

Original Research Article
Evaluation of Genetic Diversity among CLCuD-Resistant Upland Cotton Varieties Using SSR Markers

ABSTRACT

Cotton leaf curl disease (CLCuD) is a dangerous viral disease that affects the productivity of cotton crops. Since early detection, this disease has spread worldwide and has caused serious damage to the production of cotton around the world particularly in Pakistan. In this study, we evaluated the genetic diversity of CLCuD-resistant upland cotton germplasm by using microsatellite markers. For this purpose, 25 pairs of microsatellite primers were screened and 9 were found to be polymorphic. These 9 primers were used for assessing the genetic diversity of 75 upland cotton varieties. Using these markers, a total of 46 loci were amplified with an average of 5.11 loci per primer. BNL0409 and BNL2835 produced the highest number of bands, 8 bands by each primer. Mean gene diversity was 0.651 with 0.91 (BNL2835) being the highest and 0.38 (JESPR0013) being the lowest. Polymorphism information content (PIC) and major allele frequency ranged between 0.91 to 0.36 (for BNL2835 and BNL0341) and 0.77 to 0.32 (for JESPR0013 and BNL0409) respectively. Mean PIC and major allele frequency were 0.62 and 0.49 respectively. BNL2835 showed the highest allele number of 23 and the mean allele number was 11. Principal component analysis (PCA) was performed and the PCA graph was plotted. These results showed that the 75 varieties studied, using 9 microsatellite markers have an average level of genetic diversity. The findings of this study could be utilized for genetic analysis and the outcomes might be useful for cotton breeding programs.

Keywords: Molecular diversity, CLCuV, microsatellites, SSR, upland cotton, PIC, PCA

1. INTRODUCTION

The agricultural sector is a major contributor to Pakistan's economy, accounting for 22.7% of gross domestic product (GDP) [1]. Among the various crops grown in Pakistan, cotton (*Gossypium hirsutum* L.) (Malvales: Malvaceae) is of particular importance, as the country's rural-industrial -has a significant impact on utilization In fact, the economic growth of this sector is highly dependent on cotton production, which directly and indirectly affects employment [2]. These crops are susceptible to both biotic and abiotic degradation, and sustainable crop production is essential for the sustainability of Pakistan's economy.

The cotton crop faces challenges due to both biotic and abiotic factors. Fluctuating environmental conditions, such as increased temperatures and reduced rainfall, have a considerable effect on drought occurrence, which has adverse effects on the quality and yield of cotton globally. Various pathogens contribute to diseases in the cotton cultivars, including Bacterial blight, Root rot, Verticillium and Fusarium wilts, and cotton leaf curl disease (CLCuD). Among these, the Cotton crop is seriously threatened by cotton leaf curl virus (CLCuV) in the subcontinent [3]. Although *G. hirsutum*, a species of cotton, exhibits some level of tolerance against CLCuV disease [4], it still remains susceptible to these

ailments. In recent years, CLCuD has been consistently spotted in numerous countries in Africa and South Asia, particularly in Pakistan, northwestern regions of India, and more recently, in China [5].

CLCuD has caused substantial damage to the cotton productivity of China, India, and Pakistan leading to huge losses to the economy of the countries, out of which, Pakistan is among the top that faced serious losses due to this virus [6, 7]. The symptoms of this disease include thickened veins, curled leaves, and tiny enations formed on the leaves' lower side [3]. The symptoms are used for the characterization of this disease. The viruses responsible for causing this are termed CLCuD-associated *Begomoviruses* (CABs). These viruses are part of the genus *Begomovirus* in a family of viruses called Geminiviridae. The transmission of these viruses occurs through the vector whitefly called *Bemesia tabaci* [8]. There is a broader range of Geminiviridae. One of the special features of this family is that these viruses contain circular, single-stranded DNA (ssDNA) which is contained inside icosahedral twin particles. These viruses have been divided into nine genera in accordance with their range of hosts, the medium of transmission, and genome organization [9]. The most widespread and diversified of them all is the genus *Begomovirus* [10]. Microsatellites are the marker preference for many scientists when it comes to genetic diversity and structure analyses, parentage analyses, and pairing system estimations when co-dominance is crucial. Microsatellites, which are sometimes referred to as SSRs or STRs, are commonly regarded as repetitive sequences of between one and six bases that are present within the nuclear and organelles' genetic material of eukaryotic organisms [11]. SSRs offer several advantages in genetic studies. They follow a co-dominant inheritance pattern, meaning that they can be amplified and detected in both heterozygous and homozygous loci. Their advantages include broad genome coverage, abundance, and reproducibility. Their detection is easier by polymerase chain reaction (PCR) technique and offers locus-specific information. These characteristics make SSRs valuable tools in various applications such as genetic mapping, population genetics, DNA fingerprinting, and molecular breeding programs.

