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# ANALYSIS OF GENETIC DIVERSITY IN PARENTS AND HYBRIDS OF *Populus deltoides* Bartr. USING MICROSATELLITE MARKERS

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## ABSTRACT

**Molecular markers have proved a valuable source for assessing plant genetic resources by improving our understanding on the distribution and extent of genetic variability present within and among the species. In this study, genetic diversity among and between 16 parents and 12 hybrids of *Populus deltoides* were analyzed by SSRs (simple sequence repeats). The 18 selected SSR primers yielded 51 polymorphic scorable bands with PIC value of 0.77. The effective multiplex ratio and marker index were 1.82 and 0.80, respectively. Genetic similarity among the collections varied from 0.39 to 0.88 when pooled data of parents and hybrids were analyzed through UPGMA. The analysis of combined data set with SSR, clustered hybrids on dendrogram based on their relationship with parents. Thus, high polymorphism obtained indicates that SSR markers have potential for identification and characterization of genetic variation within and among *Populus* species.**

**Key words:** Genetic diversity, germplasm, hybrids, molecular markers, *Populus deltoids*, simple sequence repeat

## INTRODUCTION

The genus *Populus* is naturally distributed to forests of cold and temperate deserts of northern hemisphere. *Populus deltoids* Bartr., a fast growing deciduous tree, belongs to the family Salicaceae. It is medicinally important tree crop because its bark is used in treatment of rheumatoid, gout, scurvy, infection of chest, kidney and stomach. In India, this species is grown as block/boundary in Punjab, Haryana, Uttar Pradesh, Uttrakhand, Jammu & Kashmir and Himachal Pradesh. The tree improves the physio-chemical properties of soil and acts as alternative source of income and employment to the rural poor. *P. deltoides* is in great demand because of its fast growth on semi-alkaline soil and has rightly been designed as energy plant (Chaturvedi *et al.,* 2004). *Populus* makes an important contribution in meeting the global need of paper, timber and other wood-based products (Taylor, 2002). The clones of poplars act as unit of cultivation and breeding where each clone represents an individual cultivar. The adequate knowledge and correct identification of cultivars is required for breeding, stock handling and registration of new variety and protection of plant breeder's right. Previously, the cultivar or clone identification was done on the basis of morphological, phonological and floral characteristics which showed errors due to variation in polygenic morphological traits under different environmental conditions (Kadam, 2002). Recent advanced biotechnological tools such as molecular markers have helped to evaluate the genetic diversity among different trees species that is pre-requisite for successful breeding programme. Simple sequence repeat (SSR) markers have proved extremely useful tool in finger printing, construction of linkage maps and marker-assisted

selection as well as in establishing genetic and evolutionary relationships (Peng *et al.*, 2005; Brundu *et al.*, 2008; Shen *et al.*, 2014; Grewal *et al.*, 2014). Considering the usefulness of molecular markers in diversity analysis and need to characterize poplar germplasm, the present study was aimed to estimate genetic diversity in cultivars which may help in the improvement of poplar clones as well as in grouping diverse clones.

## MATERIALS AND METHODS

***Source of plant material***

The flowering branches of four female (G-48, $S_7C_8$, L-62/84 and $S_1$) and 12 males (82-42-5, UD-88, L-124/86, 39-N, $S_7C_{11}$, 25-N, 26-N, $S_7C_1$, $S_7C_4$, L-17/92, G-3 and $S_7C_{20}$) clones were procured from the State Forest Department, Haldwani and Shyampur, Haridwar Forest Division (India) in the month of January and February 2013, respectively. The details of genotypes used are as under:

