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# Assessment of molecular diversity in intra-*hirsutum* genotypes of cotton

**Khushpreet Kaur, Harish Kumar, Dharminder Pathak, Pankaj Rathore and Satnam Singh**

**Abstract**
In any crop plant species, for development of improved varieties requires presence of large magnitude of genetic diversity among the breeding lines. Molecular characterization of genotypes helps in precise estimation of their genetic relatedness, which can be used by breeders for creating the desired variation. In the present investigation, a set of Fifty two markers covering A and D genome of *G. hirsutum* were employed to assess the molecular diversity and genetic relationship among 44 intra-*hisutum* lines, which include a subset of 40 RILs along with parental lines namely RS 2013, PUSA 56-4, standard check LH 2108 and genetic standard TM-1. A total of 263 alleles were detected by 52 primers in the 44 lines with an average of 5.05 alleles per primer. The maximum number of alleles (9) was amplified by NAU3324 and NAU2862 markers. The polymorphism information content (PIC) providing an estimate of discriminatory power of a locus and loci, varied from 0.45 to 0.90 (BNL1421, NAU2722, BNL1421, NAU4898). A dendrogram generated by unweighted pair group with arithmetic mean (UPGMA) revealed the genetic relationships among 44 lines by dividing them into three distinct clusters. Cluster A, B and C represent 23, 11 and 10 lines, respectively. Results reveal the presence of large magnitude of molecular diversity among the studies genotypes.

**Keywords:** *Gossypium hirsutum*, Molecular diversity, SSR marker

## Introduction
Cotton (*Gossypium* sp.) is one of the most important commercial crops globally. Cotton is considered as a crop of prosperity since historic days. It provides highest employment during production, processing, spinning, weaving and marketing in textile industries throughout the world (Ranganatha *et al.* 2013) [12]. Being a rich source of earning valuable foreign exchange it is also regarded as pride of Indian agriculture (Joshi 2006) [9]. The importance of cotton (*Gossypium* sp.) in Indian economy can be judged from the fact that about 17 million farmers are dependent on its cultivation for their livelihood (Branford 2008). The textile industry having cotton as the predominant raw material, contributes about 4 per cent to the GDP and 12 per cent to the total export earnings of the country (http://www.indiantradeportal.in). Therefore, overall development of the Indian economy also depends upon growth and development of cotton industry.

Evaluation of genetic diversity among genotypes elucidate the patterns of germplasm use in breeding programmes and also gives an opportunity to take corrective steps for infusing new genes to avoid the risk associated with narrow genetic base. The studies based on morphological markers have limited use due to limited phenotyping and high genotype × environment interaction. In the recent years molecular markers are the preferential tool for diversity analysis in almost all crops. The DNA markers give a chance for specific characterization of genotypes and measuring of precise genetic relationships than other morphological and biochemical markers. Molecular characterization of cotton genotypes by DNA markers was initially carried out with RFLP (Brubaker *et al.* 1999) [4], RAPD (Mahmood *et al.* 2009) [11], AFLP (Abdalla *et al.* 2001) [1] markers but simple sequence repeats (SSRs) or microsatellite markers are being widely used and have become marker of choice, because it offer several advantages for population genetic studies such as locus specificity, multi-allelic

because of their high mutation rate, co-dominance inheritance high reproducibility, whole genome coverage and relative abundance (Varshney *et al.* 2005) [15]. It is a powerful tool for distinguishing closely related genotypes in various plant species (Gupta and Varshney 2000) [7]. Development of DNA based markers has many applications in plant breeding such as construction of high density molecular linkage maps, gene tagging, marker assisted selection, gene pyramiding, assessment of genetic diversity.

In this context reliable estimation of diversity in recombinant inbred lines at the molecular level assumes substantial importance. So, to identify the genotypes/lines which can be use as parents in hybridization program or can be valuable as varietal lines, an experiment was conducted to assess genetic diversity and relationship among 40 selected elite cotton recombinant inbred lines using SSR markers.

## Material and Methods

### Plant Material

Experimental plant material consists of recombinant inbred lines derived from the cross between American cotton lines namely, RS 2013 and PUSA 56-4A along with parental lines and standard check. PUSA 56-4 was identified for higher mean fibre strength (27.8 g/tex) at Indian Agricultural Research Institute, New Delhi. American cotton variety RS 2013 was developed by Agricultural Research Station, Sriganganagar. It was found to be having medium staple fibre (24.5 mm) and low fibre strength of about 19 g/tex. Both the parents are also contrast in their various agronomic and fiber quality traits. Genetic standard of *G. hirsutum* namely Texas Marker-1 was included in this experiment to generate additional information.

