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Assessment of genetic diversity in mulberry using morphological and molecular markers

Pooja Kala[1], Sajad Majeed Zargar[2]*, R K Bali[1], Nancy Gupta[2], R K Salgotra[2], Ajay Koul [1]

[1]Division of Sericulture, S K University of Agricultural Sciences and Technology, Jammu, Chatha, Jammu and Kashmir – 18009, India
[2]School of Biotechnology, S K University of Agricultural Sciences and Technology, Jammu, Chatha, Jammu and Kashmir – 18009, India
*****Corresponding Author: Dr. Sajad Majeed Zargar, E-mail: smzargar@gmail.com

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Abstract
The horizontal expansion of sericulture in traditional and non-traditional states has made it necessary to develop mulberry varieties specific to various agro-climatic zones. At present various improved varieties in use are mostly developed, involving exotic accessions as parents. An understanding of genetic diversity is must for selection of parental lines to gain vigor in hybrid. In the present study, an initiative was taken to assess the genetic diversity among mulberry (*Morus alba)* genotypes using morphological and PCR based molecular markers. The morphological aspects considered in the present study revealed high variability. The number of leaves per meter twig was highest in Tr-10 and Chakmajra (22). Maximum inter-nodal distance was recorded in variety MS-9 and Chinese White (5.7cm). Actual leaf area was highest in LUN-10 (225.38cm^2). The fresh weight of 100 leaves was highest in Kokuso-27 (339.0g) and dry-weight as (101.5g). Maximum leaf length observed was 23.0 cm in V-1 and maximum width of 17.3 cm was recorded in Kokuso-27. For molecular assessment of genetic diversity, nine reproducible RAPD primers were used that revealed more than 97.22 % polymorphism percentage with 0.32 average PIC value. Finally, the cluster analysis based on UPGMA divided the varieties in two major groups. Based on morphological and molecular analysis, diverse varieties having better agronomic traits can be selected as the parental source in developing new hybrids. Moreover, the RAPD profiles of each variety obtained from molecular analysis can be used to depict the authenticity of a cultivar and confirmation of hybrid variety.
Key words: Mulberry, RAPD markers, Polymorphism, Genetic diversity, PIC.

Introduction

Mulberry (*Morus* spp.) originated in the lower Himalayan belt of Indo-China region, where some species are available in natural habitat upto an elevation of 3500 meters above mean sea level. The major four indigenous species that are widely distributed in India comprise of *Morus alba, Morus indica*, *Morus laevigata*, *Morus serrata* (Hooker, 1885; Brandis, 1874) where *M. serrata* is mainly confined to North Western Himalayan belt. Morphologically, the genus *Morus* has been classified into 15 species (Pan, 2000). In order to expand the sericulture industry, development of new mulberry varieties is must. So far, mostly, exotic varieties have been used as parental lines for development of hybrids. Understanding the variation among various mulberry genotypes may help in selecting more parental lines for improving agronomic traits of the existing varieties. Knowledge of genetic diversity is must and it forms the foundation for any crop improvement program and provides a platform to exploit diverse genotypes for improving the hybrid vigour (Zargar *et al*., 2014). Various parameters can be considered for analyzing genetic diversity, however, combined morphological and molecular markers provide much better and valid results. Among molecular markers, RAPD technique (Williams *et al*., 1990) provides a convenient way and rapid assessment of the differences in the genetic composition of the related individuals and it has been employed in a large number of plants for determination and assessment of genetic diversity including mulberry (Awasthi *et al*., 2004). The genetic identities and relationships of mulberry plants including cultivars, diploids and polyploids have been determined using RAPD and other molecular markers (Xiang *et al*., 1995; Feng *et al*., 1996; Zhang *et al*., 1998; Lou *et al*., 1998: Esha *et al*., 2001). The combined morphological and molecular markers were useful in distinguishing the varieties based on agronomic traits as well as genetic basis.

Materials and Methods

Plant material

Nineteen varieties of mulberry (**detailed in Table 1**) were used for assessment of genetic diversity. Plant material was procured from different institutes of India as detailed in **Table 1**.