| S. No. | Name of genotypes | Details of genotypes | Genotype code | Source country/Originally developed | Details of sets |
|---|---|---|---|---|---|
| 1. | G-48 | Female parent | G1 | Australia | |
| 2. | 1 | G-48× S7C11 | G2 | Hybrid | |
| 3. | 12 | G-48× L-17/92 | G3 | - | |
| 4. | 14 | G-48× L-124/86 | G4 | - | Set I |
| 5. | 15 | G-48× S7C1 | G5 | - | |
| 6. | S7C11 | Male parent | G6 | USA | |
| 7. | L-17/92 | Male parent | G7 | India (Lalkuan Selection) | |
| 8. | L-124/86 | Male parent | G8 | India (Lalkuan Selection) | |
| 9. | S7C1 | Male parent | G9 | USA | |
| 10. | S-1 | Female parent | G10 | India (Shyampur, Haridwar Forest Division) | |
| 11. | 3 | S-1× 82-42-5 | G11 | - | |
| 12. | 9 | S-1× UD-88 | G12 | - | Set II |
| 13. | 11 | S-1× 39N | G13 | - | |
| 14. | 82-42-5 | Male parent | G14 | - | |
| 15. | UD-88 | Male parent | G15 | - | |
| 16. | 39N | Male parent | G16 | - | |
| 17. | S7C8 | Female | G17 | USA | |
| 18. | 4 | S7C8× 25N | G18 | - | |
| 19. | 10 | S7C8× 26N | G19 | - | Set III |
| 20. | 25N | Male parent | G20 | - | |
| 21. | 26N | Male parent | G21 | | |
| 22. | L-62/84 | Female parent | G22 | India (Lalkuan Selection) | |
| 23. | 19 | L-62/84× S7C4 | G23 | - | |
| 24. | 21 | L-62/84× G3 | G24 | - | |
| 25. | 22 | L-62/84× UD-89 | G25 | - | |
| 26. | S7C4 | Male parent | G26 | USA | Set IV |
| 27. | G3 | Male parent | G27 | - | |
| 28. | $S_7C_{20}$ | Male parent | G28 | USA | |

These materials were used in present study. Crosses among male and female were made in 2013 in Germplasm block of Naganji Nursery, Department of Tree Improvement & Genetic Resources, YSPUHF, Solan (India). Flowering branches of male clones were kept in water buckets to get abundant pollens for hybridization. Flowering branches of female clones were grafted separately on rootstock of *P. deltoides*. The grafted plants were kept moist by intermittent irrigation/watering. The flowering branches of female and male clones of *P. deltoides* were used for control crossing (hybridization). Male catkins were removed for pollen collection at anthesis for artificial pollination. All female clones were crossed with each male clone by hand pollination at stigma receptivity stage.

Pollens used in controlled crossing was tested for *in vitro* pollen viability. Every controlled cross involved single pollen and no pollen mixture was used. After pollination, flowers were bagged and tagged. The seeds of controlled crosses were harvested when mature and sown immediately.

***Experimental site***

The experimental site was located at an elevation of 1200 m masl in north-west of Himalaya and lies between 30˚ 51' N latitude and 76˚ 11' E longitude. The area is hilly, marked with elevations, depressions and has a gentle slope towards south-eastern aspect. There existed a wide variation in temperatures ranging from a minimum of 2°C in winters to a maximum of 32.6°C in summers. The soil was well drained and sandy-loam type with pH of 7.2. The experiment was conducted in nursery, whereas the assessment of paternity verification was done by using molecular marker (simple sequence repeats) technique in Forest Genetics Laboratory of YSPUHF, Solan (India).

***Reproductive biology***

For studies on reproductive biology, the male and female flowering shoots of *P. deltoides* clones were selected and marked in a glass house and field in the year 2013. Each shoot was tagged with metallic tag and data recorded.

