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Research Article

Genetic diversity analysis of selected mulberry accessions using microsatellite markers

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Abstract
The current study was carried out with a set of 44 mulberry accessions in order to evaluate the degree of distinctiveness and relatedness among the selected germplasm accessions. A total of 40 microsatellite markers were selected on the basis of earlier studies, out of which 26 primers yielded a total of 182 polymorphic bands. The PIC (polymorphism information content) values was above 0.5. Based on clusters obtained from unweighted pair group method with arithmetic mean, all the genotypes were grouped into three major cluster viz., A, B and C having sub-sub-clusters comprising of genetically different accessions. Cluster analysis displayed relatively high degree of genetic variation among the genotypes belonging to neighbouring clusters. Grouping of genotypes was further confirmed by principal component analysis which revealed similar diversification among the studied genotypes. Therefore, the current investigation can be utilized in creating unique genetic profile of mulberry genotypes.

Keywords: Mulberry, fingerprinting, microsatellite, dendrogram, diversity

INTRODUCTION
Mulberry represents one of the most diversified groups of plants belonging to genus Morus, family moraceae, division magnoliophyta, class magnoliopsida and order urticales (Arab et al., 2017; Rohela et al., 2020; Gull et al., 2021, Sathyanarayana and Sangannavar., 2021). Most of the cultivated varieties of mulberry are diploid (2n=28) and a few are polyploids (Venkatesh, 2014). Genetic identification or fingerprinting of mulberry germplasm resources is essential for the effective utilization of mulberry genotypes as parental material in breeding programmes in order to make best possible parental combinations for region and season specific genotypes or hybrids. Conventionally, morpho-physiological characterization of mulberry played significant role in phenotypic specification and differentiation (Banerjee et al., 2011; Chanotra et al., 2019; Rahman et al., 2020).

Many investigations were carried out in mulberry genome characterization aided with molecular markers. Simple Sequence Repeats (SSR) (Aggarwal and Udaykumar, 2004; Zhao et al.,2005) and Inter- Simple Sequence Repeats (ISSR) (Awasthi et al.,2004; Vijayan et al., 2006 a-b; Zhao et al.,2007 and Rohela et al., 2018) have been demonstrated as most preferred markers for diversity analysis in mulberry. They were widely used to generate molecular profile and genetic fidelity studies of mulberry genotypes to analyze the genetic relatedness...
among germplasm accessions. In view of the advantages concerned with molecular identification of mulberry over morphological identification, the present investigation was carried out with SSR markers for elucidating the genetic diversity among different mulberry cultivars so as to create genetic profile of the germplasm stock for conducting future breeding programmes.

MATERIALS AND METHODS
The current experiment was conducted at Division of Sericulture, Udheywala in collaboration with School of Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu - Jammu, Chatha-India (SKUAST- Jammu). Forty four mulberry genotypes available in mulberry germplasm bank Udheywala campus, SKUAST- Jammu were selected in order to know the extent of genetic relatedness or diversity among them (Table 1).

Young leaf samples were harvested randomly from selected genotypes (approximately 5-7g of fresh weight) for genomic DNA isolation using CTAB (Cetyltrimethyl ammonium bromide) method of Doyle and Doyle (1990) with slight modifications. Leaf lamina (500 mg) was finely grounded to powder form with the help of pre-chilled mortar and pestle by adding sufficient amount of liquid nitrogen. To the ground powder, 1 ml of extraction buffer (pre warmed) was added and incubated in water bath at 65ºC for 35 minutes with occasional stirring. To the mixture, 1:1 of phenol: chloroform was added in equal volume. The samples were subjected to centrifugation at 10,000 rotations per minute (rpm) for 15 minutes. The supernatant layer was transferred to afresh tube and mixed well with Phenol: chloroform: isoamyl alcohol in the ratio of 25:24:1(P:C:I) followed by centrifugation. To the supernatant, 24:1 of chloroform: isoamylalcohol was added and centrifuged. To this, 0.6 ml of Iso-propanol (chilled) was added to the tubes and stored at 4ºC for 1-2 hours. Centrifugation was carried under conditions of 4ºC at 10,000 rpm for 10 minutes. The supernatant was discarded and pellet was rinsed with 0.01 M ammonium acetate (200 μl-300 μl) to remove contamination. A volume of 0.6 ml of Iso-propanol was added for precipitation and the content was again subjected to centrifugation at 5,000 rpm for 10 minutes. The white pellet thus collected was again rinsed with 70 per cent ethanol and air dried at room temperature. Genomic DNA thus obtained was dissolved by adding TE buffer (200 μl) and stored at 4ºC. For further purification, 300 μl of RNase (10mg/ml) was added to the DNA sample and incubated for 1 hour in a water bath at 37ºC. The isolated DNA was treated with P:C:I (25:24:1), mixed by tilting and centrifugation was carried out at 10,000 rpm for 10 minutes. The supernatant was then collected in a fresh tube, and 0.6 ml of chilled Iso-propanol was added and centrifuged. The resultant DNA pellet thus obtained was once again rinsed with 70 per cent ethanol and kept for drying for approximately 30 minutes. Finally the dried DNA pellet was dissolved with the addition of 125 μl of TE buffer (Tris-cl, EDTA) and stored at -20ºC.