Morphological parameters

Leaf size analysis was conducted by graphical analysis. The data was recorded and statistically analyzed. Leaf shape was evaluated by visual analysis method (Lawrence, 1967). Number of leaves per meter twig was determined by counting all leaves present on a branch. Inter nodal distance was measured with the help of measuring scale. Observations for each parameter were recorded in triplicate for analysis of data. For determining the fresh leaf weight, hundred leaves were picked up randomly per replication and weighed. Observations were recorded after sixty days of sprouting in each variety viz. on leaf maturity. For estimating the dry leaf weight, hundred leaves were picked up randomly per replication and dried at 70° C in oven till constant weight was achieved to determine the dry matter gravimetrically. Ten leaves of each variety were plotted on graph to determine area. Leaf pubescence was observed using stereoscope at 40X and further categorized (Lawrence, 1967). Leaf apex, base and margin were determined visually and classified as per (Lawrence, 1967).

Genomic DNA isolation and quantification

Isolation of genomic DNA was carried out following Doyle and Doyle method, (1990) with slight modifications. About 7-8 cm young and actively growing fresh leaves from mulberry varieties grown in SKUAST Jammu, Farm were collected in liquid N_2 container and stored at -80^{0}C till further use. For extraction of genomic DNA, leaves were ground to fine powder in liquid N_2, and transferred to 1 ml of pre-warmed (at 65ºC) extraction buffer and incubated for 35 minutes. An equal volume of Chloroform: Isoamylalcohol (24:1) was added to the tube, tilted for 10 minutes and centrifuged for 15 minutes at 8,000 rpm. Supernatant was collected to which, 0.6 volume of ice-cold isopropanol was added and stored at -20^{0}C for 3-4 hours. Centrifugation was done at 10,000 rpm for 10 minutes at 4^{0}C and pellets were purified with 0.01M ammonium acetate (200µl-300µl). The pellet was washed twice with 70% chilled ethanol, air dried, dissolved in 300µl TE (10 mM Tris-Cl, 1 mM EDTA pH 8.0) buffer, treated with 3µl of RNase (10mg/ml) and finally stored at -20^{0}C for further use. The amount and quality of DNA was confirmed using Nanodrop (mySPEC, Wilmington, USA). Finally the DNA was diluted to 25ng/µl concentration for PCR amplification.

RAPD based fingerprinting

A set of 9 arbitrary random 10-mer primers (**detailed in Table 2**) were used in the present investigation. These were got synthesized from IDT (Integrated DNA Technologies, USA). The RAPD primers were diluted to 5 pmol concentration and final concentration of 25 pmol was used per reaction. Reaction mixture contained 2.5 µl of template DNA (25 ng/µl), 2.5 µl of 10× PCR Buffer, $MgCl_2$ (2 mM), 0.2mM of each dNTPs (dTTPs, dGTPs, dCTPs, dATPs), primer (5pmol) concentration with 1 U Taq polymerase per reaction. PCR tubes containing master mix and DNA template were thoroughly mixed and subjected to the thermal profile under following conditions: 4 min at 94°C, 35 cycles (1 min at 94°C, 1 min at 36-37°C, 2 min at 72°C) and 10 min at 72°C extension. PCR amplification was carried out in a 96 well Universal Gradient Thermal Cycler (PEQLAB, Deutschland and Osterrtich, United Kingdom).

The amplification products were then subjected to electrophoretic separation using horizontal agarose gel electrophoresis. 1.5% agarose gels were prepared for resolving amplified PCR products. The gel was visually examined under UV and documented using Biometra Gel documentation system.

Data analysis

RAPD-PCR bands were detected in the gel using gel documentation system. Bands were seen for their presence (1) or absence (0). All the scorable bands were considered as single locus/allele. The presence and absence of bands in all genotypes for 9 primers were used to generate binomial data using excel sheet. In order to check the informativeness and discriminatory power of RAPD primers utilized in this study, certain parameters like polymorphism percentage and polymorphic information content were evaluated.