***DNA isolation***

Genomic DNA was isolated from the leaves of parents and selected hybrids by using CTAB method (Doyal and Doyal, 1987). Young healthy leaves (2 g), taken from field growing plants, were homogenized into a fine powder in liquid nitrogen and incubated for 30 min at 65°C in extraction buffer (10% CTAB; 1 M tris-HCl; pH 8.0; 4 M NaCl; 0.5 M EDTA; 2% PVP and 0.2% β-mercaptoethanol). Nucleic acids were purified with chloroform/isoamyl alcohol (24:1) and phenol/ chloroform/isoamyl alcohol (25:24:1) precipitated with isopropanol by using a centrifuge. Total DNA was dissolved in 50-100 µL1 × TE (pH 8.0) and checked by electrophoresis on 1.0% agarose gels containing 0.5 mg $mL^{-1}$ ethidium bromide and visualized by UV light. The quality of DNA was judged on the basis whether the sample DNA formed a single high molecular weight band or smear.

***Amplification and electrophoresis***

Eighteen primers were used to screen 28 (parents and hybrids) collections of *P. deltoides*. The sequences of primers are given in Table 1. PCR were carried out in a total volume of 20 µL reaction mixture containing 1X Taq buffer A (75 mM $MgCl_2$), 10 picomoles of primer (F&R), 1 mM dNTPs mixture, 1U of Taq DNA polymerase and 50 ng genomic DNA. The SSR-PCR amplification were conducted on ProFlex$^{TM}$ thermocycler (Applied Biosystem Inc.) by using thermal profile: a hot start at 94°C for 4 min, followed by initial denaturation at 94°C for 1 min, primer annealing temperature specific to primer for 1 min, Primer amplification at 72°C for 1 min followed by final extension at 72°C for 7 min. The amplified product was separated by electrophoresis on 2% agarose gels in 1X TAE buffer at 120V for 3 h (Samriti, 2015). A 100 bp DNA ladder was used as a size standard and the gels were visualized under gel documentation system (Syngene International Ltd.).

***Effective multiplex ratio and marker index***

The effective multiplex ratio and marker index was estimated by using formula of Varshney *et al.* (2007). The formula for effective multiplex is:

$$E = n\beta$$

Where β is the fraction of polymorphic markers and n is the multiplex ratio.

Marker index was estimated by using formula:

$$MI = PIC \times E$$

Where PIC= Polymorphic information content and E= Effective multiplex ratio, as defined above.

***Data analysis***

The software NTSYS pc ver. 2.02h (GeNei$^{Tm}$) (Rohlf, 2000) was used to analyze genetic diversity in *P. deltoides*. The data on band position on agarose gel was recorded by assigning '0' for absence of band and '1' for presence of band. Dendrogram and similarity matrix were constructed by following

UPGMA function. The polymorphism information content (PIC) values, which provide an estimate of the discriminative power of a marker by taking into account not only the number of alleles at a locus but also relative frequencies of those alleles in genotypes, were calculated using the formula (Smith *et al.*, 1997):

$$PIC = 1-\Sigma pi^2,$$

where *pi* is the frequency of $i^{th}$ allele

## RESULTS AND DISCUSSION

Genetic diversity is a measure that quantifies the size of genetic variability within a population. It is important for effective conservation of *P. deltoides* (Zhu *et al.*, 2000; Meyers *et al.*, 2002). High level of genetic diversity was observed in poplar collections representing three different countries such as USA, Australia and India. In present study diversity of and among parents and selected hybrids were screened by using 18 SSR primers (Table 1). Out of 18 primers, 17 were able to produced amplification on PCR analysis hence considered as informative primer. Seventeen primers produced total of 51 bands; all were polymorphic, representing 94.45% polymorphism. Primer WPMS-03 which is denoted by 'P' alphabet produces maximum numbers of fragments i.e. 76 as compared to Primer PMGC-562 which produced minimum numbers of fragments i.e. 17 denoted by 'I' (Table 2).

