Morphological and SSR Marker Based Diversity Analysis of Lentil (Lens esculenta) Genotypes using Yield and Yield Contributing Characters

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ABSTRACT
Lentil is an important pulse crop with high nutritional value and high market price worldwide. Molecular markers have emerged as useful tools to assess the genetic diversity across crops. The study was conducted to explore genetic diversity of twenty lentil genotypes considering yield and yield attributing traits. Among all genotypes, BARI Masur-6, BARI Masur-7 followed by genotypes BD-3806 and BD-4090 showed the highest value of yield attributing traits therefore, these genotypes are considered as best performer. The results of cluster analysis based on the Ward’s method grouped the genotypes into three clusters and the genotypes of cluster III revealed the maximum value for yield per plants which indicated their importance in the selection for yield improvement program of lentil. Afterwards, 20 genotypes were evaluated through 7 sets of SSR primers to assess genetic diversity among the genotypes. Among them, four sets of primers viz., SSR 19, SSR 33, SSR 90 and SSR 213 showed high polymorphism which suggesting the greater genetic diversity in the genotypes. The unweighted pair group method of arithmetic mean (UPGMA) dendrogram based on Nei’s (1973) genetic distance led the genotypes into four major clusters which showed a bit deviation with the morphological cluster. The findings of this study will be very useful for selection of appropriate parents and the genetic understanding for the set up for future systematic lentil breeding programs.

Key words: Genetic diversity, Germplasm, Lentil, Molecular markers, Polymorphism information content.

INTRODUCTION
Lentil (Lens culinaris Medik.) is diploid (2n=2x=14) and self-pollinated cool season crop belongs to the family Fabaceae and one of the early domesticated among pulse crops (Arumuganathan and Earle, 1991; Hamweih et al., 2009). Lentil is cultivated in 4.6 million hectares of land worldwide with the production of 4.95 million ton (FAO, 2013). In Bangladesh, lentil is one of the most vital pulse crop and grain legume. It is also known as ‘masur dal’. In our country, lentil is the main source of plant protein. In the year 2015-16, lentil was grown in 1,54,449.489 ha of land and the production was 158228 metric tons in Bangladesh (BBS, 2016). But in our country, the production of lentil is not enough to meet up the demand of proper nutrition of increasing population. The reasons behind this are cultivation of lentil in traditional method, due to narrow genetic base, susceptibility to biotic and abiotic stresses, less response to fertilizer and irrigation, inaccessibility of early maturing varieties and genetic erosion.

In modern day plant breeding, choice of appropriate parent is a vital source in detecting variation of genes for hybridization activities and assist as a base for any crop improvement operation (Kushwaha et al., 2015). In case of lentil breeding, this process have been found as a proper tool in developing desirable high yielding varieties. Genetic improvement comes from genetic diversity within germplasm. Germpamlasm delivers facility for broad variability. Thus, it is necessary to assess the genetic diversity of lentil germplasm for the improvement. In case of self-pollinated crop, in hybridization operation genetic diversity is a vital factor for genetic improvement (Joshi and Dhawan, 1996). It can be assessed through advanced biometrical process such as multivariate analysis (Rao, 1952) based on Mahalanobis (1936) D² statistics and Ward’s non-hierarchical squared Euclidean distance method have become possible to quantify magnitude of diversity among lentil germplasm placing the genotypes in different clusters for their assessment in respect of breeding program which will produce heterotic combinations and wide variability in next generation.

Genetic variation is detected by Molecular markers within
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germplasm and among closely associated species (Nienhuis et al., 1995). Simple Sequence Repeats (SSRs) is an essential implement for diagnose of genetic variation like polymorphism, genetic diversity, molecular mapping, gene mapping, fingerprint construction and genetic purity test (Kumar et al., 2014). Using SSR marker in lentil genotypes, this method can be used as quick and dynamic method for genetic variation, detecting polymorphism at the DNA level and genetic diversity analysis. As superior genotypes are used as parent materials in a hybridization programs, a knowledge of the genetic diversity will be use for further improvement of productivity of lentil genotypes (Kumar et al., 2014). So it is necessary to identify the genetic diversity and characters association with yield and yield attributing traits which is of great importance to the breeders for the development of genotypes with desired qualitative and quantitative characters and wider genetic base which would help the plant breeder in picking the exact parents in breeding operation for desired high yielding varieties. Therefore, the research work was conducted to evaluate the diversity of lentil genotypes at molecular level through SSR markers.

