



ORIGINAL ARTICLE

Evaluation of genetic diversity in some promising varieties of lentil using karyological characters and protein profiling



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Abstract Somatic chromosome study from root tip cells using the squash technique of the cytological method and seed protein profile of 5 varieties of *Lens culinaris* (Lentil) through SDS-PAGE were investigated. Karyotype analysis showed gross uniformity in morphology. Somatic chromosome number $2n = 14$ is constant for all the varieties. Chromosomes are mostly long to medium in length with secondary constrictions in one pair of chromosome. Primary constrictions in chromosome ranged from nearly median to nearly submedian in most of the cases. Notwithstanding the gross homogeneity, karyotype analysis revealed minute differences in details. Each lentil variety is thus characterized by its own karyotype, serving as one of the identifying criteria. The seed protein profile revealed that varieties are very close to each other with respect to similarity index that ranged from 0.594 to 0.690. With regard to seed protein banding patterns, slight polymorphism (14.285%) indicating low genetic diversity has been identified among the 5 varieties. A dendrogram indicates one variety is plesiomorphic and rest varieties are apomorphic. All the experimental varieties of lentil studied here show low genetic diversity due to their similar genetic base, indigenous genetic resources and conservative nature of the seed protein.

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1. Introduction

Lentil (*Lens culinaris* Medik.) belonging to the family Fabaceae is considered as one of the ancient, domesticated, economically important winter legume crop agriculturally cultivated worldwide as human food [32]. The seeds of this plant are commonly used as edible pulse. Lentils are valued

for their high protein content (as much as 30%) and good source of vitamins and other important nutrients.

Seed protein profiles obtained by gel electrophoresis have been successfully used not only to resolve taxonomic and evolutionary problems of several crop species but also to distinguish cultivars of a particular crop species [26,11,22]. In particular, seed protein profiles produced by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) have been successfully used for the identification or discrimination of various crop species, even at the varietal levels [18,39,20,42,8,40,44,2,35,43]. The technique is economical,

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simple, and rapid and generally free from environmental effects compared with the traditional morphological and other descriptive criteria derived from field trials [9,14,30,31,44]. Moreover, it is reported that SDS-PAGE of seed proteins is an extensively used technique for describing and assessing the seed protein diversity of many crop germplasm [12,21] and is potentially a useful identifier and descriptor for the purpose of seed identification and Plant Variety Rights [13,30,10].

Knowledge of cytological and molecular relationships between plant species is very useful in planning effective breeding strategies designed to transfer desirable genes or gene clusters from one species into another, thereby producing fruitful genomic reconstructions and disease free plants. Determination of genetic diversity of any given crop species is a suitable precursor for improvement of the crop because it generates baseline data to guide selection of parental lines and design of a breeding scheme. It is a valuable technique to get knowledge closeness between investigated genera (i.e., through similarity index) [23]. The aim of the present study was to find out genetic diversity of 5 different varieties of lentil using cytological characters and protein profiling.

2. Materials and methods

The seeds of 5 different varieties of lentil namely WBL 81 (Suvendu), WBL 256 (Ranjan), WBL 58 (Subrata), WBL 77 (Moitree) and B 77 (Asha) were officially procured from Pulses and Oilseeds Research Station, Berhampore, West Bengal, India. Characteristics of lentil varieties (Fig. 1) used in this work are presented in Table 1.

2.1. Karyological analysis

2.1.1. Study of somatic chromosome

Somatic chromosomes were studied from root-tip cells. Fresh healthy roots (November and December months are suitable for seed germination within a day), showing peak mitotic activity from 11 AM to 12.00 Noon, were collected and washed in distilled water. For scattering and clarification of chromosome morphology, pretreatment of root tips with mixture of saturated solution of pDB and aesculine for 3–3.15 h at 12–14 °C was found to be very effective for different varieties of lentil. The root tips putting in pre-treating solution were initially chilled at 0–5 °C for 4–6 min and then kept at 12–14 °C. For the sake of comparative karyological analysis, the same pre-treatment chemical was used for all the varieties. Root-tips were then carefully washed in distilled water and transferred to a suitable fixative such as, glacial acetic acid and ethanol (1:3) and kept overnight. The materials were then kept in 45% acetic acid for 3–5 min, subsequently warm over a flame in 2% acetic-orcein:HCl (1 N) mixture (9:1) for 3–4 s and finally kept for 2–3 h. Root-tips were squashed in 45% acetic acid for microscopic observation.