The concentration of DNA samples was estimated by agarose gel electrophoresis. Presence of fairly pure DNA was detected and thus subjected to amplification by polymerase chain reaction (PCR) with 40 microsatellite markers (Table 2). Clustering of the chosen genotypes was performed through UPGMA analysis based on scoring of data as 1 for presence of band and 0 as absent for generating binomial data matrix (Sneath and Sokal, 1973) and dendrogram based on Jaccard’s Similarity Coefficient were generated by using NTSYS program version 2.0 software. Analysis of various parameters including major allele frequency, gene diversity, heterozygosity, polymorphism percentage (PP%) and polymorphic information content (PIC) were derived with the help of R-software.

RESULTS AND DISCUSSION
The capability of SSR primers to discriminate genetic diversity among different genotypes of mulberry was assessed based on characters like major allele frequency, gene diversity, heterozygosity, polymorphism percentage (PP%) and polymorphic information content (PIC) as presented in Table 3. Molecular study yielded 182 highly polymorphic bands with 26 primers for forty four mulberry genotypes with selected primers (Fig.1). Results presented higher values for major allele frequency of 0.558 for primer M2 at band size of 190 bp, high heterozygosity of 0.870 for primer Mul3SSR105 at 240 bp. The average value for major allele frequency was recorded as 0.351 and primer M2 presented the highest value as 0.558 at 190-210 bp. The highest value for genetic diversity was recorded as 0.909 for primer M6 at 310 bp and highest range of heterozygosity was recorded as 0.870 for primer Mul3SSR105 at 240 bp. The results depicted presence of high polymorphism percentage and PIC value above 0.5, revealing the great amount of genetic variation among forty four different genotypes of mulberry. Hence, the genotypes could be believed to have different genetic makeup with considerable variation in their expression. Similar studies have been reported earlier by Ramesh et al. (2004) and Wani et al. (2010) utilized similar microsatellite markers for characterization of mulberry genotypes and reported presence of genetic divergence among different mulberry accessions. Kala et al. (2016) utilized nineteen mulberry genotypes from the same germplasm bank and conducted assessment of divergence on the basis of morphological and molecular analysis. The results of the study showed close similarity with the present investigation as they too mentioned the mulberry accessions to exhibit wide range of divergence. In addition to this, Wang et al. (2017) also recorded presence of genetic diversity in Morus spp. with the help of SSR and ISSR markers.