**Table 1: The primers and their sequences used**

| Primer names | Sequences |
|---|---|
| WPMS-03 | FP – TTTACATAGCATTTAGCCTTTAGA |
| | RP - TTATGATTTTGGGGGTGTTATGGA |
| WPMS-05 | FP - TTCTTTTT CAA CTG CCT AACTT |
| | RP – TGATCCAATAACAGACAGAACA |
| ORPM-015 | FP – CGTGAGTTTTGAGGCCATTT |
| | RP – CATGGAAAGGATCACCCACT |
| PMGC-451 | FP - AATTACAACCACTTTAGCATATTC |
| | RP – TGCCGACACATCACACATACC |
| PMGC-325 | FP – CGATTTATGACAGACAGCTTG |
| | RP – GTACCGTTGAGGTGGCTAG |
| PMGC- 333 | FP – CTTAGTGGTGAAGTATTC |
| | RP - GAG TGG GTG CTGATTCATCC |
| PMGC- 409 | FP – ACGTATATGAAGTTCTTGATTGC |
| | RP – GACAGATCATTATGATTACTACAG |
| PMGC- 420 | FP – ATGGATGAGAAATGCTTGTG |
| | RP – ACTGGCACACGCTTTAACTGG |
| PMGC- 422 | FP – AACCTCGAATTAAGAATAACCC |
| | RP – GTCTCGGTTAAGGTATTGTCGC |
| PMGC- 433 | FP – GCAGCATTGTAGAATAATAAAAG |
| | RP – AAGGGGTCTATTATCCACG |
| ORPM-026 | FP - GCTGCAGTCAAATTCCAAAA |
| | RP – CGAGCGTCTTCTTCATGGAT |
| PMGC- 562 | FP – TTTTGGGAGGGGAGTCGAG |
| | RP – ACAACTCTCAACTTCCTAAC |
| PMGC- 571 | FP – CTGGTACCGATGGAGAAGAC |
| | RP – CAAACCAACAACTCACCGTAC |
| PMGC- 2020 | FP – TAAGGCTCTGTTTGTTAGTCAG |
| | RP – GAGATCTAATAAAGAAGGTCTTC |
| PMGC- 2060 | FP – CTCTCAAATGCTGATTTACCG |
| | RP – TCTTCAGTTGCAGTATTCAAAG |
| PMGC- 2140 | FP – GCTGTCAGAATCAAACACTTC |
| | RP – AAGCAGATAACTAAGACATGCC |
| PMGC- 2143 | FP – TCATCATCCATTACTCAACTTG |
| | RP – TCATCATCCATTACTCAACTTG |
| PMGC- 2163 | FP – CAATCGAAGGTAAGGTTAGTG |
| | RP – CGTTGGACATAGATCACACG |

One the basis of the SSR markers, unique DNA fingerprint profile for each genotype was developed to depict relationship among tested collections. The combine plotted dendrogram based on microsatellite molecular markers showed clear pattern of interpretation of the results. Among diversity analysis, high levels of polymorphism produced by 17 primers, out of 18; which separated the 28 collections into two main clusters with 39% similarity (Fig.1). The first major cluster (I) includes 27 collections,

**Table 2: Details of amplification given by 18 SSR primers in 28 *Populus* collections**

| Primer | Primer code | PIC values | Polymorphic bands (No.) | Monomorphic bands (No.) | Unique bands (No.) | Name of collection |
|---|---|---|---|---|---|---|
| ORPM-15 | A | 0.13 | 3 | 0 | 0 | - |
| ORPM-026 | B | 0.59 | 3 | 0 | 0 | - |
| PMGC-325 | C | 0.45 | 3 | 0 | 0 | - |
| PMGC-333 | D | 0.58 | 4 | 0 | 0 | - |
| PMGC-409 | E | 0.48 | 2 | 0 | 0 | - |
| PMGC-422 | F | 0.53 | 3 | 0 | 0 | - |
| PMGC-433 | G | 0.55 | 3 | 0 | 1 | 10 |
| PMGC-451 | H | 0.50 | 2 | 0 | 0 | - |
| PMGC-562 | I | 0.36 | 2 | 0 | 0 | - |
| PMGC-571 | J | 0.53 | 4 | 0 | 1 | S7C8 |
| PMGC-2020 | K | 0.73 | 4 | 0 | 0 | - |
| PMGC-2060 | L | 0.69 | 4 | 0 | 0 | - |
| PMGC-2140 | M | 0.5 | 2 | 0 | 0 | - |
| PMGC-2143 | N | 0.36 | 3 | 0 | 0 | - |
| PMGC-2163 | O | 0.67 | 3 | 0 | 0 | - |
| WPMS-F03 | P | 0.75 | 3 | 0 | 0 | - |
| WPMS-F05 | Q | 0.67 | 3 | 0 | 0 | - |
| PMGC-420 | R | 0 | 0 | 0 | 0 | - |
| Total | | | 51 | 0 | 2 | |