MATERIALS AND METHODS

Plant materials
Twenty lentil genotypes were used in the experiment were collected from Plant Genetic Resources Centre (PGRC), BARI, Gazipur, Bangladesh. The identity of 20 lentil genotypes are presented in Table 1.

Experimental details and estimation of quantitative traits
The experiment was carried out during Rabi season. The land was prepared properly by 5-6 ploughing and cross ploughing. The clods were broken into small pieces and leveled by ladder. All the stubbles and weeds were removed from the plot properly. Urea, triple super phosphate and muriate of potash were used as source of nitrogen, phosphorus, potassium at the rate of 32, 77, 32 kg ha⁻¹ respectively at the time of final land preparation. The experiment was conducted following randomized complete block design (RCBD) with three replications. The seeds of the 20 lentil genotypes were sown in the field on 8th November 2016. The length of line was 1.2 m and line to line distance was 30cm.

Generally different genotypes matured at different times. Therefore, harvesting was done at least 90% of the pods when turned brown and plants turned in yellow or straw color. Five plants were selected from each replication and uprooted to collect data of different traits viz., days to first flowering, days to fifty percent flowering, days to maturity, plant height (cm), total number of primary branches, total number of pods peduncle¹, total number of pods plant¹, the number of seeds pod¹, the number of seeds plant¹, 100-seed weight (g) yield plant¹ (g).

Genomic DNA extraction of plant materials
Genomic DNA has been extracted from young 21 days old fresh leaves of the 20 genotypes using the Cetyl Trimethyl Ammonium Bromide (CTAB) mini-prep method. Total, seven primers namely SSR 19 (forward 5'-GACTCATATTGGTTCTTAGCAG-3’ and reverse 5'-GAAGGACGGGTCCATAG-3’), SSR 33 (forward 5'-CAAGCATAGGCCCATGAA G-3’ and reverse 5'-CTTTACCTACTACCTACTCCT-3’), SSR 48 (forward 5'-CATTGAGGATAGTGATGGC-3’ and reverse 5'-CTCAGATACCTACCTACTC-3’), SSR 90 (forward 5'-CCGTGACACCTCTAC-3’ and reverse 5'-CGTCTTAAAGAGATGACAC-3’), SSR 156 (forward 5'-GTACATTGAAACGATCTAC-3’ and reverse 5’-CAAATGGGGCA TGAAGGAG-3’), SSR 207 (forward 5’-GAGAGACGTCAGAGTAG-3’ and reverse 5’-GATTGTGCTTCGTG GTTC-3’) AND SSR 213 (forward 5’-CAGCCACCTCTTATG-3’ and reverse 5’-GAATTGTCTTCTTACGAC-3’) were used to estimate genetic diversity among lentil genotypes.

PCR reactions were performed on each DNA sample in a 10 μl reaction mixture consists of Taq polymerase buffer (1 μl), primer forward (0.5 μl), primer reverse (0.5 μl), maxim Taq DNA polymerase (4 μl), dNTPs (0.2 μl), MgCl₂ (1 μl), genomic DNA (1 μl) and suitable amount of sterile deionized water. DNA amplification was performed in an oil free thermal cycler and PCR amplification process consist denaturation at 94°C for 3 min, 32 cycles of denaturation at 94°C for 45 seconds, annealing (60°C and 62°C) for 45 seconds and extension at 72°C for 6 min at last. The amplified products were scored as bands on visualization on gel on UV illuminator. Only the reliable bands were included in analysis. The pattern of bands obtained after amplification with the primers was scored using Alpha Viewer (Version 3.2.8) to identify the molecular weight of DNA band comparing with known size DNA ladder. The size (in nucleotide base pairs) of the amplified band for each microsatellite marker was determined based on its migration relative to a molecular weight size.