The dendrogram analysis for 44 mulberry genotypes resulted into three major clusters (A, B and C) validating presence of genetic divergence between them (Fig. 2). Cluster-A comprised of twenty one (21) genotypes namely; BC-259, Tr-10, C-763, Asayuki,
| S.No. | Name of genotype | Donor Name      | Nature of breeding | Genetic Nature |
|-------|------------------|-----------------|--------------------|----------------|
| 1     | Asayuki          | CSR & TI, Mysore| Cross Pollinated Hybrid | Exotic        |
| 2     | Enshutukasuka    | CSR & TI, Mysore| Collection         | Exotic        |
| 3     | Fukushima        | CSR & TI, Berhampore | Collection     | Exotic        |
| 4     | Goshyerami       | CSR & TI, Mysore| Selection          | Exotic        |
| 5     | Ichinose         | RSRS, Kodathi   | Cross Pollinated Selection | Exotic        |
| 6     | Kairyoroso       | CSR & TI, Mysore| Cross Pollinated Hybrid | Exotic        |
| 7     | Kamabori         | CSR & TI, Mysore| Cross Pollinated Hybrid | Exotic        |
| 8     | Kokuso-20        | CSR & TI, Mysore| Mutation           | Exotic        |
| 9     | Kokuso-27        | CSR & TI, Mysore| Cross Pollinated Hybrid | Exotic        |
| 10    | Limencina        | CSR & TI, Mysore| Collection         | Exotic        |
| 11    | Miuraso          | CSR & TI, Mysore| Collection         | Exotic        |
| 12    | Rokokyoso        | RSRS, Kodathi   | Clonal Selection   | Exotic        |
| 13    | Shimamouchi      | CSR & TI, Mysore| Cross Pollinated Hybrid | Exotic        |
| 14    | BC-259           | CSR & TI, Berhampore | Back Cross Selection | Indigenous   |
| 15    | Bhrem C-776      | CSR & TI, Pamapore | Cross Pollinated Selection | Indigenous   |
| 16    | Behrampur        | CSR & TI, Berhampore | Clonal Selection   | Indigenous   |
| 17    | C-763            | CSR & TI, Mysore| Cross Pollinated Hybrid | Indigenous   |
| 18    | Chakmajra        | DOS, J&K Govt.  | Natural Selection  | Indigenous   |
| 19    | Chinese white    | CSR & TI, Mysore| Collection         | Indigenous   |
| 20    | Dhar local       | DOS, J&K Govt.  | Open Pollinated Hybrid | Indigenous   |
| 21    | Kanva-2          | CSR & TI, Mysore| Cross Pollinated Hybrid | Indigenous   |
| 22    | KNG              | CSR & TI, Mysore| Clonal Selection   | Indigenous   |
| 23    | LF-1             | CSR & TI, Mysore| Clonal Selection   | Indigenous   |
| 24    | LF-2             | CSR & TI, Mysore| Clonal Selection   | Indigenous   |
| 25    | NS-1             | SKUAST-Jammu.   | Open Pollinated    | Indigenous   |
| 26    | NS-2             | SKUAST-Jammu.   | Open Pollinated    | Indigenous   |