### Genomic DNA extraction

Genomic DNA from the RILs, parental lines (PUSA 56-4 and RS 2013), and standard check (LH 2108) was extracted following protocol given by Saghai-Maroof *et al.* (1984) [14]. About four week old leaves were collected, frozen and was crushed to a fine powder adding in a pestle and mortar. The powder was transferred immediately to a 50 ml autoclaved Oakridge tube containing 20 ml of pre-warmed (60ºC) CTAB extraction buffer(1.5% CTAB, 100mM Tris HCL,1.4M NaCL, 20mM EDTA, 2% β-Mercaptoethanol, 2% Polyvinlpyrolidone) and incubated at 60ºC for one hour in a water bath. After incubation, 15ml of chloroform: isoamyl alcohol (24:1) was added and tubes were shaked on a rotary shaker for 30 minutes and then centrifuged at 10,000 rpm for 10 minutes at room temperature. The supernatant was transferred to a clean sterile 50 ml Falcon tube. Following centrifugation, the upper aqueous phase was transferred to a clean strile 50 ml Falcon tube and about 0.8 volume of chilled isopropyl alcohol was added and the tubes were inverted gently several times. The DNA formed white cotton like precipitate and good quality DNA floated atop. The floating DNA was hooked out using a sterile hooked or pelleted by centrifugation and then transferred into a clean sterile 2.0 ml microfuge tubes and was rinsed with 70 per cent ethanol for five minutes so as to remove any residual salts followed by re-centrifugation. Pellet was collected and the leftover ethanol was dried up completely by turning down microfuge tubes on a bloating paper and was allowed to air dry (at room temperature) for one hour. Then 500-800 µl volume of 1X TE (Tris EDTA buffer-10mM Tris HCl, 1Mm EDTA, pH 8.0) was added. The tubes were left for few hours at room temperature to allow DNA to dissolve. The samples containing DNA were stored at 4ºC until used.

For the purification, the DNA samples were thawed to room temperature and an equal volume of Tris-saturated phenol: chloroform (1:1) was added and centrifuged for about 5 minutes at 12000 rpm. The aqueous phase was piped out in a fresh tube and two chloroform: isoamyl alcohol extractions were performed as before. The upper phase was again pippeted out and 0.1 volume of 3M sodium acetate and 2.5 times the total volume of chilled ethanol were added to it. The contents were mixed gently and the precipitated DNA was spooled out. Then two washings with 70% ethanol were performed to remove extra salts and the pellet was dried at room temperature. The pellet was dissolved in appropriate volume of 1X TE.

### SSR Markers, PCR amplification and gel electrophoresis

A total of Fifty two markers covering A and D genome of *G. hirsutum* were employed to assess the molecular diversity and genetic relationship among 44 intra-*hisutum* lines, which include a subset of 40 RILs along with parental lines namely RS 2013, PUSA 56-4, standard check LH 2108 and genetic standard TM-1(Table 1). The polymerase chain reaction was performed using thermal cycler. 40 ng of genomic DNA was used in 15 µl of total volume with a final concentration of 1X taq buffer, $MgCl_2$ (16mM), Primers (1.0µM), dNTPs (200 µM) and Taq DNA polymerase (1U).

Amplified products were resolved using 6% polyacrylamide gel prepared in 0.5 X TBE buffer. From the total product, 10 µl of each sample was loaded in 6 % polyacrylamide gel and was resolved by running the gel at 300 V for about 90 minutes. The gels were visualized under UV light and photographed using photo gel documentation system (Alphaimager HP, Alpha Innotech).