2.1.2. Karyomorphometrical analysis

The total length as well as the short arm length of all the chromosomes of the 5 varieties of lentil was measured accurately. In all the karyotypes, ratio of the short arm to the total length of the chromosome in percentage, F% (form percentage or centromeric percentage) was determined after Krikorian et al. [24].

The *centromeric index* (F%) i.e. the position of centromere of each chromosome was calculated using the following formula:

$$\text{Centromeric index (F\%)} = \frac{\text{Length of the short arm}}{\text{Whole length of the chromosome}} \times 100$$

Total centromeric index (TF%) was also determined in each taxa following Huziwara [19] by the formula:

$$\begin{aligned} \text{Total centromeric index (TF\%)} \\ = \frac{\text{Sum of the short arm length}}{\text{Sum of total chromosome length}} \times 100 \end{aligned}$$

Disparity index (DI%) of chromosomes in a karyotype was calculated according to Mohanty et al. [28] by the formula:

$$\text{DI} = \frac{\text{Longest chromosome} - \text{shortest chromosome}}{\text{Longest chromosome} + \text{shortest chromosome}} \times 100$$

During the preparation of karyotypes at least 4–5 well spread metaphase plates were compared and analyzed. Photomicrographs were taken from the well spread preparations with the help of Olympus digital SLR camera and LM digital SLR adaptor fitted with Olympus CX 41 microscope.

2.2. Extraction of seed proteins

0.2 g dry seed of each variety was taken in pre-chilled pestle and mortar and homogenized in chilled 2 ml of 0.2 M phosphate buffer (pH-8.2). The extracts were centrifuged at 10,000 rpm for 15 min at 4 °C. The extracted crude proteins were recovered as supernatant which was used for protein profiling.

Protein concentration of extracts was measured immediately and directly from the supernatant by dye binding assay as described by Bradford [7]. A standard curve of absorbance at 595 nm versus 10–80 µg of BSA was also drawn and from this curve, the amount of protein in sample was calculated and finally expressed as mg per g of seed. Repetition of same experiment was done 3 times in order to check the reproducibility of the method.

2.3. SDS-PAGE

Just before starting electrophoresis process, supernatant was mixed (1:1) with 2X sample buffer [27] and heated in a 1.5 ml eppendorf tube in water bath at 85 °C for 3 min to denature the protein. After that, the protein samples were subjected to one dimensional SDS-PAGE in a gel slab of 1 mm thickness (4% stacking gel = 2.5 cm height and 10% resolving gel = 5.5 cm height). Total size of the gel was 8 × 7.3 cm².

Electrophoresis was carried out in the discontinuous buffer system in a vertical electrophoresis apparatus (Bio-Tech India Pvt. Ltd) according to the method of Laemmli [27]. Using micro-pipette 20 µl protein samples were loaded to each well of the gel. In one well of the same gel, protein molecular weight marker (molecular weight range = 14–97 kDa) of Chrommas Biotech, India, was applied. 0.02% bromophenol blue (BPB) was added in the protein sample as tracking dye to see the movement of protein in the gel. The gel was run at 10 mA constant current mode. Then, the gel was stained for overnight in 0.025% Coomassie brilliant blue (CBB) R-250.