Polymorphism percentage was calculated by dividing the number of polymorphic bands by the total number of scored bands. PIC was calculated as proposed by Roldan-Ruiz *et al*., (2000), PIC = 2fi (1-fi), where fi = frequency of bands present and 1-fi = frequency of bands absent. Bi-nomial data matrix of all genotypes generated from 9 primers was subjected to the UPGMA (un-weighted pair group method with arithmetic averages) analysis and a dendogram was constructed using STATISTICA software.

Results

Divergence among genotypes/ existing varieties is the cornerstone in developing improved varieties with desired traits. Diverse varieties can be exploited by a breeder to enhance productivity by predicting cross combinations to produce good F_1 hybrids. Nineteen genotypes/ varieties used in the present investigation as a study material to assess genetic variation.

Morphological assessment for genetic variation

Various morphological parameters (detailed in Materials and methods section) were considered. The leaf morphology studies revealed an inter nodal distance ranged between 5.7 cm in Chinese White and Tr-10 variety & 4.1 cm in S-36. Most of the varieties showed a strigose type of pubescence. Fresh leaf weight showed a great variation ranging from 339 g in variety Kokuso-27 and to 89g in S-36 whereas dry leaf weight varied from 101.5 g in Kokuso-27 to 28 g in S-36. The leaf length was found highest in V-1 (23 cm) and the lowest in variety S-36 (10.2 cm). The leaf width ranged from 17.3 cm in Kokuso-27 to 7.5 cm in variety S-36. Maximum varieties showed a serrate type of apex with a cordate leaf base. Leaf shape in most of the varieties showed an ovate type with an acuminate type leaf margin. Number of leaves per meter twig were counted and found to be the maximum (22 leaves) in Chakmajra and Tr-10 whereas MS-9 showed the lowest (10 leaves).The sprouting period of the varieties varied from 16th January to 27th March. The moisture percentage was found to be highest (62.3%) in S-1531 and lowest (78.2%) in Sujanpur (**details are given in Table 3, Table 4 and Table 5**). Varieties with good yield parameters can be selected for further improvement.

Molecular marker based assessment of genetic variation

Nine reproducible RAPD primers detailed in Table 2, were used to study the polymorphism among 19 mulberry genotypes/ varieties. We considered different parameters to know the discriminatory power of these RAPDs for genetic variation study in case of mulberry. The polymorphism percentage of these RAPDs was 100 percent except OPA-03 having an average of 97.22 per cent per primer. Further, the PIC value for each primer was calculated. It ranged from 0.095 (OPC-02) to 0.45 (OPA-07) with an average found to be 0.32. The polymorphic information content was high in case of OPA-07 (0.45), OPA-05 (0.44), and OPA- 11 (0.43) whereas the primers OPC-02 (0.095), OPA-03 (0.16), OPA-10 (0.17) showed the lowest PIC. Certain primers such as OPB-10 (0.33) and OPC-08(0.39) showed the medium level of PIC. **Figure 1** depicts the RAPD fingerprint profile of OPA-10 primer. Results indicated that these RAPDs can be used as genetic resource for studying polymorphism among mulberry genotypes in future studies.

Further, cluster analysis was done using STATISTICA program, based on UPGMA. The genotypes/ varieties were grouped in 2 major clusters having linkage distance of 78 Units. The cluster II as represented in **Figure 2** was further divided into sub-cluster at linkage distance of 48 Units.

Discussion

Heterozygosity among the mulberry varieties is due to out breeding behavior and it has been well recorded in case of most of tree species (Tikader *et al.*, 1999). The evolution of homozygous lines through selfing has not successful due to dioecious nature of mulberry. Even in case of monoecious varieties, selfed lines could not be maintained beyond early generations probably because of the expression of deleterious glues and intrinsic requirement of heterozygosity for mulberry growth and development. Based on these facts molecular markers can be used as a tool to study its inheritance pattern for understanding variation among varieties, which is an important step for crop improvement (Zargar *et al.*, 2014).