Data analysis
Data was analyzed by two-way analysis of variance (ANOVA) using R studio software. The data were presented as means with different alphabetical letters in the same column indicates significant differences among the treatments and cultivars at P <0.05 according to a least significant difference (LSD) test. The cluster analysis was determined by Ward’s Method based on Euclidean distance and hierarchical cluster analysis using R software. The summary statistics including the number of alleles per locus, major allele frequency, gene diversity and Polymorphism Information Content (PIC) and NeI’s genetic identity and genetic distance values were determined using Power Marker version 3.23 (Liu and Muse, 2005), a genetic analysis software.

RESULTS AND DISCUSSION

Analysis of variance
Knowledge of genetic diversity in germplasm is essential for active germplasm collection, conservation, utilization and strategies in and crop improvement programs (Alghamdi et al., 2014). In the current investigation, analysis

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Table 1: List of 20 lentil genotypes used in this experiment with their characters.

| Genotypes       | Characters                                |
|-----------------|-------------------------------------------|
| BARI Masur-6    | Bushy plant, medium leaflet size and violet color flower |
| BARI Masur-7    | Bushy plant, large leaflet size and violet color flower |
| BD-3804         | Semi-erect plant, medium leaflet size and white color flower |
| BD-3806         | Erect plant, medium leaflet size and violet color flower |
| BD-3808         | Semi-erect plant, medium leaflet size and white color flower |
| BD-3810         | Semi-erect plant, medium leaflet size and white color flower |
| BD-3945         | Semi-erect plant, medium leaflet size and white color flower |
| BD-3948         | Bushy plant, medium leaflet size and white color flower |
| BD-3975         | Bushy plant, medium leaflet size and white color flower |
| BD-3985         | Semi-erect plant, large leaflet size and white color flower |
| BD-3986         | Bushy plant, large leaflet size and white color flower |
| BD-3995         | Bushy plant, large leaflet size and white color flower |
| BD-4028         | Semi-erect plant, large leaflet size and violet color flower |
| BD-4088         | Bushy plant, medium leaflet size and violet color flower |
| BD-4090         | Semi-erect, medium leaflet size and white color flower |
| BD-4095         | Bushy plant, large leaflet size and white color flower |
| BD-4134         | Bushy plant, medium leaflet size and white color flower |
| BD-5958         | Semi-erect, small leaflet size and white color flower |
| BD-5959         | Semi-erect, medium leaflet size and white color flower |
| BD-5983         | Semi-erect, medium leaflet size and white color flower |

Table 2: Analysis of variance for different traits of 20 lentil genotypes.

| Traits                        | Sources of variation |
|-------------------------------|----------------------|
|                               | Replication (df=2)   | Genotype (df=19) | Error (df=38) |
| Days to first flowering       | 0.050                | 29.104***        | 0.436         |
| Days to fifty percent flowering| 0.866                | 27.139***        | 0.621         |
| Days to maturity              | 0.150                | 30.943***        | 0.500         |
| Plant height (cm)             | 0.521                | 23.360***        | 1.121         |
| Primary branches plant\(^{-1}\) (no.) | 0.580              | 2.7816***        | 0.425         |
| Pods peduncle\(^{-1}\) (no.) | 0.043                | 0.336***         | 0.037         |
| Pods plant\(^{-1}\) (no.)    | 4.00                 | 1853.06***       | 10.31         |
| Seeds plant\(^{-1}\) (no.)   | 2.6                  | 6652.5***        | 34.4          |
| Seeds pod\(^{-1}\) (no.)     | 0.001                | 0.040***         | 0.001         |
| 100-seed weight (g)           | 0.003                | 0.064***         | 0.002         |
| Seed yield plant\(^{-1}\) (g) | 0.001                | 2.334***         | 0.015         |