remaining collection G2 (G-48 × S7C11) grouped separately. The cluster I was further divided into 2 sub-clusters. The sub-cluster I contained 15 collections and sub-cluster II contained 12 collections.

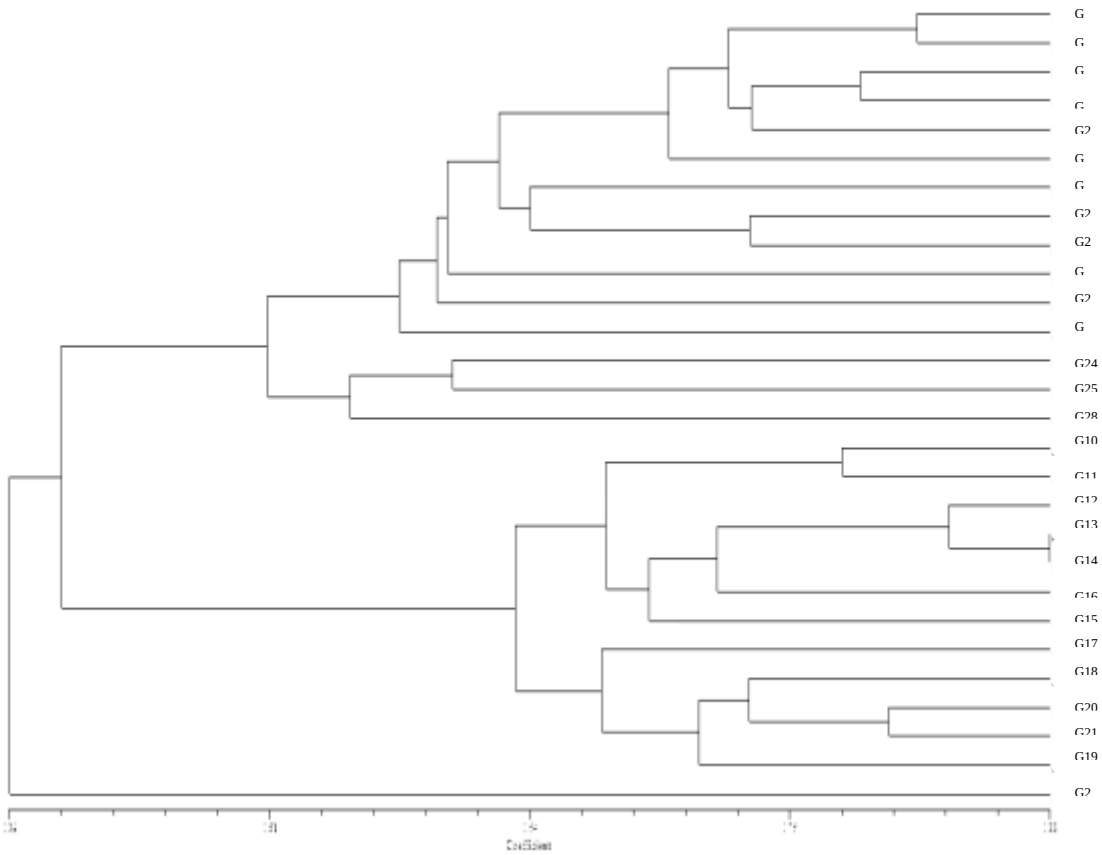


**Fig. 1: Dendrogram of 28 collections of *Populus deltoides* based on SSR analysis**

Out of nine, 8 collections of set I (placed in sub-cluster IV) showed similarity of 59%. Maximum similarity (82%) was found between g1 (G-48 female parent) and g3 (G-48 × L-17/92 hybrid). Seven collections of set II (placed in sub-cluster V) showed similarity of 68% between parents and hybrids. Maximum similarity of 88% were found in '11' (S-1 × 39N hybrid) and 82-42-5 (male parent). The collections of set III placed in sub-cluster VI in dendrogram showed 68% similarity among parents and hybrids. Maximum similarity of 82% was found among 25N and 26N. Seven collections of set IV clustered in sub-cluster I show maximum similarity 42%. The maximum similarity of 73% was found between 19 (L-62/84 × S7C4 hybrid) and $G_{27}$ (G-3 male parent).

Twenty eight collections of *P. deltoides* were placed in 4 set on the basis of parent and hybrid combinations. On similarity basis values, Individual dendrograms were constructed to assess the relationship between parents and hybrids. From individual dendrograms, maximum similarity (82%) was found between G-48 and 12 (G-48 × L-17/92) in set I, 88% between 11 (S-1× 39N) and 82-42-5 in set II, 81% between 25N and 26N in set III and 74% between 19 and S7C1 in set IV, respectively (Fig. 2-5). DNA marker-based studies have been carried out in a number of crop species to assess genetic diversity (Ganopoulos *et al.,* 2011: Singh *et al.,* 2015). In present study, 94.45% polymorphism obtained with SSR markers in *Populus* collections suggested presence Jaccard's similarity coefficient values for SSR markers ranged from 0.39 to 0.88, with average

