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ESTIMATION OF GENETIC DIVERSITY IN WALNUT

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Abstract

Juglans regia L. belonging to the family Juglandaceae inhabits the northern parts of Pakistan. Biochemical analysis of the plant is not well documented in the country. Present research was the first documented attempt to study total seed storage proteins in 20 genotypes of *Juglans regia* collected from Swat, Dir and Chitral areas. A protocol was optimized for extraction and separation of seed storage protein from unprocessed seeds. Comparatively simple banding pattern (as compared to legumes and cereals) was observed in *Juglans regia* L. A total of 114 protein loci were detected in 20 genotypes giving an average of approximately 7 alleles per genotype. Genetic distances estimated during present study range from "0" to "60". Medium values of Genetic Distances (GD_{max}= 60%) was estimated among three comparisons. Twenty genotypes were grouped in three clusters based on dendrogram analysis. Genotypes collected from Chitral valley were predominantly grouped in one cluster.

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.

In Pakistan, it is cultivated in most villages between 925-3000 m altitude and it also occurs as wild. Wild trees of walnut are found commonly in mixed deciduous and coniferous forests at altitude ranging from 1550 m to 3000 m. The wild walnuts are found in Kaghan valley. Ayubia National Park, Swat, (especially the region of Kalam and up the 2 tributaries of the Swat River beyond Kalam i.e., along the roads to Ushu and to Utror). In some regions walnut trees grow to enormous sizes. The largest one that is located in Bumborait valley (Chitral) measures 6.8m in girth. The height of the largest trees ranges between 40-50 m (Rashid, 1998). *Juglans regia* is a long lived species and is not difficult to find trees of 100-200 years old, and even some 1,000 years old (Leslie & McGranahan, 1998).

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The nuts of the wild trees are smaller, rounder, and have a much thicker shell. There is an enormous variability in nut traits e.g., nut sizes (small to very large), shape, shell thickness (very thin to very thick), the degree of shell seal, the colour of kernals, and the taste and appearance of kernels (Rashid, 1998).

Over 1,000,000 metric tons walnuts are produced annually in the world (Anon., 2004). In Pakistan Walnut Production is about 20,000 tons per year. Walnuts contain 16.66% protein and 66.90% lipids on a dry weight basis. Albumin, globulin, prolamin and glutelin are the major proteins which accounts for 6.81, 17.57, 5.33 and 70.11%, respectively. (Sze-tao & Sathe, 1995).

For studies on population genetic structure, protein (especially seed storage proteins) assays have been widely used for a number of commercially important species. The technique of Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is widely used for separation of seed storage protein variants due to its reliability and simplicity in describing the genetic structure of crop germplasm (Murphy *et al*., 1990). Seed storage protein profiles have also been used to study evolutionary relationship of several crop plants (Rao *et al*., 1992; Das & Mukharjee, 1995). Although some work has been reported in literature describing protein analysis in walnut but unfortunately no such work has been done in Pakistan. Specific objective of the present research was estimation of Genetic diversity in local collection of walnut based on total storage protein.

Materials and Methods

Plant material: Twenty unprocessed seed germplasm of *Juglans regia* were collected from three different areas of northern NWFP. Ten germplasm were collected from the upper areas of District Swat, (seven germplasm from Senay, (Miandam), (35^0 03" 17.76"N and 72^0 33' 15.59"E) one each from Shin, Nawakaley (35^0 01' 32.61"N and 72^0 28'34.76"E) and Gulibagh (34^0 52' 29.00" N and 72^0 26' 05.00" E). Three more germplasm were collected from Dir ($35^0$20'36.00"N and $71^0$50' 21.78"E), and seven collected from Chitral (35^0 53' 15.00" N and $71^0$48' 01.00"E) areas. The description of germplasm is given in Table 1.

Method of protein extraction: For the estimation of genetic diversity among 20 local *Juglans regia* germplasm, the seed from each genotype were ground and converted into a fine powder (0.2g) with the help of needle in the eppendorf tube. Protein was extracted from grinded powder by adding 1500 µl extraction buffers (2M KPO_4. 0.5 M EDTA, 80% Glycerol, 1mM DTT) to each Eppendorf tube and vortexed. The solution was incubated at room temperature for 30 minutes. The homogenate sample was centrifuged at 6000rpm for 20 minute at room temperature. After centrifugation three layers were formed in the eppendorf tube. The middle layer was collected in a separate Eppendorf tube and stored at 4ºC.

Gel Electrophoresis: Genetic diversity of 20 germplasm of *Juglans regia* was investigated using Sodium Dodecyle Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The electrophoretic procedure was carried out using slab type SDS-PAGE, with 12.5% polyacrylamide gel. Three µl of the extracted protein was loaded with the help of micropipette into the wells of the gel. Gel was run at a constant voltage of 70V for 2.5 hrs. After electrophoresis the gel was stained with 2% Commassie blue solution for an hour at room temperature. Gels were distained in distilled water. After distaining the gels were photographed using gel documentation system "Uvitech".