Genetic variation based on morphological parameters

In the present study we observed that most of the varieties/ genotypes showed ovate shape leaves except few such as MS-9, S-36, Tr-8, S-1708, S-146 and Kokuso-27 having wide type of leaf shape. This may be due to the fact that most varieties are developed as selection from similar stock and in general mulberry leaf ovate in shape. Similar observations were made by Gupta (2006) and Mazal (2009). The inter-nodal distance varied from 4.06 to 6.32. The shortest inter nodal that has been observed in S-36 shows the superiority of the varieties amongst other varieties. Anonymous (1990) reported that variety Chakmajra had inter nodal distance (4.3 cm); whereas, Gupta (2006) reported that variety Sujanpur has inter nodal distance of 4.01 cm. Further, leaf area of the varieties showed a wide variation. The largest leaf was observed in LUN-10 (225.38 cm^2). Gupta (2006) observed that leaf area range from 171.70 cm^2 (C-763) to 267.30 cm^2 (BC-259). Number of leaves per meter branch is inversely related to inter-nodal distance. It did fluctuate significantly and showed highest value in variety of Tr-10 and Chakmajra. The number fell drastically to 10.0 in MS-9. The fresh

leaf weight of the varieties did not show a wide fluctuation except in S-36 wherein a weight of 89.0 g was observed in it whereas the highest leaf weight was observed in Kokuso-27 as 339.0 g. Gargi *et al*., (1997) reported fresh leaf variation from 511.7 to 394.5 g. The dry leaf weight was calculated and found more in variety Kokuso-27 (101.5 g) similar results were observed by Gupta (2006).

Studying polyamorphism as revealed by RAPD markers

Here we considered certain parameters like polymorphism percentage, PIC (Roldan- Ruiz *et al*., 2000), to depict the discriminatory power of RAPD primers. These parameters have been calculated in other crop plants such as in Sesame (Salazar *et al*., 2006), *Brassica* (Gupta *et al*., 2014) where informative capacity of primers has been detected by calculation of these parameters. The polymorphism ranged from 75-100 percent with an average PIC value of 0.32 per primer and polymorphism of 97.22 per primer. A total of 53 bands were generated. Bhattacharya and Ranade (2001) reported a polymorphism percentage of 85 percent using the RAPD primers. reported a polymorphism percentage of 72 percent using 45 mulberry varieties which belonged to twelve species using the RAPD markers. The dendrogram was constructed using the UPGMA analysis grouped the genotypes into two major clusters. The cluster I, have further set of sub clusters. The first sub-cluster included three varieties (Tr-4, Chakmajra and LF-1). The other sub-cluster also included three varieties (S-36, S-1708 and Chinese white). Thus RAPD markers proved to be very informative in the assessment of genetic variation on the basis of higher polymorphism percentage (97.22%). The analysis with RAPD markers disclosed wide variation within mulberry that reflected a high level of diversity within these species. The cluster II, also possess sub clusters. The first sub-cluster involved nine varieties (LUN-10, S-1635, S-146, S-1531, Tr-8, MS-9, Sujanpur, V-1 and Kokuso-27). Combined morphological and molecular data will be very useful in identifying the parental lines for improvement of yield traits in mulberry by development of new hybrids.

Conclusions

The present study helped us to explore the diversity among mulberry genotypes and identifying the highly polymorphic RAPD primers. The diverse genotypes can be employed as parents in future breeding programs and the informative primers can be utilized further for evaluation of diversity among the crop. However, there is further need of involving larger number of genotypes as well as markers to have better picture of germplasm structure. The morphological studies conducted in these varieties/ genotypes also give information about the efficiency of the varieties. As such this study can be considered as a first and a major step for improving various yield traits in future mulberry varieties. Moreover the RAPD fingerprint profile of these 19 varieties / genotypes can be used for parental identification in F1 hybrids.