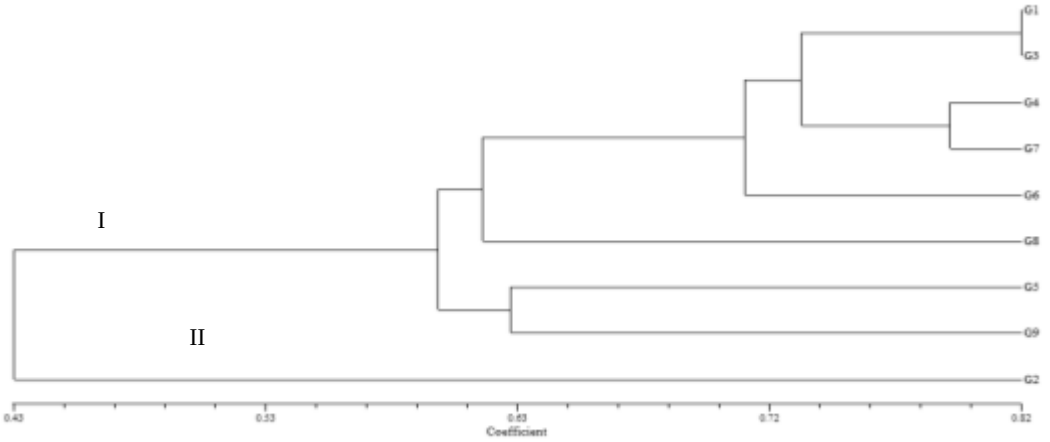


**Fig. 2: Dendrogram of set I based on SSR analysis**

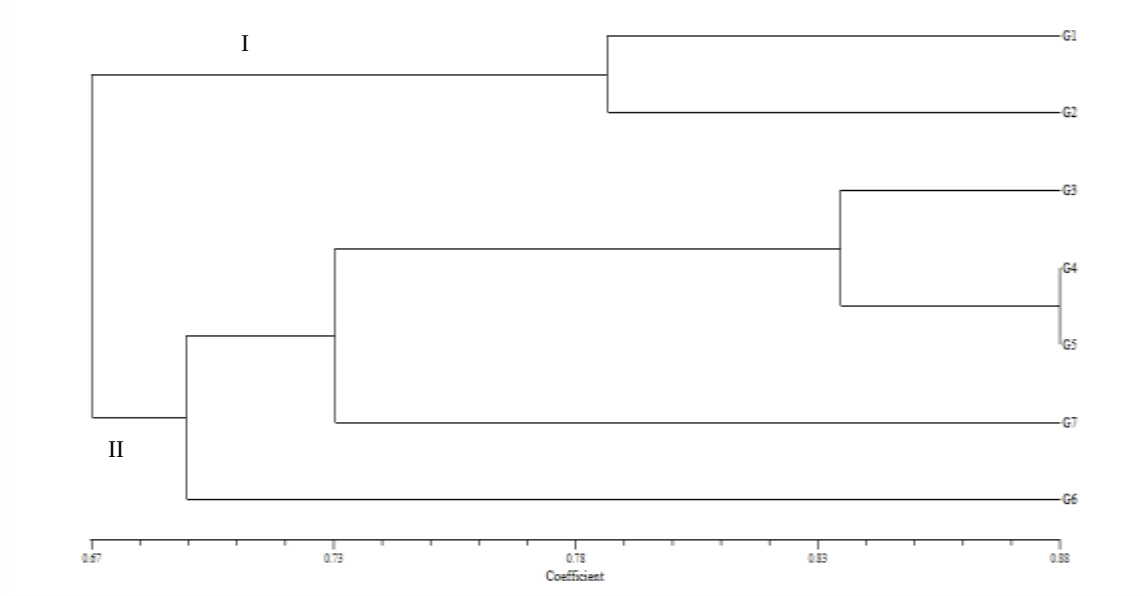


**Fig. 3: Dendrogram of set II based on SSR analysis**

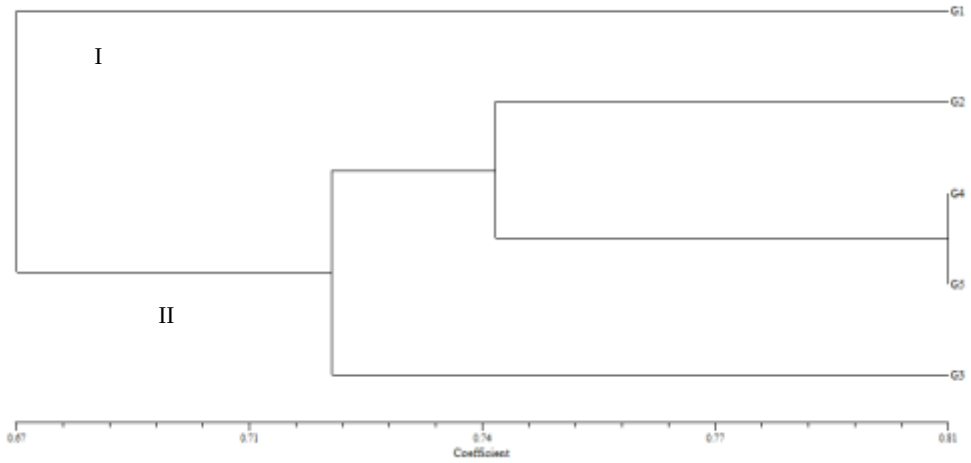


**Fig. 4: Dendrogram of set III based on SSR analysis**

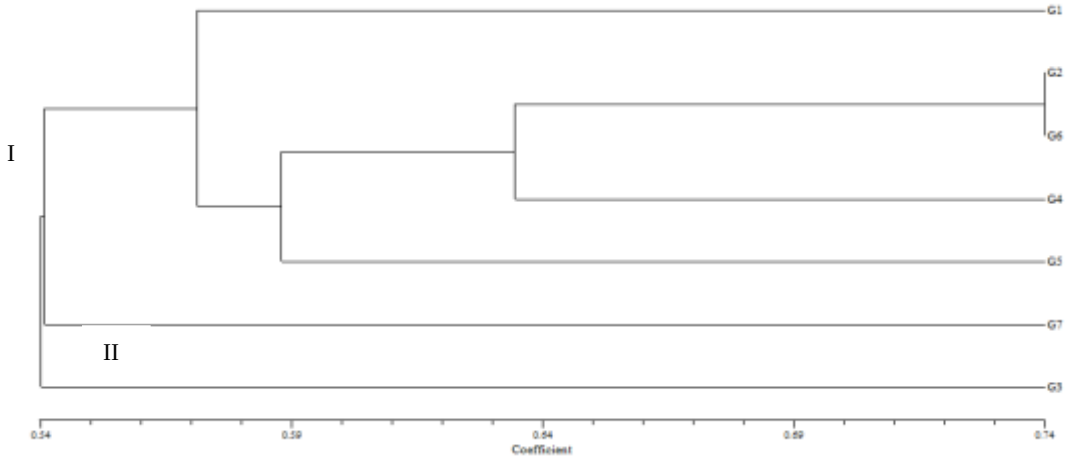


**Fig. 5: Dendrogram of set IV based on SSR analysis**

similarity value of 0.66, showed considerable diversity among the studied collections. At 0.39 similarity coefficient value, the collections of *P. deltoides* are diverged into different clusters. The PIC is a measure of allelic diversity at a locus and ranged from 0.13 to 0.75, with an average of 0.53. Of 18 markers, four markers (24%) revealed PIC value above 0.6. Primer WPMS-F03 amplified 76 alleles and had the highest PIC value of 0.75 and hence had high power of discrimination. The effective multiplex ratio and marker index were 1.82 and 0.80, respectively.