| 27    | NS-3             | SKUAST-Jammu.   | Open Pollinated    | Indigenous   |
| 28    | S-1              | CSR & TI, Mysore| Clonal Selection   | Indigenous   |
| 29    | S-30             | CSR & TI, Mysore| Mutation           | Indigenous   |
| 30    | S-36             | CSR & TI, Mysore| Mutation           | Indigenous   |
| 31    | S-41             | CSR & TI, Mysore| Mutation           | Indigenous   |
| 32    | S-54             | CSR & TI, Mysore| Mutation           | Indigenous   |
| 33    | S-146            | RSRS, Kodathi   | Open Pollinated Selection | Indigenous   |
| 34    | S-799            | CSR & TI, Mysore| Open Pollinated Hybrid | Indigenous   |
| 35    | S-1531           | CSR & TI, Mysore| Open Pollinated Selection | Indigenous   |
| 36    | S-1608           | CSR & TI, Berhampore | Open Pollinated Hybrid | Indigenous   |
| 37    | S-1635           | CSR & TI, Berhampore | OPH Selection    | Indigenous   |
| 38    | S-1708           | CSR & TI, Berhampore | Open Pollinated Selection | Indigenous   |
| 39    | Sujanpur         | DOS, J&K Govt.  | Open Pollinated Collection | Indigenous   |
| 40    | Tr-1             | CSR & TI, Berhampore | Colchiploid      | Indigenous   |
| 41    | Tr-4             | RSRS, Kodathi   | Polyploid         | Indigenous   |
| 42    | Tr-8             | RSRS, Kodathi   | Polyploid         | Indigenous   |
| 43    | Tr-10            | RSRS, Kodathi   | Polyploid         | Indigenous   |
| 44    | V-1              | CSR & TI, Mysore| Cross Pollinated Hybrid | Indigenous   |
| S.No. | Primer | Sequence | Amplicon size (bp) |
|------|--------|----------|-------------------|
| 1    | Mul3SSR4 F | GGAGCAGTCAATCCTCTTG | 314 |
|      | Mul3SSR4 R | CTGGGGTTCAACTAAGCTC | |
| 2    | Mul3SSR9 F | GACCCAGCATGAGCCTAC | 365 |
|      | Mul3SSR9 R | GTTCAAACACAAATCTCC | |
| 3    | Mul3SSR16 F | CTAGTAGCAGATCACCAC | 207 |
|      | Mul3SSR16 R | GTTCCTCTCCTAATCC | |
| 4    | Mul3SSR17 F | GTCTTGCACTAGGAGAGG | 345 |
|      | Mul3SSR17 R | CTCACAGGAAGACACACC | |
| 5    | Mul3SSR19 F | CCAAGTCTCTCCTCAG | 170 |
|      | Mul3SSR19 R | GTTTTGTGACTTGCCG | |
| 6    | Mul3SSR20 F | CTAGCAGATCCTGAGGAT | 252 |
|      | Mul3SSR20 R | CTCCGCCCAAATATCACAC | |
| 7    | Mul3SSR50 F | CTAGCAGATCCACCAAGAC | 161 |
|      | Mul3SSR50 R | GTTTGTTGACTCTGACAG | |
| 8    | Mul3SSR53 F | CGCCTATGACGATTACGAC | 124 |
|      | Mul3SSR53 R | GGACCTTGATGGCCATTG | |
| 9    | Mul3SSR65 F | CTGGAAGTAACAGAAGCAGC | 210 |
|      | Mul3SSR65 R | GCCCTCCACATTGAACTAGA | |
| 10   | Mul3SSR70 F | GAGAGGGGAAGGAGAGAGA | 170 |
|      | Mul3SSR70 R | CAAAGAGATCCAAATAGAAGC | |
| 11   | Mul3SSR71 F | GGATACTACCTGTTGGTTGACTG | 360 |
|      | Mul3SSR71 R | ATCCCTCTCCTAAGC | |