In this study, we aimed to evaluate the genetic diversity among seventy-five upland cotton varieties using SSR markers.

2. MATERIALS AND METHODS

This study was conducted at the Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University Multan from June 2022 to June 2023. In this study, genetic diversity analysis of cotton cultivars was done against whitefly resistance using simple sequence repeat (SSR) markers. A total of 75 CLCuD-resistant cotton cultivars were used for evaluating genetic diversity. The names of the varieties used in this study are given in Table 1. A total of 25 SSR markers were screened, out of which, 9 markers were found to be polymorphic (Table 2). These 9 markers were proceeded with for further study. DNA from fresh leaves was extracted using a modified CTAB extraction protocol [12]. The markers were used to amplify alleles of the 75 cotton varieties. PCR was performed using the standard procedure and the PCR products were subjected to agarose gel electrophoresis for band visualization. The bands were visualized and recorded using GelDoc XR+ in

combination with ImageLab software (<https://www.bio-rad.com/en-pk/product/image-lab-software>).

Band data were recorded in the form of a binary data matrix using Excel. This binary data matrix was used for phylogenetic and statistical analysis. The data was imported in PowerMarker V 3.25 (<http://statgen.ncsu.edu/powermarker/>) and different parameters were analyzed including major allele frequency, polymorphism information content (PIC), gene diversity, and allele number [13]. Following that, genetic distance among the varieties was calculated and a neighbor-joining (NJ) phylogenetic tree was constructed. Principal component analysis (PCA) was performed using OriginPro (https://www.originlab.com/demodownload_st.aspx) and PC1 and PC2 were plotted.

Table 1: Cotton varieties utilized for genetic diversity assessment in current research work

| Sr. No. | Variety Name | Sr. No. | Variety Name | Sr. No. | Variety Name |
|---------|--------------|---------|-------------------|---------|---------------|
| 1 | Barhi-M1 | 26 | Cris-580 | 51 | CIM-482 |
| 2 | CIM-443 | 27 | CIM-473 | 52 | CIM-496 |
| 3 | Cris-583 | 28 | DPL New Cotton-33 | 53 | Cris-587 |
| 4 | Cris-599 | 29 | SLH-13 | 54 | CIM-446 |
| 5 | Cris-613 | 30 | FH-142 | 55 | Sitar-008 |
| 6 | SLH-119 | 31 | S-14 | 56 | SLH-317 |
| 7 | VH-282 | 32 | B-021 | 57 | VH-305 |
| 8 | VH-363 | 33 | CIM-448 | 58 | NIAB-2009 |
| 9 | SI Okra1-23 | 34 | TARZAN-2 | 59 | GM-90 |
| 10 | FH-152 | 35 | NIAB-2010 | 60 | IR-3701 |
| 11 | Samaru-72 | 36 | Cris-635 | 61 | CA-325 IRABLT |
| 12 | AA-802 | 37 | FVH-53 | 62 | Cris-562 |
| 13 | RH-112 | 38 | CIM-499 | 63 | CIM-1100 |
| 14 | NIAB-112 | 39 | Hari-Dost | 64 | SLH-12 |
| 15 | FBS-30 | 40 | SLS-90/2 | 65 | SLH-06 |
| 16 | CIM-506 | 41 | LB-391 | 66 | Cris-625 |
| 17 | Malmal | 42 | VH-300 | 67 | FH-113 |
| 18 | Cris-541 | 43 | Chilala-76/2 | 68 | CIM-632 |
| 19 | CEMB-33 | 44 | SLS-B7/175 | 69 | Barberton |
| 20 | LaOkra-541 | 45 | TARZAN-1 | 70 | FBS-37 |
| 21 | FH-87 | 46 | Gomal-105 | 71 | MNH-886 |
| 22 | Bt.CIM-602 | 47 | DP-Acala-90 | 72 | Cyto-179 |
| 23 | Cris-601 | 48 | Cris-628 | 73 | IUB-222 |
| 24 | BH-160 | 49 | CIM-612 | 74 | Cyto-124 |
| 25 | S-32 | 50 | MNH-329 | 75 | CIM-554 |