**Table 1:** Microsatellite primers used in present study

| S. No. | Primer name | Chromosomal location | S. No. | Primer name | Chromosomal location |
|---|---|---|---|---|---|
| 1 | BNL1434 | 1 | 27 | NAU2869 | 10, 20 |
| 2 | BNL409 | s1, 13 | 28 | NAU3426 | 12 |
| 3 | BNL1679 | 12 | 29 | NAU2038 | 3 |
| 4 | BNL834 | 17 | 30 | NAU1014 | 3, 11 |
| 5 | BNL1047 | 22, 25 | 31 | NAU1066 | 10, 20 |
| 6 | BNL3800 | 8 | 32 | NAU3021 | 3 |
| 7 | BNL3281 | 1, 12, 13,18 | 33 | NAU2323 | 10 |
| 8 | BNL3147 | 3, 9, 11, 21 | 34 | NAU2665 | 8, 24 |
| 9 | BNL3948 | 9, 20 | 35 | NAU3325 | 5 |
| 10 | BNL1421 | 1, 13, 18 | 36 | NAU1278 | 9, 12 |
| 11 | BNL530 | 4, 22 | 37 | NAU3489 | 6 |
| 12 | BNL448 | 20, 22 | 38 | NAU3402 | 5 |
| 13 | BNL3792 | 2, 8, 20 | 39 | NAU2862 | 7, 16 |

| | | | | | |
|---|---|---|---|---|---|
| 14 | BNL1122 | 16 | 40 | NAU2348 | 9 |
| 15 | BNL2440 | 1, 10, 15 | 41 | NAU3433 | 1, 15 |
| 16 | BNL1694 | 7, 16 | 42 | NAU5373 | 6, 25 |
| 17 | BNL3189 | 2, 14, 26 | 43 | NAU2896 | 2 |
| 18 | BNL3449 | 11, 21 | 44 | NAU3127 | 4 |
| 19 | BNL3359 | 6, 13, 25 | 45 | NAU3083 | 3 |
| 20 | BNL2652 | 1,13, 18 | 46 | NAU2373 | 15 |
| 21 | NAU5347 | 5, 19 | 47 | NAU3284 | 11 |
| 22 | NAU5384 | 2 | 48 | NAU2714 | 6, 25 |
| 23 | NAU920 | 2, 8 | 49 | NAU5289 | 3 |
| 24 | NAU3324 | 8, 24 | 50 | NAU3839 | 3 |
| 25 | NAU4898 | 5 | 51 | NAU3690 | 1, 15 |
| 26 | NAU2722 | 1 | 52 | MUSS500 | 8, 24 |

**Scoring of SSR alleles and Analysis**

Scoring of the SSR alleles was performed manually with respect to location of the bands sequentially from the smallest to the largest-sized bands. Banding pattern of each of the microsatellite marker was recorded for all the genotypes as per the requirements of software. In binomial format diffused bands or bands showing ambiguity in scoring were taken as missing data and designated as '9' in comparison with '1' for the presence of a band and '0' for the absence of a band in data matrix. Finally, the data were entered in an excel sheet for required statistical analyses.

The genetic diversity among the lines was computed using computer software programme – DARwin (Perrier and Jacquemond–Collet 2006). The data were subjected to unweighted pair group method with arithmetic mean (UPGMA) analysis to generate dendogram. Polymorphic information content (PIC) that provides an estimate of the discriminatory power of a locus or loci, by taking into account not only the number of alleles that are expressed, but also relative frequencies of those alleles, was estimated using following equation of Botstein *et al.* (1980).

$$PIC = 1- \sum_{i=1}^{n} (P_{ij})^2 - \{ \sum_{i=1}^{n} (P_{ij})^2 \}^2 + \sum_{i=1}^{n} \{(P_{ij})^2 \}^2$$

Where $P_{ij}$ is the frequency of $j^{th}$ allele in $i^{th}$ primer and summation extends over 'n' patterns.

**Results and Discussion**

Assessment of genetic relationships among the diverse genotypes is prerequisite to get success in any crop's breeding programmes. Genetic diversity analyses based on molecular markers is more effective in the assessment of genetic relationship among the genotypes as compared to the morphological markers. In the present investigation, a set of 52 SSR markers were used to assess the molecular diversity among 44 lines, which include a subset of 40 RILs along with parents, standard check LH 2108 and genetic standard TM-1.

Summarized data for number of alleles detected per marker and polymorphic information content (PIC) values for each of the 52 SSR markers are presented in Table 2. Representative gels showing SSR polymorphism is presented in figure 1 and 2. Based on the specificity of genotype of a plant, a particular DNA profile can be ascribed to a particular plant. The number of alleles detected per marker and diversity level depends upon the number and origin of genotypes. A total of 263 alleles were detected by 52 primers in the 44 lines with an average of 5.05 alleles per primer. The maximum number of alleles (9) was amplified by NAU3324 and NAU2862 markers whereas minimum number of alleles (3) was amplified by NAU2323, BNL448, NAU3083, NAU2373, NAU5384, BNL3359, NAU1014, NAU3489, NAU3433, BNL3948, BNL3948, BNL3189 and NAU3839 primers. Elci *et al.* 2014 [6] detected 103 alleles employing 40 markers in 96 (94 *G. hirsutum* L, 1 *G. herbaceum* L, and 1 *G. barbadense* L.) cultivars with an average of 2.57 alleles per primer. Dahab *et al.* (2013) [5] also observed 241 alleles employing 70 SSR markers with an average of 3.44 alleles per primer in 50 representative cotton cultivars. A total of 66 alleles were obtained by Bertini *et al.* (2006) [2] by using 31 SSR primer pairs with an average of 2.13 alleles in 53 cotton cultivars.