| 12   | Mul3SSR74 F | CCCATTGAGGTTTTTCTGAG | 400 |
|      | Mul3SSR74 R | ATGTAAGCTCGGATTTGAC | |
| 13   | Mul3SSR80 F | GAGCCGTGGTTCATTTCCGT | 140 |
|      | Mul3SSR80 R | CAACGGCTGGGAAAGAC | |
| 14   | Mul3SSR91 F | CAGTGAACGTTGAGCACAG | 227 |
|      | Mul3SSR91 R | ATCCCAAGATCCAAATACC | |
| 15   | Mul3SSR93 F | CAGCCAATGCACCTTTTACG | 340 |
|      | Mul3SSR93 R | GTGGAAGCTCTTGTGAGC | |
| 16   | Mul3SSR94 F | CTCCTCATTGGTCTTCACTC | 195 |
|      | Mul3SSR94 R | CAAGATACAGCCCGAGGGAAG | |
| 17   | Mul3SSR95 F | GATCACGTCGCAAATAAGC | 209 |
|      | Mul3SSR95 R | TAAGAGCTGAGGGGGAAGG | |
| 18   | Mul3SSR97 F | TTCACACTGAAACAAATC | 292 |
|      | Mul3SSR97 R | ATTCGAGTTGTGACAGC | |
| 19   | Mul3SSR102 F | TTGGTTGCTGAGAAATGCA | 225 |
|      | Mul3SSR102 R | TTGTCAGTGGAAAAAAGC | |
| 20   | Mul3SSR103 F | GTCGACATGCTTTGCTGC | 235 |
|      | Mul3SSR103 R | GTAAGACGTGAGGGGAAG | |
| 21   | Mul3SSR105 F | GCAGAATCCACATTTATGCC | 240 |
|      | Mul3SSR105 R | CCTCATAGATCGACAGAC | |
| 22   | Mul3SSR114 F | GCAACCTCTGCTTGTGTTT | 102 |
|      | Mul3SSR114 R | TGGTCGCCCTAGACAGAC | |
| 23   | Mul3SSR116 F | CCAAGAAAGTGAAATCC | 277 |
|      | Mul3SSR116 R | CTGAAGCCCGTGGATACAG | |
| 24   | Mul3SSR122 F | GGTGATGGCCTTTGATG | 219 |
|      | Mul3SSR122 R | GGTGATCTGAGGGGAAG | |
| 25   | Mul3SSR131 F | ACTGTGCTCTGAGGATG | 300 |
|      | Mul3SSR131 R | GAGAGCTCTGAGGGGAAG | |
| 26   | Mul3SSR187 F | GGACATTTCCACACCCCTG | 324 |
|      | Mul3SSR187 R | AACTGCAAGTGGCAGAAC | |
| No. | Primer | Forward | Reverse | Length |
|-----|--------|---------|---------|--------|
| 27  | Mul3SSR197F | GGTGAAAAGTTGCTGTGAGTCC | CAGCAACTAGAGGACTTTT | 180 |
| 28  | Mul3SSR203F | GACCGTAGGAGAGAGTGCC | GGTATCCGCTAAACACAC | 440 |
| 29  | Mul3SSR229F | CTTATAGCGATTTGCAGGCC | GAAATTCGGACTCCTGTGTKC | 240 |
| 30  | Mul3SSR230F | CCGGTAGCTGTTTGGTTTC | CAGCCCAAAATCCCATCCTACT | 380 |
| 31  | SS05    | F: TCCAGCAAAATGAGGACAAAGTT | R: TTGCCTTCCCGATTATGCTG | 350 |
| 32  | SS02    | F: GCTTCTGATCAATCTAGCTTCCC | R: GCAAAACTACGCACCCCG | 355 |
| 33  | SS04    | F: CGAGGGAGGGATGAAGGAGGC | R: CACATTCATCCACCTCTTATA | 190 |
| 34  | SS17    | F: TACAGGGCTCGGGCAATG | R: TGGATCCTAAGCAGTTGGTCT | 220 |
| 35  | SS06    | F: ACTCAAATGAGAAGGAAAGGATATAC | R: TTTACTAAATCCCAAGCCACA | 180 |
| 36  | SS19    | F: TTCTGTGCTGTCTCCGTTACA | R: TGAGAACATACAACTAGGATGGAAAC | 300 |
| 37  | SS09    | F: AGAACCTTCTCCCGCTTATG | R: CCTTGGCGTAGGCAAGAT TG | 200 |
| 38  | SS18    | F: TTCTGCCCCCTGTGGTC | R: AGCAATTCTCTTTCAACTCACCTTCT | 180 |
| 39  | M2      | F: CGTGGCTGTCGTCGGTACAG | R: CACACCTACTCTCTCTCTCTCAG | 190 |
| 40  | M6      | F: TCCCTAGGTTTGGCGGTCGTTTACAT | R: CCTTATTCTCCCTCTACTATTGCTTG | 310 |