Table 2: SSR primers' names and sequence used for genetic diversity assessment

| Name | Annealing Temp. °C | Direction | Sequence 5' to 3' |
|-----------|--------------------|-----------|----------------------------|
| JESPR0013 | 54.7 | F | GCTCTCAAATTGGCCTGTGT |
| | | R | GGTGGAGGCATTCCTGCTAAC |
| BNL2835 | 57.8 | F | AAGATAATCGCCGGCTAGCT |
| | | R | CCGCCAGTTTGCCATAATAT |
| JESPR0220 | 54.7 | F | CGAGGAAGAAATGAGGTTGG |
| | | R | CTAAGAACCAACATGTGAGACC |
| JESPR0274 | 57.8 | F | GCCCACTCTTTCTTCAACAC |
| | | R | TGATGTCATGTGCCTTGC |
| BNL0409 | 55.9 | F | AGAAGTCGGACGTGGAAAGA |
| | | R | GCCGTTTTCTCAGTGATGT |
| BNL0387 | 54.7 | F | GAAGGGGAATTTATAGCGGG |
| | | R | AGAGACTCCCGCACTTGAAA |
| BNL0341 | 55.9 | F | ACCTGGGGTACTTGTCCACA |
| | | R | CCATCCCATTTGTGATACCC |
| BNL0243 | 54.7 | F | GGGTTTTCTGGGTATTTATAACAACA |
| | | R | TCATCCACTTCAGCAGCATC |
| JESPR0042 | 57.8 | F | CGTTGCCGTCTTCGACTCCTT |
| | | R | GTGGGTGGCTAATATGTAGTAGTCG |

3. RESULTS AND DISCUSSION

To analyze the genetic diversity of upland cotton germplasm, in this research work, we used 9 sets of SSR primers on 75 cotton varieties. These 9 markers amplified 46 loci in selected cotton cultivars. The highest number of amplified by a single primer was 8 which was demonstrated by two markers, BNL2838 and BNL0409 respectively (Table 4). The second highest number of loci per marker was 7 which were amplified by BNL0243 and the lowest number of loci amplified was 3 by BNL0341 and JESPR0013 respectively.