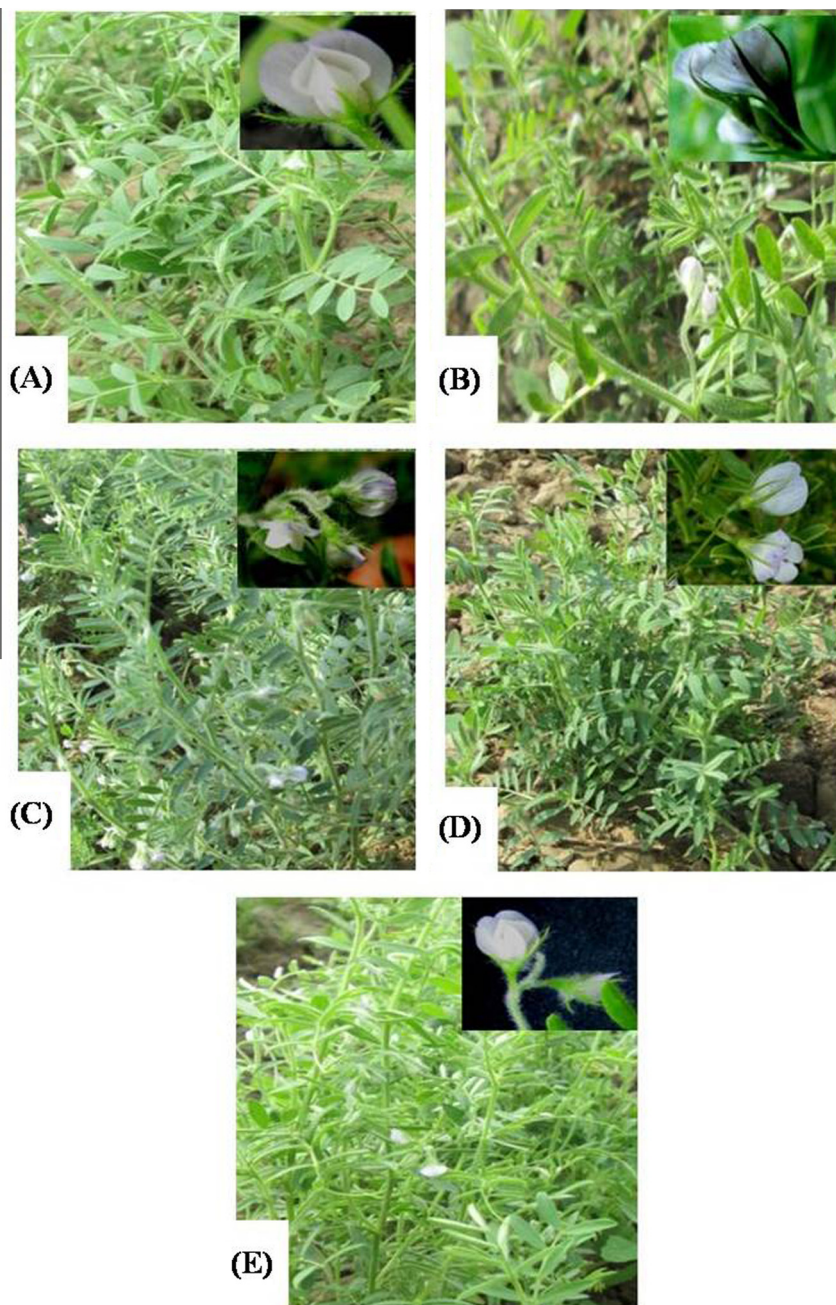


Figure 1 Showing field grown plant of (A) Variety Suvendu (WBL 81), (B) Variety Ranjan (WBL 256), (C) Variety Subrata (WBL 58), (D) Variety Moitree (WBL 77), (E) Variety Asha (WBL 77). With flower (inset).

2.4. Analysis of gel documentation

Finally, gels were photographed and scanned using Bio-Rad, USA made Gel-Doc™ XR+ system. Detailed analysis of protein band patterns in terms of band number, mobility of protein bands, staining intensity, band percentage, lane percentage and the determination of molecular weight of each band were done by Image Lab™ software (version 5.0). The presence and absence of the bands were entered in a binary data matrix. The similarity matrix thus generated was used to construct dendrogram using SEAVIEW version 2.6 Software.

3. Results

3.1. Analysis of somatic chromosome

Mitosis in root tip cells of 5 varieties of lentil showed regular cell division. No apparent chromosomal abnormalities were found. The chromosome number counted from the mitotic metaphase plate was constant i.e., $2n = 14$ for all varieties (Fig. 2A–E). The chromosome complements of all 5 varieties of lentil showed gross morphological similarities. In general, chromosomes were short and bi-armed.