Fig. 1. PCR amplification obtained with primer Mul3SSR9: M; molecular ladder, 1-44; mulberry genotypes

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Table 3. Major allelic frequency, gene diversity, heterozygosity, polymorphism percentage and PIC content for the studied primers

| S.No. | Primer       | Band size (bp) | Major allele frequency | Gene diversity | Heterozygosity | PP  | PIC   |
|-------|--------------|----------------|------------------------|----------------|----------------|-----|-------|
| 1.    | Mul3SSR9     | 365            | 0.289                  | 0.850          | 0.137          | 50.00 | 0.836 |
| 2.    | Mul3SSR19    | 170            | 0.173                  | 0.850          | 0.057          | 33.30 | 0.832 |
| 3.    | Mul3SSR65    | 210            | 0.428                  | 0.752          | 0.125          | 60.00 | 0.726 |
| 4.    | Mul3SSR70    | 170            | 0.277                  | 0.827          | 0.034          | 100.00 | 0.805 |
| 5.    | Mul3SSR74    | 400            | 0.266                  | 0.773          | 0.036          | 100.00 | 0.737 |
| 6.    | Mul3SSR80    | 140            | 0.314                  | 0.813          | 0.159          | 14.20 | 0.790 |
| 7.    | Mul3SSR91    | 227            | 0.454                  | 0.578          | 0.045          | 33.30 | 0.486 |
| 8.    | Mul3SSR93    | 340            | 0.423                  | 0.742          | 0.108          | 20.00 | 0.710 |
| 9.    | Mul3SSR94    | 195            | 0.500                  | 0.648          | 0.066          | 33.30 | 0.592 |
| 10.   | Mlrl3SSR97   | 292            | 0.256                  | 0.820          | 0.850          | 14.20 | 0.796 |
| 11.   | Mul3SSR102   | 225            | 0.400                  | 0.700          | 0.026          | 33.30 | 0.645 |
| 12.   | Mul3SSR103   | 235            | 0.318                  | 0.801          | 0.580          | 40.00 | 0.775 |
| 13.   | Mul3SSR105   | 240            | 0.268                  | 0.855          | 0.870          | 25.00 | 0.840 |
| 14.   | Mul3SSR114   | 102            | 0.285                  | 0.836          | 0.022          | 100.00 | 0.818 |
| 15.   | Mul3SSR122   | 219            | 0.342                  | 0.788          | 0.186          | 16.60 | 0.759 |
| 16.   | Mul3SSR131   | 300            | 0.500                  | 0.580          | 0.770          | 33.30 | 0.493 |
| 17.   | Mul3SSR197   | 180            | 0.548                  | 0.626          | 0.237          | 25.00 | 0.580 |
| 18.   | Mul3SSR203   | 440            | 0.428                  | 0.704          | 0.037          | 66.60 | 0.657 |
| 19.   | Mul3SSR229   | 240            | 0.333                  | 0.753          | 0.062          | 25.00 | 0.711 |
| 20.   | Mul3SSR230   | 380            | 0.291                  | 0.815          | 0.079          | 60.00 | 0.792 |
| 21.   | SS05         | 350            | 0.294                  | 0.807          | 0.023          | 16.60 | 0.781 |
| 22.   | SS02         | 355            | 0.447                  | 0.720          | 0.023          | 40.00 | 0.684 |
| 23.   | SS19         | 300            | 0.333                  | 0.780          | 0.072          | 20.00 | 0.748 |
| 24.   | SS18         | 180            | 0.275                  | 0.78           | 0.228          | 20.00 | 0.744 |
| 25.   | M2           | 190            | 0.558                  | 0.634          | 0.264          | 20.00 | 0.600 |
| 26.   | M6           | 310            | 0.136                  | 0.909          | 0.107          | 36.30 | 0.901 |

Mean       -        -       0.759          0.200          39.84          0.724
Fig. 2. Cluster dendrogram of 44 mulberry genotypes based on Jaccard's Coefficient

Fig. 3. Centroids of 44 mulberry genotypes according to the 2-D PCA based on similarity matrix
under PCA. Such results had earlier been advocated by Wang et al. (2017), where they described combining of genotypes of Group-III and Group-IV to form Cluster-V. Similar reports on PCA was also demonstrated by Boeing et al. (2014) and Das and Mandal, (2018) for describing the divergence among mulberry collections.

Estimation of genetic variability among germplasm collections is useful tool which facilitates efficient management and utilization of available resources. As such, it is prudent to have the genetic characterization of mulberry by using microsatellite markers to elucidate the genetic diversity and to eliminate duplicate accessions through DNA fingerprinting so as to create genetic profile of the available germplasm accessions. Molecular analysis of 44 mulberry accessions available at mulberry germplasm bank of SKUAST-Jammu comprising of mulberry genotypes commonly cultivated in temperate and tropical areas of the country with the help of SSR marker yielded 182 highly polymorphic bands with 26 primers. Results showed higher values for major allele frequency of 0.558 for primer M2 at band size of 190 bp and high heterozygosity of 0.870 for primer Mul3SSR105 at 240 bp. Whereas, value for highest genetic diversity was recorded as 0.909 for primer M6 at 310 bp. High polymorphism percentage and PIC value above 0.5, revealing the great amount of genetic variation among forty four different genotypes of mulberry was recorded in case of molecular analysis. Further the genotypes were subjected to clustering based on Jaccard’s similarity coefficient which categorised 44 genotypes into three main cluster with genetically different accessions. The current investigation thus provides a platform for selection of diverse parental stock for mulberry breeding programmes aimed at improvement of the mulberry genetic stock.

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