Table 1. List of twenty germplasm of *Juglans regia* used during present study.

S. No.	Name	Collection place
1.	Sw-1	Senay, (Miandam), Swat
2.	Sw-2	Senay,(Miandam), Swat
3.	Sw-3	Senay, (Miandam), Swat
4.	Sw-4	Senay,(Miandam), Swat
5.	Sw-5	Senay, (Miandam), Swat
6.	Sw-6	Senay, (Miandam), Swat
7.	Sw-7	Senay, (Miandam), Swat
8.	Sw-8	Shin, Swat
9.	Sw-9	Nawakaley, Swat
10.	Sw-10	Gulibagh, Swat
11.	Ch-1	Garamchashma, Chitral
12.	Ch-2	Chitral, city
13.	Ch-3	Chitral, city
14.	Ch-4	Chitral, city
15.	Ch-5	Chitral, city
16.	Ch-6	Snowghar, Chitral
17.	Ch-7	Chitral, city
18.	Dir-68	Manegatta, Dir
19.	Dir-66	Dogal, Shahikot, Dir
20.	Dir-45	Shahikot, Dir

Sw= Swat, Ch= Chitral, Dir= Dir.

Statistical analysis: Only scorable bands were included in analysis. Faint/unscorable bands were not considered for analysis, each individual band was considered as a single locus/allele. Alleles/loci (bands) were scored as present (1) or absent (0). Genetic diversity was estimated using following formula (Nei & Li, 1979).

$$GD = 1 - dxy/dx + dy\text{-}dxy$$

where GD = Genetic distance between two genotypes, dxy=number of common bands in 2 genotypes, dx=total number of bands in genotype 1 and dy =total number of bands in genotype 2. The bi-variant 1-0 data matrix was also used to construct a dendrogram using computer program "Popgene 32".

Results and Discussion

Total seed storage proteins extracted from 20 *Juglans regia* germplasm were separated using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). For

genetic analysis, only scorable bands were considered. Alleles/loci were scored as present (1) or absent (0) and bivariate data matrix (1-0) was generated. A procedure called "Unweighted Pair Group of Arithmetic mean" (UPGMA) developed by Nei & Li (1979) was used to estimate Genetic Distances (GD) among all the possible combinations (Table 2). Genetic distances estimated during present study ranged from 0 and 60%. Maximum genetic distance (GD=60%) was estimated for 6 comparisons viz., Sw-1:Ch-2, Sw-5:Ch-2, Sw-4:Ch-3 Sw-5:Ch-3, Sw-4:Ch-6, Sw-4:Ch-7. Five comparisons viz., Sw-1:Dir-68, Sw-7:Sw-8, Ch-2:Ch-3, Ch-4:Ch-5, Ch-6:Ch-7, showed complete homozygosity for seed storage protein profile (GD=0). Seventeen, Fiftysix, Seventythree, Fifteen and Eighteen, comparisons showed 50%, 40%, 30%, 20% and 10% genetic distance, respectively. It is evident that the (190 comparisons) showed low-medium genetic distances (GD= 0-60%). No such work has been reported in the country for *Juglans regia* L. Hence present study is the first documented attempt to estimate genetic distances among species.

Bivariate data obtained for 20 genotypes of *Juglans regia* using SDS-PAGE was also used to construct dendrogram. Twenty *Juglans regia* genotypes were grouped in 3 clusters "A", "B" and "C" (Fig. 1). Group "A" was largest group comprised of 8 genotypes (Sw-1, Dir-68, Ch-4, Ch-5, Ch-2, Ch-3, Ch-6, Ch-7), while group "C" was smallest comprised of 5 genotypes (Sw-5, Dir-66, Sw-2, Sw-3, Sw-4). Comparison "B" consisted of 7 genotypes (Sw-6, Ch-1, Sw-7, Sw-8, Sw-9, Sw-10, Dir-45). Most of the genotypes collected from Chitral valley were listed in group "A". In general, results of dendrogram analysis were similar to those presented in Table 2 (based upon Genetic Distance estimates). It is concluded from the dendrogram (Fig. 1) that genotypes "Sw-1" and "Sw-4" are most different among the group of 20 genotypes of *Juglans regia*. Hence it is recommended that these 2 genotypes Sw-1, and Sw-4, should be used in future breeding programs to create higher amount of genetic variability in Pakistani germplasm of *Juglans regia* L.