*** indicates significant at 0.001 statistical level.

of variance showed statistically significant difference among the genotypes for all traits under study viz., days to first flowering, days to 50% flowering, days to maturity, plant height, number of primary branches plant\(^{-1}\), number of pods peduncle\(^{-1}\), number of pods plant\(^{-1}\), number of seeds plant\(^{-1}\), number of seeds pod\(^{-1}\), 100-seed weight and seed yield plant\(^{-1}\) (Table 2) indicating the existence of genetic diversity in the genotypes. Hence, selection could better be employed considering these traits in practical lentil breeding program and the broadening of genetic base (Gupta and Sharma, 2006). This results were in consistent with Gautam et al. (2013) and Roy et al. (2013).

Trait wise mean performance of the genotypes

The mean performances of the lentil genotypes for different yield and yield attributing traits are presented in Table 3. The genotypes displayed considerable amount of difference in their mean value and this indicating the presence of variability among the genotypes for the characters studied (Table 3). Considering the traits days to first flowering, days to fifty percent flowering and days to maturity, the genotype BD-5983 was earliest followed by the genotype BD-3975, BD-3808 and BD-3810 reflected that this material could be used to develop early maturing variety, which is the vital need for lentil improvement program in Bangladesh context (Roy et al., 2013). Maximum plant height (39.94 cm) was observed in genotype BD-3995 which was followed by BD-4095 (39.89 cm) while minimum (28.27 cm) was found in genotype BARI Masur-6. The maximum number of primary branches plant\(^{-1}\), number of pods plant\(^{-1}\), Number of pods peduncle\(^{-1}\) and number of seeds plant\(^{-1}\) was observed in the
The largest cluster III consists of the maximum number of genotypes were grouped into three separate clusters (Fig 1). Euclidean distance following Ward’s method, 20 lentil genotypes were categorized into three clusters (Table 4) which indicates that this genotype contained maximum value mean value for different traits such as number of pods peduncle, number of pods plant, 100-seed weight, and seed yield plant (Table 4) which indicates that this genotype could get the major priority for the yield improvement. Earlier Gautam et al. (2014) observed moderate to high yield donating traits in cluster III and II in the lentil genotypes. The members of cluster I were BD-3810, BD-3945, BD-3948, BD-3985 and BD-4134, the members of cluster II were BD-3804, BD-3808, BD-4095, BD-5958 and BD-5959. The short duration genotypes were grouped into cluster II whereas cluster I included long duration genotypes indicating maximum contribution of this character towards the divergence between cluster II and I. Gautam et al. (2014) also reported early maturing genotypes in cluster II and late maturing genotypes in cluster III. The genotypes which are grouped into the same cluster probably disperse very little from one to another (Roy et al., 2013). Many researchers exploited that cluster analysis could be a powerful tool to screen a large number of germplasms on the basis of similarity (Chunthaburee et al. 2016; Siddiqui et al., 2017).

Diversity analysis through SSR primers

Genetic similarity analysis using UPGMA

UPGMA dendrogram revealed the 20 genotypes were categorized into four major clusters considering their

Table 3: Mean performance of twenty genotypes based on different quantitative traits related to yield.