**Table 2:** Primers with their specific number of alleles and PIC values

| S. No. | Marker Name | No. of alleles | PIC | S. No. | Marker no. | No. of alleles | PIC |
|---|---|---|---|---|---|---|---|
| 1 | NAU2869 | 8 | 0.81 | 27 | BNL1434 | 4 | 0.78 |
| 2 | BNL409 | 6 | 0.80 | 28 | NAU3426 | 7 | 0.83 |
| 3 | BNL1679 | 5 | 0.77 | 29 | NAU2038 | 5 | 0.75 |
| 4 | NAU1066 | 6 | 0.80 | 30 | NAU1014 | 3 | 0.57 |
| 5 | BNL1047 | 5 | 0.73 | 31 | BNL834 | 4 | 0.67 |
| 6 | NAU3324 | 9 | 0.84 | 32 | BNL3800 | 4 | 0.67 |
| 7 | NAU4898 | 4 | 0.90 | 33 | BNL3281 | 6 | 0.79 |
| 8 | BNL3147 | 5 | 0.45 | 34 | NAU2665 | 5 | 0.75 |
| 9 | NAU3021 | 4 | 0.70 | 35 | NAU3325 | 4 | 0.80 |
| 10 | NAU2323 | 3 | 0.70 | 36 | NAU1278 | 4 | 0.66 |
| 11 | BNL530 | 7 | 0.81 | 37 | NAU3489 | 3 | 0.58 |
| 12 | BNL448 | 3 | 0.61 | 38 | NAU3402 | 8 | 0.86 |
| 13 | BNL3792 | 5 | 0.76 | 39 | NAU2862 | 9 | 0.88 |
| 14 | NAU5373 | 7 | 0.84 | 40 | NAU2348 | 6 | 0.90 |
| 15 | NAU2896 | 5 | 0.78 | 41 | NAU3433 | 3 | 0.70 |
| 16 | NAU5347 | 7 | 0.85 | 42 | BNL3948 | 3 | 0.54 |
| 17 | MUSS500 | 6 | 0.75 | 43 | BNL1421 | 4 | 0.90 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 18 | NAU3127 | 7 | 0.84 | 44 | BNL1122 | 7 | 0.82 |
| 19 | NAU3083 | 3 | 0.67 | 45 | BNL2440 | 8 | 0.81 |
| 20 | BNL3449 | 6 | 0.83 | 46 | BNL1694 | 5 | 0.80 |
| 21 | NAU2373 | 3 | 0.78 | 47 | BNL3189 | 4 | 0.64 |
| 22 | NAU3284 | 4 | 0.81 | 48 | NAU2714 | 3 | 0.74 |
| 23 | NAU5384 | 3 | 0.74 | 49 | NAU5289 | 5 | 0.81 |
| 24 | BNL3359 | 3 | 0.72 | 50 | NAU3839 | 3 | 0.88 |
| 25 | BNL2652 | 8 | 0.87 | 51 | NAU3690 | 4 | 0.70 |
| 26 | NAU2722 | 6 | 0.90 | 52 | NAU920 | 4 | 0.68 |

The PIC value of each marker was used to assess their in formativeness. Selecting only highly polymorphic markers may result in overestimating the genetic diversity, therefore to reduce bias, all the markers were used for diversity assay. The polymorphism information content (PIC) value, varied from 0.45 to 0.90 indicates a considerable efficiency of markers for studying the polymorphism level in recombinant inbred lines. The highest PIC value was shown by BNL1421, NAU2722, BNL1421, NAU4898 (0.90) primers which is followed by NAU3839 (0.88), NAU2862 (0.88), BNL2652 (0.87) and NAU3402 (0.86). PIC value depends upon the nature of various genotypes being analyzed; as closely related genotypes were shown less polymorphism in comparison to genetically diverse genotypes (Hoque *et al*. 2015) [8]. Elci *et al.* (2014) [6] reported the PIC value ranging from 0.00 (NAU1369) to 0.749 (CIR246). PIC values observed by Bertini *et al.* (2006) [2] and Liu *et al.* (2000) [10] were 0.18 to 0.62 and 0.05 to 0.82 respectively.