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Table 1: Details of Mulberry genotypes/ varieties with their origin and genetic nature

S.No	Genotypes/ Varieties	Origin	Genetic nature
1.	LUN-10	J&K Government	Polyploid
2.	LF-1	CSRTI, Mysore	Clonal Selection
3.	LF-2	CSRTI, Mysore	Clonal Selection
4.	Tr-10	RSRS, Kodathi	Polyploid
5.	MS-9	CSRTI, Mysore	Collection
6.	NS-1	Division of Sericulture, SKUAST-Jammu	Open Pollinated
7.	S-36	CSRTI, Mysore	EMS Treatment of Berhampore Local.
8.	Chakmajra	J&K Government	Natural Selection
9.	Tr-8	RSRS, Kodathi	Polyploid
10.	S-1708	CSRTI, Berhampore	Open Pollinated Selection
11.	Chinese White	CSRTI, Mysore	Collection
12.	S-30	CSRTI, Mysore	Mutation
13.	S-146	RSRS, Kodathi	Open Pollinated Selection
14.	Tr-4	RSRS, Kodathi	Polyploid
15.	V-1	CSRTI, Mysore	Cross Pollinated Hybrid
16.	S-1531	CSRTI, Mysore	Open Pollinated Hybrid Selection
17.	S-1635	CSRTI, Berhampore	Open Pollinated Hybrid Selection
18.	Kokuso-27	CSRTI, Mysore	Cross Pollinated Hybrid
19.	Sujanpur	DOS, J&K Govt.	Open Pollinated Collection

Table 2: Details of RAPD primers with various parameters revealing their resolving nature

Primer	NB	NPB	NMB	PIC =2fi (1-fi)
OPA-02	12	12	0	0.41
OPA-03	4	3	1	0.16
OPA-05	6	6	0	0.44
OPA-07	5	5	0	0.45
OPB-10	4	4	0	0.33
OPA-10	6	6	0	0.17
OPA-11	7	7	0	0.43
OPC-02	3	3	0	0.095
OPC-08	6	6	0	0.39
Average	5.9	5.8	0.1	0.32

NB: Total number of Bands; **NPB**:Number of Polymorphic Bands; **NMB**: Number of monomorphic Bands**; PIC**: Polymorphic Information Content

Table 3: Morphological variations among various characters of leaves

S. No.	Genotypes/ Varieties	Apex	Base	Margin	Shape	Pubescence
1	LUN-10	Serrulate	Truncate	Acuminate	Ovate	Scabrous
2	LF-1	Serrate	Cordate	Acuminate	Ovate	Puberulous
3	LF-2	Serrate	Cordate	Acuminate	Ovate	Strigose
4	Tr-10	Serrate	Cordate	Acuminate	Ovate	Puberulous
5	MS-9	Serrate	Cordate	Acuminate	Wide Ovate	Hirsute
6	NS-1	Serrate	Cordate	Acuminate	Ovate	Strigose
7	S-36	Serrate	Lobate	Acuminate	Wide Ovate	Hirsute
8	Chakmajra	Serrate	Truncate	Acuminate	Ovate	Strigose
9	Tr-8	Dentate	Cordate	Acuminate	Wide Ovate	Strigose
10	S-1708	Serrate	Cordate	Acuminate	Wide Ovate	Strigose
11	Chinese white	Crenate	Cordate	Acuminate	Ovate	Strigose
12	S-30	Serrate	Cordate	Acuminate	Ovate	Strigose
13	S-146	Serrate	Cordate	Acuminate	Wide Ovate	Strigose
14	Tr-4	Serrate	Truncate	Acuminate	Ovate	Hispid
15	V-1	Serrate	Truncate	Acuminate	Ovate	Strigose
16	S-1531	Serrate	Truncate	Acuminate	Ovate	Strigose
17	S-1635	Crenate	Cordate	Acuminate	Ovate	Strigose
18	Kokuso-27	Dentate	Cordate	Acuminate	Wide Ovate	Puberulous
19	Sujanpur	Serrate	Cordate	Acuminate	Ovate	Strigose