| Genotypes     | DF  | DFF | DM  | PH  | PBP | PP  | SP  | SPD | TSW | SY  |
|---------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| BARI Masur-6  | 61 h| 73.33| 112.3| 28.27| 6.78b-e|1.89bc|174 c|271 cd|1.56 fg|1.89 a| 4.96 b|
| BARI Masur-7  | 63.33| 76.67| 113 c-a| 31.5| 7.67 ab|2.0 b | 188 a|303.7| 1.61 df|1.76 bc| 5.25 a|
| BD-3804       | 60.33| 30.33| 108.3| 33.55| 6.7 b-e|2.33 a|145.9|241 hi|1.65 d|1.64 de|4.16 d|
| BD-3806       | 62 gh| 72.67| 110.3| 37.2 b-d| 7.89 a|2.0 b | 180.9|278 cd|1.54 f |1.68 cd|4.84 b|
| BD-3808       | 60.67| 71.67| 107.3| 35 e-h| 7.33 a-c|1.67 cd|146.2|236.4|1.62 de|1.58 ef|3.56 hi|
| BD-3810       | 61.67| 72.33|110.8| 32.14| 6.8 a-d|2.33 a|129.8|186.6|1.44 f |1.54 f |3.34 jk|
| BD-3945       | 63.67| 76 bc| 113-b-d| 36.4 c-e| 5 f   |1.56 d|107.9|168.1|1.56 g |1.42 hi|2.55 m|
| BD-3948       | 65.67| 78.67| 114 ab| 38.94| 6.44 c-e|1.5 d | 129.4|178.8|1.38 j |1.59 ef|2.84 l|
| BD-3975       | 58 k | 69.67| 104.3| 35.2 e-h| 7.67 ab|1.5 d | 158 ef|249 gh|1.5 e-g|1.41 hi|3.23 k|
| BD-3985       | 61.67| 72 gh| 109.3| 37.78| 5.11 f |2.17 ab|111.7|159.9|1.43 hi|1.39 hi|2.11 n|
| BD-3986       | 64.67| 74.67| 112 c-e| 35.61| 7.78 ab|2.33 a|166 d|291.1|1.75 ab|1.38 i|3.74 f-h|
| BD-3995       | 63.33| 74.33| 112 c-e| 39.94| 7.67 ab|1.56 d|153.4|261 ef|1.70 bc|1.63 de|3.95 e|
| BD-4028       | 69.33| 79.33| 114.7| 35 e-h| 5.78 ef|1.56 d|177 bc|262 ef|1.48 h |1.63 de|4.25 d|
| BD-4088       | 68 b | 78.67| 114 ab| 35 e-h| 7.11 a-c|2.17 ab|157 ef|261 c|1.78 a |1.45 hi|3.66 g-i|
| BD-4090       | 62 e-g| 73 fg| 109.7| 32.69| 0.97 f |2.33 a|166.9|275 cd|1.65 cd|1.79 b|4.61 c|
| BD-4095       | 63.33| 74.33| 111.7| 39.89| 5.89 d-f|1.5 d|172.8|269 de|1.56 g |1.54 fg|3.93 ef|
| BD-4134       | 65.33| 75.33| 113-b-d| 34.7 e-h| 5.10 f |1.61 cd|105.3|143.9|1.37 j |1.45 hi|1.96 n|
| BD-5958       | 68.33| 78.33| 113 a-c| 34.66 f-h| 7.55 ab|2.17 ab|162 de|256 fg|1.5 e-g|1.45 hi|3.62 g-i|
| BD-5959       | 62.33| 72.67| 108.3| 33.83| 6.7 b-e|1.61 cd|154.4|249 gh|1.62 de|1.46 gh|3.51 ij|
| BD-5983       | 57.67| 69.67| 104.3| 35.3 e-g| 6.33 c-e|1.56 d|177 bc|258 fg|1.44 h |1.41 hi|3.7 e-g|

Notes: DF denotes days to first flowering; DFF denotes days to fifty percent flowering; DM denotes days to maturity; PH denotes plant height; PBP denotes primary branches plant; PP denotes pods peduncle; PP denotes pods plant; SP denotes seeds plant; SPD denotes seeds pod; TSW denotes 100-seed weight and SY denotes seed yield plant.
similarity (Fig 2) which somewhat failed to match the earlier dendrogram (Fig 1) based on Euclidean distance using R software, summarizing the data on differentiation among 20 genotypes of lentil according to Ward’s method.