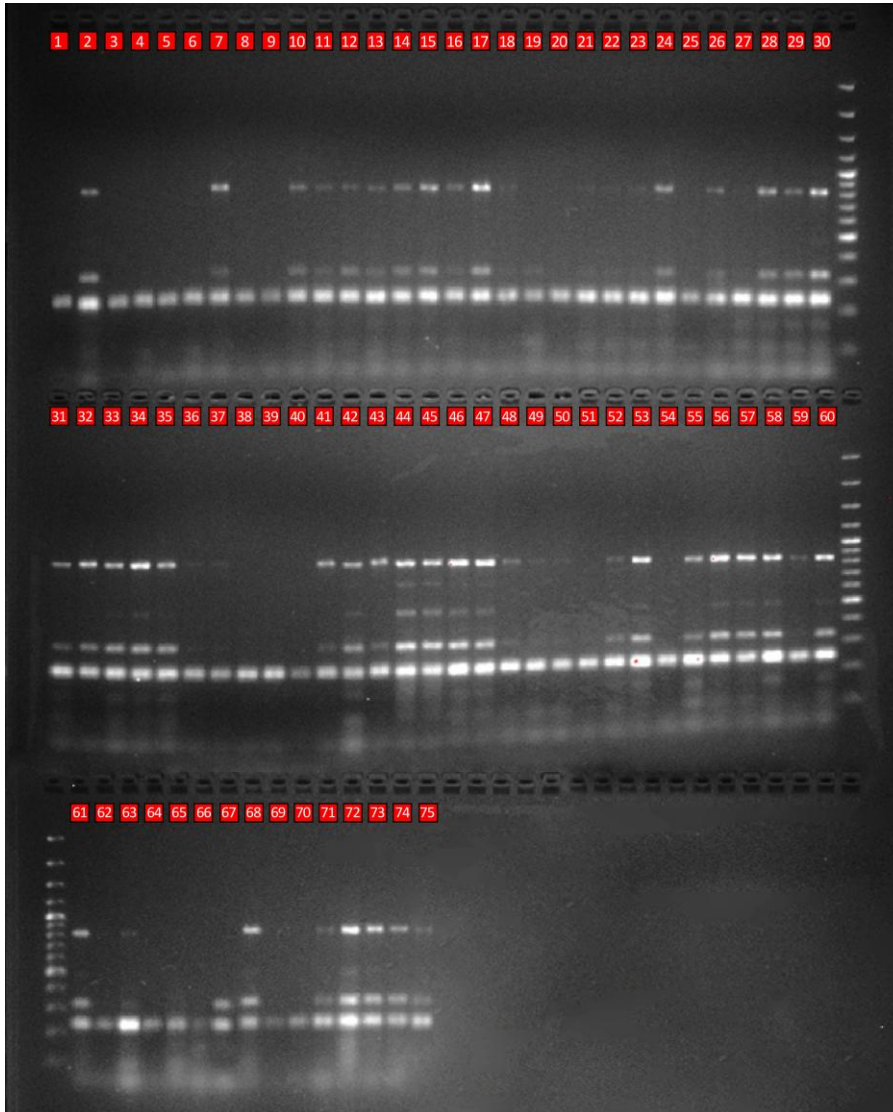


Figure 1: Agarose gel visualization for BNL2835 on 75 samples

A binary data matrix of gel results was used. Details of the amplified bands along with other analyzed parameters are provided in Table 3.

Table 3: Summary statistics of results including major allele frequency, allele number, genetic diversity, PIC, and number of unique bands.

| Marker | Major Allele Frequency | Sample Size | Allele No. | Gene Diversity | PIC | Number of Unique Bands |
|-----------|------------------------|-------------|------------|----------------|--------|------------------------|
| BNL0409 | 0.3200 | 75 | 20 | 0.8469 | 0.8347 | 8 |
| JESPR0220 | 0.5867 | 75 | 5 | 0.5607 | 0.4937 | 3 |
| JESPR0274 | 0.6800 | 75 | 9 | 0.5077 | 0.4799 | 5 |
| BNL2835 | 0.1467 | 75 | 23 | 0.9170 | 0.9113 | 8 |
| BNL0341 | 0.6933 | 75 | 4 | 0.4405 | 0.3648 | 3 |
| BNL0243 | 0.2933 | 75 | 17 | 0.8473 | 0.8328 | 7 |
| JESPR0013 | 0.7733 | 75 | 6 | 0.3897 | 0.3749 | 3 |
| JESPR0042 | 0.2800 | 75 | 11 | 0.8245 | 0.8043 | 4 |
| BNL0387 | 0.6667 | 75 | 7 | 0.5312 | 0.5091 | 5 |
| Total | | | | | | 46 |
| Mean | 0.4933 | 75 | 11 | 0.6517 | 0.6228 | 5.1111 |

OriginPro was used to perform PCA. Results of PCA are presented in Figures 2 and 3. PC1 and PC2 had an eigenvalue of 5.098 and 3.535 and 10.85 and 7.52% of the variance. Comparatively higher eigenvalues of PC1 and PC2 represent the accuracy of PCA and represent their contribution in variance.