The 44 lines were broadly divided into three clusters (A, B and C) (fig. 3). Cluster A consist of 22 lines. Which is further sub divided in two sub clusters. Sub cluster A1 comprised of 10 lines (81, 78, 149, 89, 9, 86, 14, 6, 1, 74). Line 81, 78 and 149 exhibited highest similarity in this sub cluster. Sub cluster A2 included 12 lines (52, 4, 63, 72, 188, 173, 176, 53, 131, 119, 148 and 113). The maximum similarity in this sub cluster was recorded between line 188 and 173. Cluster B consist of 11 lines that also had two sub clusters. Standard check, LH 2108 included in Sub cluster B2, having highest similarity with line 191. Cluster C consist of 10 lines including two parents (line 139, 7, 38, 62, 92, 12, 73, 138, RS 2013 and PUSA 56-4). Parent PUSA 56-4 exhibited similarity with line 12 and 73, whereas RS 2013 exhibited similarity with line 138. The genetic standard TM-1 was observed to be in cluster A. The lines present in this cluster are more similar to genetic standard.

It is evident from the present investigation that substantial variation is available among the RILs. The population may be used for the mapping of some economically important quantitative trait loci and their interaction with the environment. As the study was done with limited set of markers, so, more molecular exploration with more number of markers need to be done. Thus, the genetic variability assessment based on quantitative characters and genetic diversity based on SSRs present a perfect picture to build upon future prospects. Such preliminary studies are very crucial for planning and executing successful projects utilizing the mapping populations.

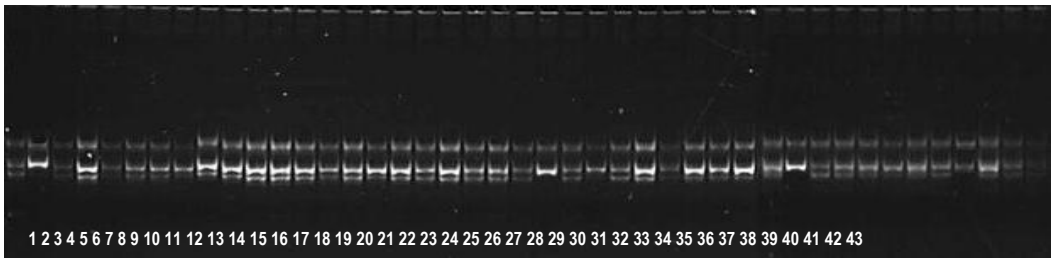


**Fig 1:** PCR amplification products obtained with BNL3147 primer

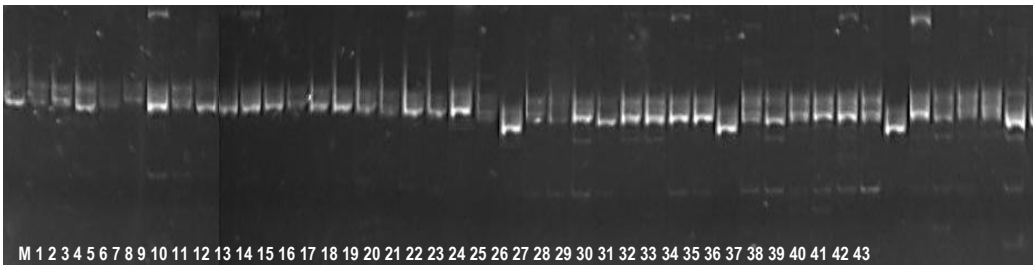


**Fig 2:** PCR amplification products obtained with BNL3558 primer

**Table 3:** Description of plates

| **1–PUSA56-4** | **2–RS2013** | **3–Line1** | **4–Line4** | **5–Line6** | **6–Line7** | **7–Line9** |
|---|---|---|---|---|---|---|
| b–Line12 | 9–Line14 | 10–Line18 | 11–Line26 | 12–Line32 | 13–Line35 | 14–Line38 |
| 15–Line41 | 16–Line52 | 17–Line53 | 18–Line62 | 19–Line67 | 20–Line68 | 21–Line72 |
| 22–Line73 | 23–Line74 | 24–Line78 | 25–Line81 | 26–Line86 | 27–Line89 | 28–Line92 |
| 29–Line113 | 30–Line119 | 31–Line121 | 32–Line131 | 33–Line138 | 34–Line139 | 35–Line148 |
| 36–Line149 | 37–Line173 | 38–Line176 | 39–Line181 | 40–Line186 | 41–Line188 | 42–Line191 |
| 43–Line2108 | | | | | | |