Table 1 Characteristics of lentil varieties used in the study.

Varieties	Origin/Pedigree	Characters phenotypic	Phenoty-pic seed colour	Type varietal	Maturity	Year of release
WBL 81 (SUBHENDU)	Selection from chemical mutation [EMS 0.2%] of Asha (B-77)	The variety is erect with profuse branching habit; foliage dark green, flowers are blue and 2 per cluster, flowering early (60 days), and cotyledon orange. Seeds are small in size and oval in shape. It takes seedling to flowering in 65–70 days, and matures in 110–115 days	Seed coat dark mottled	Microsperma (small sized seed)	110–115 days	2009
WBL 256 (RANJAN)	Selection from X-ray irradiated material of Asha (B-77)	Plant height ranges 30–35 cm, typically spreading, and foliage color light green. Flowers are white. 57–62 days are required to flower, small in size	Seeds grey with black dots all over	Microsperma	120–125 days	1982
WBL 58 (SUBRATA)	The proposed variety has been developed from a cross between JLS-2 and T-35 which are more or less resembled to Moitree (WBL 77) and Subhendu (WBL 81)	This variety is semi-erect with profuse branching habit. The plant height is about 40–45 cm at ripening stage. Foliages are hairy; light green and tendrillous with persistent anthocyanin pigmentation. Flowers are violet and 2–3 in number per cluster. Cotyledon is red in color and lustrous. Seed is small in size and oval in shape	Seed coat is dark mottled	Microsperma	132–136 days	1998
WBL 77 (MOITREE)	The proposed variety has been developed from a cross between ILL 7723xBLx84176. Varieties are most closely resembled the varieties of Asha (B 77), Ranjan (WBL 256) and Subrata (WBL 58)	The plant is erect in nature with 40–45 cm height, profuse branching habit with absence of stem pigmentation. Foliage with medium size leaflet is green in color with medium intensity. Leaf pubescence and tendril are absent. Flowers are blue and 2 per cluster. Seeds are small in size and oval in shape. Cotyledons are shining lustrous and orange in color	Grey mottled seed coat.	Microsperma	118–120 days	2009
B 77 (ASHA)	Developed by single plant selection from a locally grown landraces material	Stem has semi-spreading growth habit with plant height of 35–40 cm. Stem and foliage is light green in color. Flower is white in color. 2 pods are on an average per axil. Seeds are small in size	Seed coat is ash colored having dark spots all over	Microsperma	120–125 days	1980

The chromosomes of different varieties of lentil were arranged and grouped into 4 types according to the length of chromosome, position of the centromere and presence of number of chromosomes with secondary constriction (SC) for the preparation of karyograms (Fig. 3A–E).

Type A: chromosome between 5.63 and 6.88 μ in length with 2 constrictions, one primary and the other secondary and both are located at the nearly median position at the opposite ends of the chromosomes. The middle segment is much smaller than the end ones.

Type B: chromosomes with 4.79–7.1 μ in length with nearly median constrictions.

Type C: chromosomes within 3.98–7.26 μ in length with nearly submedian constrictions.

Type D: chromosomes within 4.05–4.37 μ in length with nearly subterminal constrictions.

Types A–C were found in the varieties WBL 58 and B 77, while Types A–D were found in the varieties WBL 81, WBL 256 and WBL 77 of lentil.

In lentil total chromosome length varies between 70.90 and 83.00 μ in the varieties. There is one pair of chromosome with secondary constriction (SC) in all 5 varieties. The mean of total F% varies within 33.23–35.19% (Table 2).