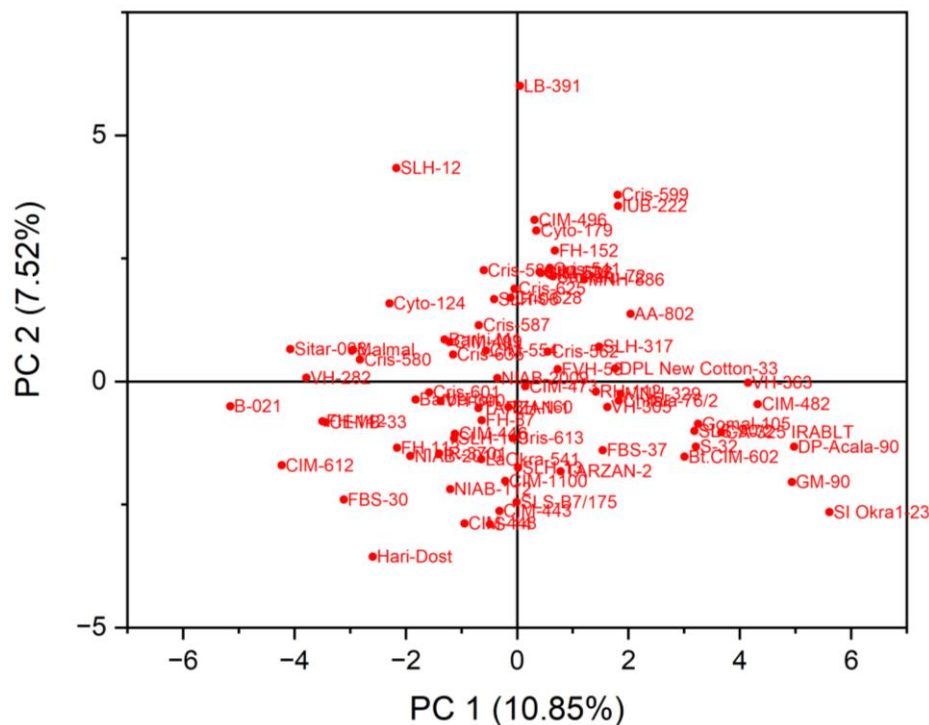


Figure 2: Score plot of PC1 and PC2 from PCA analysis

This phylogenetic tree is divided into three main clusters A, B, and C, each one consisting of 20, 21, and 34 varieties respectively. Cluster A holds two sub-clusters and in this cluster, SAMARU-72 showed the most distant relationship with a genetic distance of 0.35. Following this two varieties, MNH-886 and IUB-222, showed the second-highest genetic distance of 0.28 in cluster A. In cluster B, SIOkra1-23 displayed the highest genetic distance of 0.30 followed by 0.29 for VH-305. Finally in cluster C, having 34 varieties, Cyto-124 showed the highest genetic distance of 0.34 in the first sub-cluster and 0.48 cumulative genetic distance.

Studies like this have been performed in the past which evaluated the genetic diversity of cotton germplasm using SSR markers [14, 15]. In this study, we used a higher number of varieties which gave more insight into the genetic diversity of upland cotton. On average, our findings demonstrated higher gene diversity and PIC and lower major allele frequency than the findings of Ali et al. [14] and Hussain et al [15]. This suggests that the available upland cotton germplasm is more diverse than previous considerations.

Obtained results demonstrate that many varieties of the available cotton germplasm have considerable levels of genetic diversity and can be helpful in breeding programs to develop new better varieties that may not only show resistance to pests and viral infections but may also have favorable characteristics like high yield and stress tolerance. Further genomic analysis, sequencing, transcriptomic, and proteomic studies are required to validate these results.

4. CONCLUSION

The average gene diversity and PIC values along with PCA analysis conclude higher levels of genetic diversity than what previous studies suggest. This can be helpful for developing improved varieties. Breeders can make use of this genetic diversity to develop new varieties with better morphological traits as well as improved resistance to diseases like CLCuD and other biotic and abiotic stresses. Cross-breeding of varieties with high yield with those showing resistance to CLCuD can produce new varieties with beneficial features from both. Further studies are required to expand the scope of current results.

