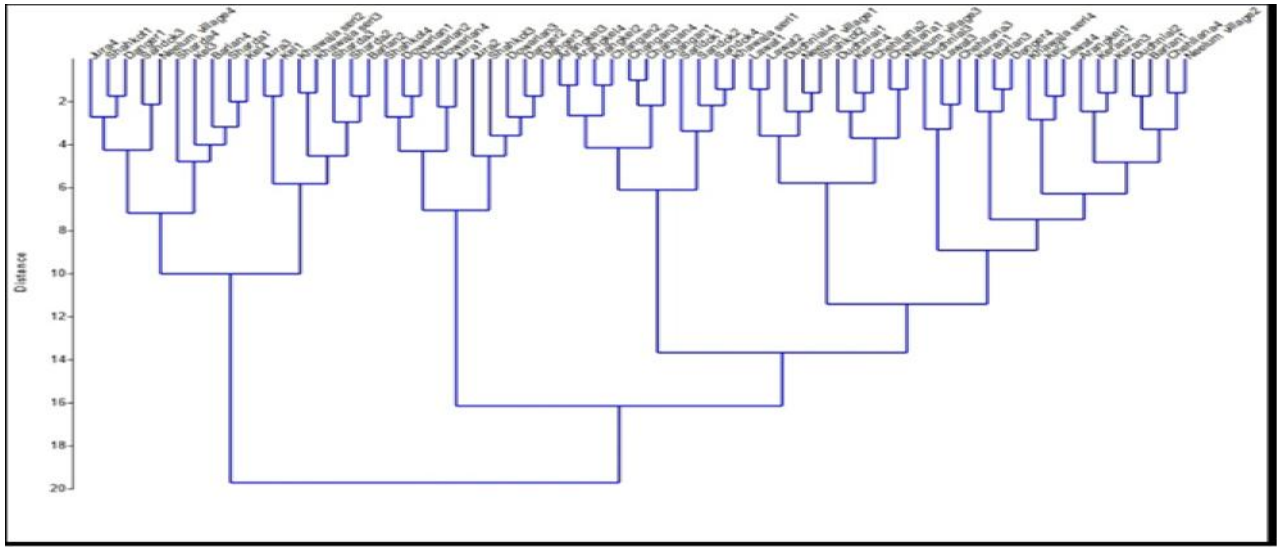

Figure 3.1: Dendrogram revealing genetic variation among walnut cultivars of Neelum valley Azad Jammu and Kashmir based on the SSR and cluster analysis (Wards).

Principal Component Analysis (PCA): In the Current study PCA determined the likeness of the obtained information about the genetic diversity and population structure among walnut cultivars using SSR technique. The analysis with SSR seemed to be efficient in verifying the cultivars status of walnut. In PC 1 with eigenvalue of 0.653659 accounted for 10.607% of the total variability followed by PC 2 with eigenvalue 0.6069 accounted for 9.8483% of the total variation observed among the 64 walnut cultivars. PC 3 had eigenvalue of 0.501985 with total variability of 8.1459%. PC 4, PC 5 and PC 6 on the other hand had eigenvalues of 0.435051, 0.383548 and 0.331439 respectively and accounted for 7.0597%, 6.224% and 5.3784% of the total variability. Eigenvalue 0.31227, 0.295471, 0.252449 and 0.242178 exhibited as 5.0673 percent in WGA349, 4.7947 percent in WGA071, 4.0966 percent in WGA225 and 4.0966 percent in WGA331. Components WGA376, WGA276, WGA321, WGA332, WGA027, VAM-1 and VAM-8 with eigenvalue of 0.200129, 0.197398, 0.183519, 0.176056, 0.159996, 0.144514 and 0.140442 showed total variability of 3.2476%, 3.2032%, 2.978%, 2.8569%, 2.5963%, 2.3451% and 2.279% respectively. JM59276, JM5272, JM78331, JM68820, JM3882 showed 1.6858, 1.5481, 1.3888, 1.3442 and 1.1698% of variance with eigenvalue 0.103889, 0.095401, 0.085585, 0.082838 and 0.072091 respectively. In JM77590 with eigenvalue of 0.059314 accounted for 0.96251% of the total variability, JM9158 with eigenvalue 0.056697 accounted for 0.92003% of the total variation observed among the 64 walnut cultivars. JM2039 had eigenvalue of 0.052121with total variability of 0.84578%. Total variance with 0.69612%, 0.54878%, 0.52235%, 0.47359% and 0.4004% with eigenvalue 0.042898, 0.033819, 0.03219, 0.029185 and 0.024675 exhibited by JM1672, JM7515, JM5446, JM8463 and JM7109. In JM3979 with epigenic value of 0.022297 accounted for 0.36182% out of the total variability, JM5969 with eigenvalue 0.015831 accounted for 0.25689% of the total variation observed among the 64 walnut cultivars. JM9040 had eigenvalue of 0.013478 with total variability of 0.21871%. JM7090 with eigenvalue 0.012408 with total variance 0.20136%.