**Table 4:** Cluster mean for yield and yield related characters of 20 genotypes.

| Characters                          | Cluster Mean                  |
|------------------------------------|-------------------------------|
|                                    | I                             | II                           | III                          |
| Days to first flowering            | 63.60 (H)                     | 62.17 (L)                    | 63.56 (I)                    |
| Days to fifty percent flowering    | 74.87 (H)                     | 72.83 (L)                    | 74.70 (I)                    |
| Days to maturity                   | 111.58 (H)                    | 109.1 (L)                    | 111.38 (I)                   |
| Plant height (cm)                  | 36.01 (H)                     | 35.37 (I)                    | 34.92 (L)                    |
| Primary branches plant\(^1\) (no.)| 5.69 (L)                      | 7.30 (H)                     | 6.99 (I)                     |
| Pods peduncle\(^1\) (no.)          | 1.83 (I)                      | 1.81 (L)                     | 1.93 (H)                     |
| Pods plant\(^1\) (no.)             | 116.82 (L)                    | 153.43 (I)                   | 173.46 (H)                   |
| Seeds plant\(^1\) (no.)            | 167.46 (L)                    | 249.05 (I)                   | 276.87 (H)                   |
| Seeds pod\(^1\) (no.)              | 1.43 (L)                      | 1.62 (H)                     | 1.60 (I)                     |
| 100-seed weight (g)                | 1.48 (L)                      | 1.53 (I)                     | 1.61 (H)                     |
| Seed yield plant\(^1\) (g)         | 2.56 (L)                      | 3.67 (I)                     | 4.34 (H)                     |

The research work of Singh et al. (2016) showed the genetic distance of the cluster ranged from fifty to seventy percent with an average of fifty four percent. Genotypic variations based on molecular characterization indicated that genotypes fit in different clusters due to their genetic components itself. Therefore, it will be used for further lentil breeding program, especially for hybridization and genotype that selected from different clusters will provide maximum heterosis as favors yield. Overall allelic diversity and polymorphic information content (PIC) value

The seven SSR primer sets were employed in the present study, among this four SSR primer sets were polymorphic and produced varying number of alleles with different size.
Among the 20 lentil genotypes, entirely 33 alleles were identified with an average of 8.25 alleles per locus. The maximum number of alleles per locus produced in SSR 19 (10) whereas minimum number of alleles per locus was displayed by SSR 90 (7) (Table 5). The similar result was recorded by Kushwaha et al. (2015) who observed the minimum SSR loci in SSR 130 and maximum in SSR 191 markers. Major allele frequency was highest in SSR 90 and lowest frequency was found in two marker namely, SSR 19 and SSR 33. Yadav et al. (2016) also found maximum allelic frequency in SSR 99, SSR 113 and SSR 124 and lowest in SSR 90.

Polymorphism information content (PIC) value is a reflection of allele diversity and frequency among the
Table 5: Details of SSR markers showed polymorphism, number of alleles detected, major allele frequency, allele size range, gene diversity and polymorphism information content (PIC).

| Primer  | No. of allele | Allele size range (bp) | Major allele frequency | Gene diversity | Polymorphism information content (PIC) |
|---------|---------------|------------------------|------------------------|----------------|---------------------------------------|
| SSR19   | 10.00         | 235-254                | 0.20                   | 0.88           | 0.86                                  |
| SSR33   | 8.00          | 257-270                | 0.20                   | 0.84           | 0.82                                  |
| SSR90   | 7.00          | 184-191                | 0.30                   | 0.82           | 0.79                                  |
| SSR213  | 8.00          | 180-188                | 0.25                   | 0.84           | 0.82                                  |
| Mean    | 8.25          |                        | 0.24                   | 0.84           | 0.82                                  |

**CONCLUSION**

The performance of 20 lentil genotypes for eleven yield and different yield contributing traits were revealed significant variations among the genotypes for all the traits. Considering the traits pods plant⁻¹, seeds plant⁻¹, seeds pod⁻¹, 100-seed weight and seed yield plant⁻¹ BARI Masur-6, BARI Masur-7 followed by genotypes BD-3806 and BD-4090 were best performer. The genotypes with high yielding traits were placed in cluster III. The SSR technique revealed that markers SSR 19 would be best in screening lentil genotypes. The results of this study also showed that microsatellite markers are linked to genes which is suitable tools for genetic understanding for the setup of future systematic lentil breeding programs.

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