REFERENCES

1. Imtiaz Ahmad HMM, Naila Abbas Dar, Shuaib Malik. *Pakistan Economy Survey 2021-22*, F.D. Economic Adviser's Wing, Editor. 2022
2. Mayee C, Monga D, Dhillon S, Nehra P, and Pundhir P. *Cotton-wheat production system in South Asia: A success story*. 2008
3. Farooq A, Farooq J, Mahmood A, Shakeel A, Rehman KA, Batool A, et al. *An overview of cotton leaf curl virus disease (CLCuD) a serious threat to cotton productivity*. Aust J Crop Sci, 2011. **5**(13): p. 1823-1831
4. Zafar Y, Bashir A, Mansoor S, Saeed M, Asad S, Saeed N, et al. *Cotton leaf curl virus epidemic in Pakistan: virus characterization, diagnosis and development of virus resistant cotton through genetic engineering*. in *Proceedings of the Technical Seminar at the 56th Plenary Meeting of the International Cotton Advisory Committee, Washington DC*. 1997.

5. Cai JH, Xie K, Lin L, Qin B, Chen B, Meng J, et al. *Cotton leaf curl Multan virus newly reported to be associated with cotton leaf curl disease in China*. Plant Pathol, 2010. **59**(4): p. 794-795.doi: 10.1111/j.1365-3059.2010.02266.x
6. Mansoor S, Briddon RW, Zafar Y, and Stanley J. *Geminivirus disease complexes: an emerging threat*. Trends Plant Sci, 2003. **8**(3): p. 128-134.doi: 10.1016/S1360-1385(03)00007-4
7. Zubair M, Zaidi SS-e-A, Shakir S, Amin I, and Mansoor S. *An insight into Cotton leaf curl Multan betasatellite, the most important component of cotton leaf curl disease complex*. Viruses, 2017. **9**(10): p. 280.doi: 10.3390/v9100280
8. Briddon RW and Markham P. *Cotton leaf curl virus disease*. Virus Res, 2000. **71**(1-2): p. 151-159.doi: 10.1016/S0168-1702(00)00195-7
9. Varsani A, Roumagnac P, Fuchs M, Navas-Castillo J, Moriones E, Idris A, et al. *Capulavirus and Grablovirus: two new genera in the family Geminiviridae*. Arch Virol, 2017. **162**: p. 1819-1831.doi: 10.1007/s00705-017-3268-6
10. Fauquet C, Briddon R, Brown J, Moriones E, Stanley J, Zerbini M, et al. *Geminivirus strain demarcation and nomenclature*. Arch Virol, 2008. **153**: p. 783-821.doi: 10.1007/s00705-008-0037-6
11. Zane L, Bargelloni L, and Patarnello T. *Strategies for microsatellite isolation: a review*. Mol Ecol, 2002. **11**(1): p. 1-16.doi: 10.1046/j.0962-1083.2001.01418.x
12. Doyle JJ and Doyle JL. *A rapid DNA isolation procedure for small quantities of fresh leaf tissue*. Phytochem Bull, 1987
13. Liu K and Muse SV. *PowerMarker: an integrated analysis environment for genetic marker analysis*. Bioinform, 2005. **21**(9): p. 2128-2129.doi: 10.1093/bioinformatics/bti282
14. Ali I, Khan NU, Gul S, Khan SU, Bibi Z, Aslam K, et al. *Genetic diversity and population structure analysis in Upland cotton Germplasm*. Int J Agric Biol, 2019. **22**(4): p. 669-76.doi: 10.17957/IJAB/15.1114
15. Hussain SB, Farooq L, and Zubair M. *Assessment of Genetic Diversity for Cotton Leaf Curl Disease (CLCuD) and Qualitative Traits among Elite Cotton Cultivars*. Asian J Biotechnol Genet Engineering, 2022.doi: 10.1007/s00299-021-02669-6