The ordination of the walnut revealed that cultivars WGA202, WGA069, WGA005, WGA032 and WGA118 were distinct for the total percentage of variance. The ordination of the characters on PC1 and PC2 showed that some of the variables were highly inter-correlated as seen in the overlapping pattern of the variables. Also, the variables accounting for most of the variation can be seen on the right and left hand quadrant of the plot. Present finding were in harmony with (Jonah *et al*., 2014).

Meanwhile, in the lower two quadrate of the projections a compact group of *J. regia* accessions as compared to upper two quadrates. PC1 and PC2 elucidated greater variations among loose groups of walnut cultivars in quadrate projections whereas compact group of *J. regia* cultivars exhibited less variations. These results in agreements with the previous study (Mujaju and Chakuya, 2008).

The SSR results attained led to the inference that 64 walnut cultivars were divergent. In the actual use, the measurements of genetic variations among existing walnut genotypes is mandatory to monitor the germplasm conservation and breeding program of walnut while ensuring that the extensive diversity of germplasm is well-kept in the study areas.

Table 3.2. Principal Component Analysis (PCA) and cluster analysis of 64 Walnut Cultivars based on principal component.

Principal Components Axis	Eigenvalue	% Variance	Principal components Axis	Eigenvalue	% Variance
PC1	0.653659	10.607	PC19	0.103889	1.6858
PC2	0.6069	9.8483	PC20	0.095401	1.5481
PC3	0.501985	8.1459	PC21	0.085585	1.3888
PC4	0.435051	7.0597	PC22	0.082838	1.3442
PC5	0.383548	6.224	PC23	0.072091	1.1698
PC6	0.331439	5.3784	PC24	0.059314	0.96251
PC7	0.31227	5.0673	PC25	0.056697	0.92003
PC8	0.295471	4.7947	PC26	0.052121	0.84578
PC9	0.252449	4.0966	PC27	0.042898	0.69612
PC10	0.242178	4.0966	PC28	0.033819	0.54878
PC11	0.200129	3.2476	PC29	0.03219	0.52235
PC12	0.197398	3.2032	PC30	0.029185	0.47359
PC13	0.183519	2.978	PC31	0.024675	0.4004
PC14	0.176056	2.8569	PC32	0.022297	0.36182
PC15	0.159996	2.5963	PC33	0.015831	0.25689
PC16	0.144514	2.3451	PC34	0.013478	0.21871
PC17	0.140442	2.279	PC35	0.012408	0.20136
PC18	0.11073	1.7969			

Figure 3.9. PCA (Principal Component Analysis) describing the total cultivars among walnut by using SSR analysis data.

Conclusion: The data about genetic diversity is necessary for the development of suitable germplasm, ecology and existence in different set of environment. The data obtained from the present study is valuable for the walnut germplasm development as the molecular technique described the important differences of walnut genotypes in the study area and walnut populations. Therefore, this data and knowledge provide new visions concerning the amount of genetic diversity between and within populations. The present study specifies the genetic diversity, relationships of walnut using SSRs and obtained data on population structure. The SSRs analysis proved to be an effective technique in authenticating the variety rank of walnut genotypes. In actual practice, the quantification of genetic diversity in walnut populations is required to lead the on-going rising of *J. regia* while ensuring that an extensive genetic variations are well-kept in the study areas. For the establishment of *J. regia* nursery industry, this data and knowledge can be useful to reduce replication of the clones and approve the diversity of the promulgated clones for growth on *J. regia*.

Acknowledgements: We gratefully acknowledge the Higher Education Commission of Pakistan for awarding an NRPU research to fund this research. Also, we thank the University of Azad Jammu and Kashmir for every support and facilitation for this research. The authors are thankful to Dr. Ghulam M. Ali (senior scientific officer (SSO) Director NIGAB, Member coordination and monitoring PARC, Islamabad) for providing collaborative support from his organization.

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