ORPM series (Tuskan e*t al.,* 2004) markers were first designed for *Populus trichocaspa* and WPMS for *P. nigra* (Smulders *et al.,* 2001). With these primers lower amounts of genetic divergence observed between collections was 0.39. Low amount of genetic diversity has also been observed in Tibetan poplar (Shen *et al.,* 2014). Microsatellite technique focuses on the detection of hybridization in natural (Lexer *et al.,* 2005, 2007; Smulders *et al.,* 2008; Castiglione *et al.,* 2010) or artificial (Broeck *et al.,* 2004) poplar stands. SSR loci help to reveal mechanisms of inter-specific crossing and reproductive isolation (Zhang *et al.,* 2009; Lexer *et al.,* 2010; Sanz *et al.,* 2011). In this study we concentrated on variations in hybrids and parents by using SSRs and observed one elite hybrid. All the applications where reliable individual identification required SSRs have shown high effectiveness (Gomez *et al.,* 2003; Fossati *et al.,* 2005). The future conservation management plans should aim to protect as many individuals as possible, as each individual transfer's pollen and seeds. Rapid deforestation may greatly affect gene exchange between individuals and hinder pollen and/or seed

dispersal, thereby leading to high level of genetic differentiation between populations. Long-term geographic barriers increase the probability of extinction so may lead to locally adapt populations (Sharma *et al.,* 2016). Hence, long-term conservation should focus on protecting genetic diversity, not only in individuals but also in populations.

**Conclusion:** In present study "Hybrid 1", a cross derived between G-48 × S7C11, formed a separate group in dendrogram and is considered an elite genotype. This suggests that the variation at genetic level may be due to different combinations of genes present in parents. Thus, this high level of genetic diversity and relationship of *Populus* collections could be used to establish a base line to assist future conservation and breeding programmes. The information obtained could be valuable for devising strategies for getting poplar plants with high vigour and softwood content by making crosses among contrasting individual in future. The SSR markers are highly effective against estimating genetic diversity of *P. deltoides*.

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