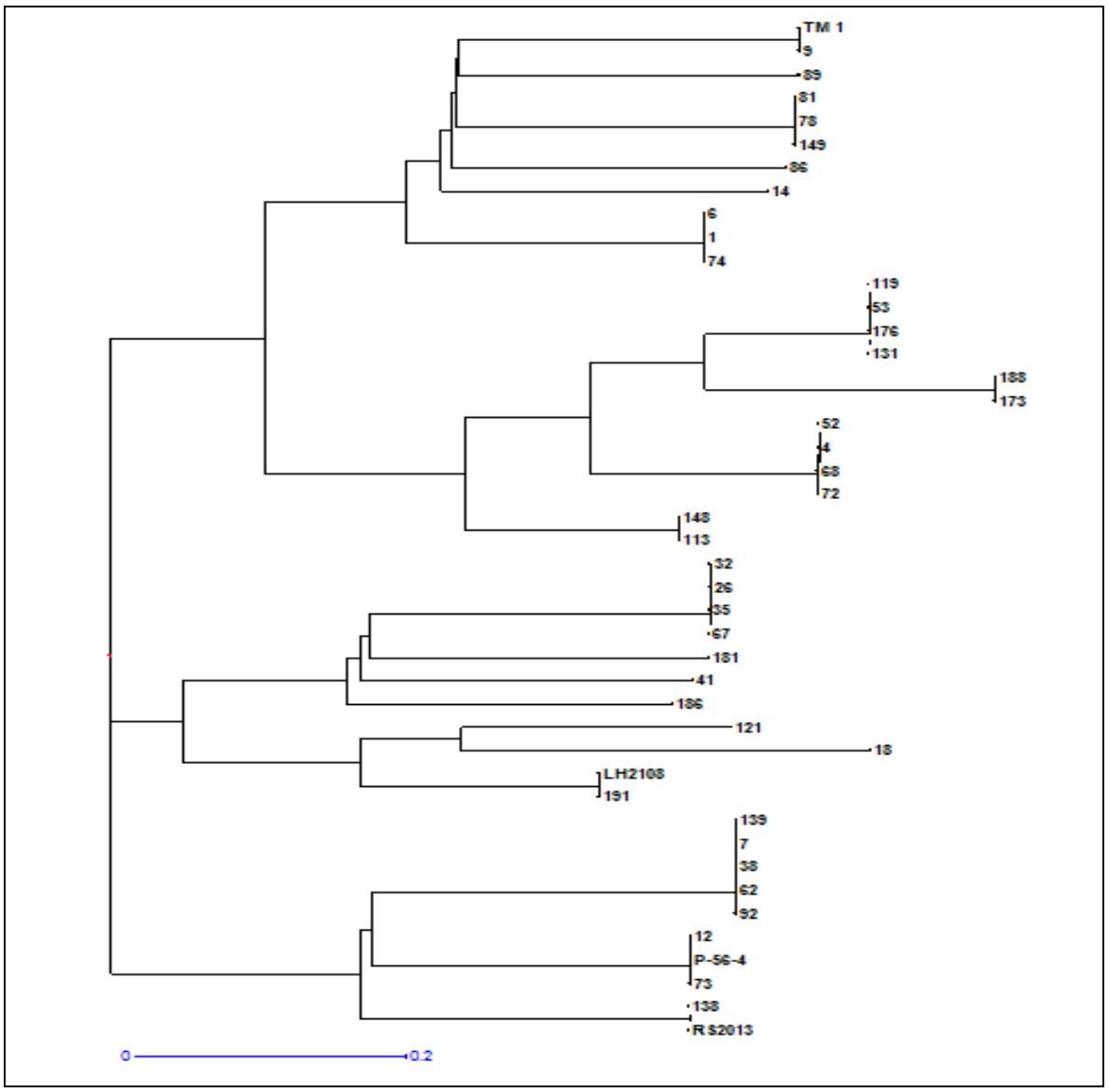


**Fig 3:** Dendrogram obtained by SSR marker analysis using DAR win 5.0

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