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Table 4: Variation in leaf weight, number of leaves per meter twig, inter nodal distance and moisture percentage

S.No.	Genotypes/ Varieties	Inter nodal Distance(cm)	Leaf weight		Number of leaves per meter twig	Moisture percentage
			Fresh Weight (g) (100 leaves)	Dry Weight(g) (100 leaves)		
1	LUN-10	5.2	292.5	85.5	15	70.8
2	LF-1	4.3	233.0	68.0	14	70.8
3	LF-2	4.5	187.0	58.0	16	69.0
4	Tr-10	4.5	191.5	59.5	22	68.9
5	MS-9	5.7	160.0	51.0	10	68.1
6	NS-1	4.8	156.5	45.0	12	71.2
7	S-36	4.1	89.0	28.0	14	68.5
8	Chakmajra	4.5	220.0	52.0	22	76.4
9	Tr-8	4.7	157.5	37.0	21	76.5
10	S-1708	5.0	234.0	87.0	20	62.8
11	Chinese white	5.7	164.5	39.0	18	76.3
12	S-30	4.8	202.5	47.5	21	76.5
13	S-146	4.7	167.5	58.0	21	65.4
14	Tr-4	4.8	193.0	59.5	21	69.2
15	V-1	4.3	137.0	48.0	16	65.0
16	S-1531	4.4	151.0	57.0	13	62.3
17	S-1635	4.9	126.0	38.0	20	69.8
18	Kokuso-27	4.5	339.0	101.5	15	70.1
19	Sujanpur	4.3	202.0	44.0	14	78.2
	C.D.(p=0.05)	0.1	1.5	0.6	1.4	1.7
	S.E.(m)	0.04	0.50	0.22	0.51	0.59

Table 5: Morphological variations in length, width, leaf area and sprouting time

S.No.	Genotypes/ Varieties	Length (cm)	Width (cm)	Actual leaf area(cm^2)	Sprouting time
1	LUN-10	22.8	14.80	225.4	16 January
2	LF-1	19.9	16.50	218.2	27 January
3	LF-2	17.4	12.10	137.7	21 March
4	Tr-10	20.5	14.40	184.8	16 January
5	MS-9	16.1	12.20	123.8	15 January
6	NS-1	17.8	13.20	152.7	16 January
7	S-36	10.2	07.50	052.7	27 January
8	Chakmajra	18.1	12.70	121.7	15 January
9	Tr-8	14.1	11.43	112.0	17 January
10	S-1708	17.6	13.00	129.0	19 January
11	Chinese white	19.9	13.60	156.3	22 January
12	S-30	18.8	15.10	151.7	20 January
13	S-146	18.7	13.70	172.1	15 January
14	Tr-4	15.5	16.70	162.2	17 January
15	V-1	23.0	12.80	175.2	24 January
16	S-1531	16.8	11.70	121.1	22 January
17	S-1635	13.8	12.00	110.9	20 January
18	Kokuso-27	21.9	17.30	216.1	27 March
19	Sujanpur	17.5	13.60	141.9	17 January
	C.D.(p=0.05)	1.7	1.7	1.6	-
	S.E(m)	0.59	0.57	0.56	-