Little information is available in the literature regarding studies on genetic diversity in walnut. Malvolti *et al*., (1993) studied genetic differences in Italian walnut based on isozyme analysis and found non-significant differences for the genetic parameters studied (number of alleles per locus, polymorphic loci per locus and mean hetrozygosity. Nicese *et al*., (1998) studied genetic diversity in walnut population from California, USA using Randomly Amplified Polymorphic DNA primers and found that the genotypes sharing common parents tend to group together. Sharma & Sharma (2001) studied genetic diversity in Indian walnut using kernel characters. They grouped 229 accessions in 16 clusters. The clustering pattern of the genotypes from the same location revealed their distribution in more than one cluster indicating non-parallelism between geographic and genetic diversity. Dogan *et al*., (2005) in Turkey found high amount of genetic diversity in walnut based on fruit characters. Foroni *et al*., (2005) characterized walnut germplasm using Simple Sequence repeat primers (SSR) and detected 33 putative alleles in 22 walnut genotypes, nine of which were unique to one genotype. Present study provides first report to document walnut germplasm in Pakistan based on protein markers. A medium amount of genetic diversity (ranging from 0-60%) based on total seed storage proteins was observed in the material studied. The information will be helpful to establish a gene bank of genetic resources of walnut found in northern parts of Pakistan.

Table 2. Genetic Distances (GD) estimated among 20 *Juglans regia* germplasm used during present study.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1																			
2	0.4																		
3	0.3	0.1																	
4	0.4	0.2	0.1																
5	0.3	0.5	0.4	0.3															
6	0.3	0.4	0.3	0.4	0.4														
7	0.3	0.4	0.3	0.4	0.4	0.3													
8	0.3	0.4	0.3	0.4	0.4	0.3	0.0												
9	0.3	0.5	0.4	0.3	0.3	0.3	0.1	0.1											
10	0.4	0.4	0.3	0.3	0.4	0.4	0.2	0.2	0.3										
11	0.4	0.5	0.4	0.5	0.5	0.1	0.1	0.1	0.3	0.3									
12	0.3	0.4	0.5	0.6	0.6	0.3	0.3	0.3	0.4	0.4	0.2								
13	0.3	0.4	0.5	0.6	0.6	0.3	0.3	0.3	0.4	0.4	0.2	0.0							
14	0.1	0.3	0.4	0.5	0.5	0.4	0.1	0.1	0.3	0.3	0.3	0.2	0.2						
15	0.1	0.3	0.4	0.5	0.5	0.4	0.1	0.1	0.3	0.3	0.3	0.2	0.2	0.0					
16	0.3	0.4	0.5	0.6	0.4	0.5	0.3	0.3	0.4	0.4	0.4	0.3	0.3	0.2	0.2				
17	0.3	0.4	0.5	0.6	0.4	0.5	0.3	0.3	0.4	0.4	0.4	0.3	0.3	0.2	0.2	0.0			
18	0.0	0.4	0.3	0.4	0.4	0.3	0.3	0.3	0.3	0.4	0.4	0.3	0.3	0.1	0.1	0.3	0.3		
19	0.3	0.4	0.3	0.4	0.2	0.3	0.3	0.3	0.3	0.4	0.4	0.5	0.5	0.4	0.4	0.3	0.3	0.3	
20	0.3	0.3	0.4	0.4	0.3	0.3	0.1	0.1	0.2	0.1	0.3	0.4	0.4	0.3	0.3	0.4	0.4	0.3	0.3

1=Sw-1, 2=Sw-2, 3=Sw-3, 4=Sw-4, 5=Sw-5, 6=Sw-6, 7=Sw-7, 8=Sw-8, 9=Sw-9, 10=Sw-10, 11=Ch-1, 12=Ch-2, 13=Ch-3, 14=Ch-4, 15=Ch-4, 16=Ch-6, 17=Ch-7, 18=Dir-68, 19=Dir-66, 20=Dir-45.

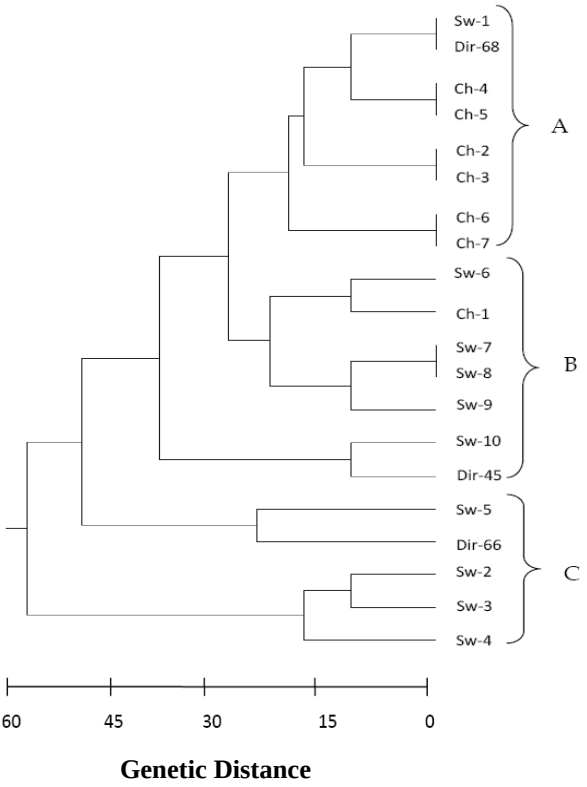

Fig. 1. Dendrogram constructed for 20 *Juglans regia* using protein profile generated from SDS-PAGE data.

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