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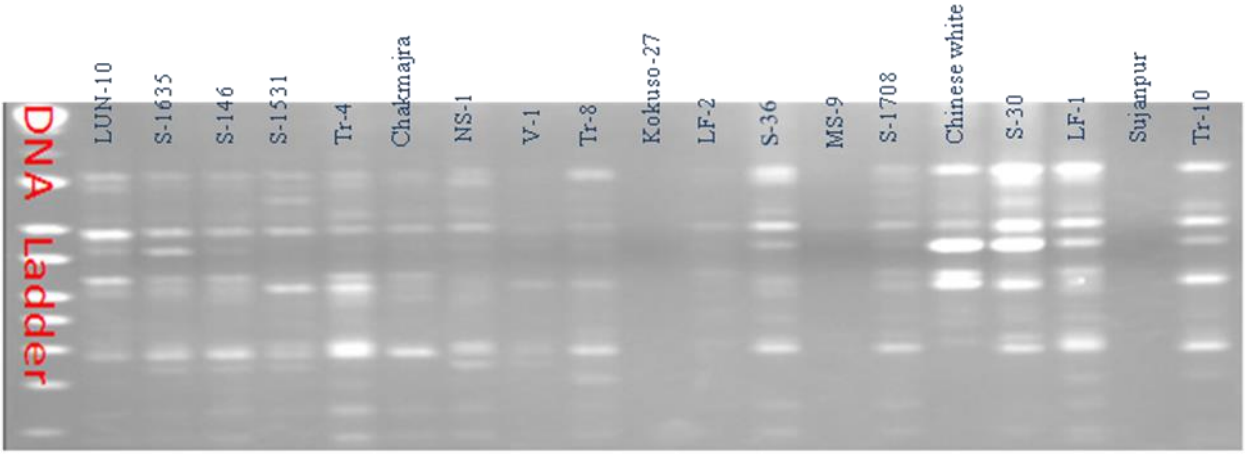

Figure 1 A: RAPD profile of primer OPA-10

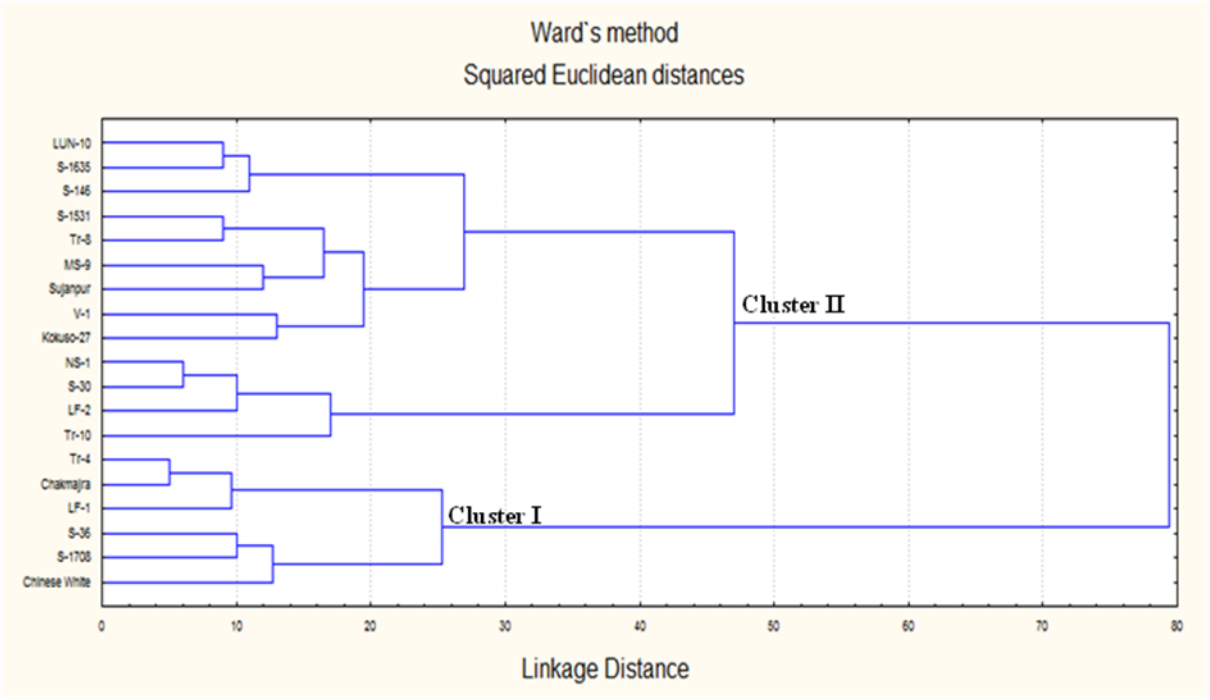

Figure 2. Dendrogram illustrating genetic relationship based on UPGMA analysis