3.2. Analysis of seed storage proteins

The total seed protein of 5 varieties of lentil obtained by one dimensional denaturing SDS-PAGE were studied and

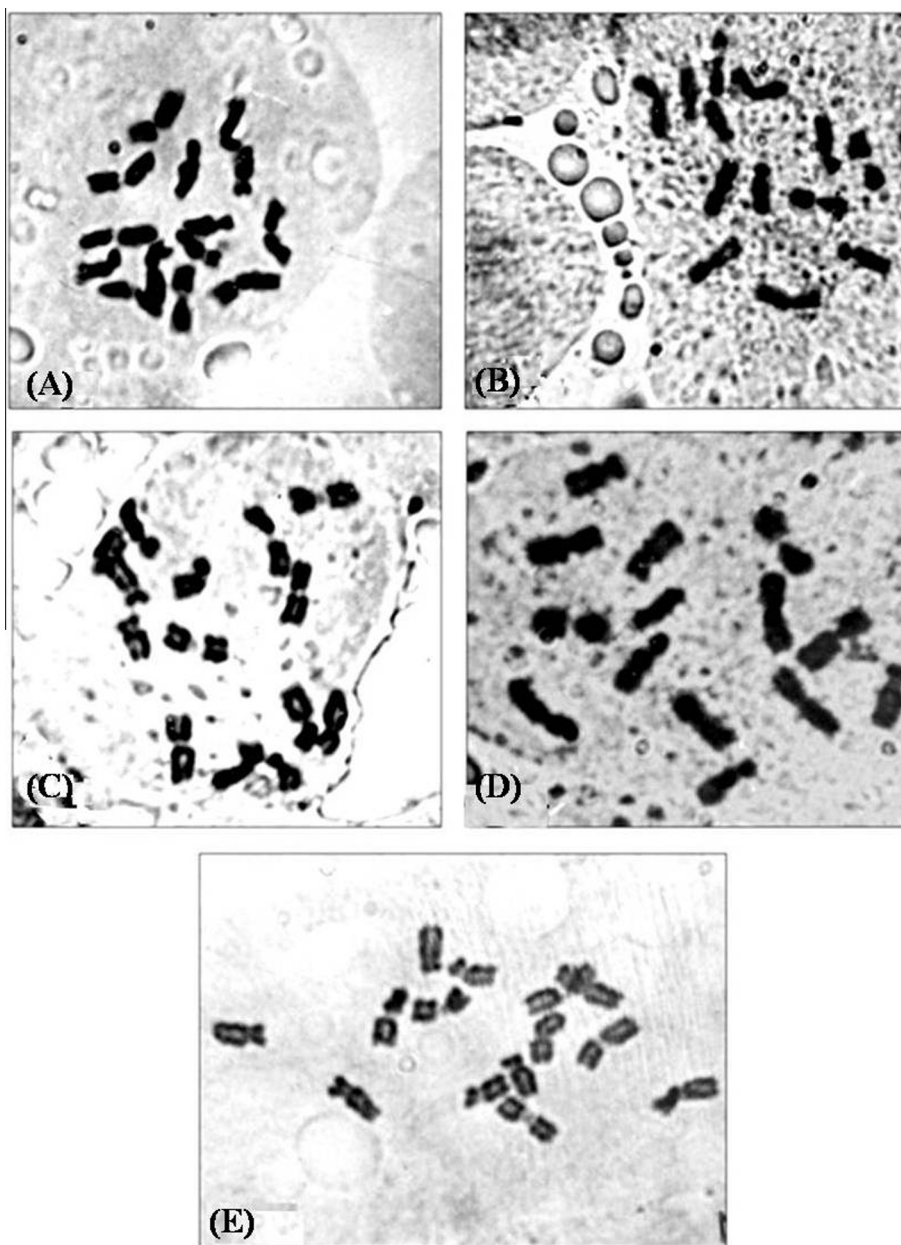


Figure 2 Showing mitotic metaphase plate of (A) Variety Suwendu (WBL 81), (B) Variety Ranjan (WBL 256), (C) Variety Subrata (WBL 58), (D) Variety Moitree (WBL 77), (E) Variety Asha (B 77).

revealed qualitative and quantitative intervarietal differences in terms of total number of protein band, position, thickness, staining intensity, relative mobility of bands and molecular weight (Figs. 4 and 5). Varieties showed variation in total number of protein bands ranging from 21 to 26. During protein profiling of total proteins of experimental varieties, WBL 58 and WBL 77 showed maximum number (26) of protein bands while minimum number (21) of protein band was found in WBL 81. The electrophorogram of WBL 256 and B 77 showed intermediate number (23–24) of protein bands. The results of relative mobility (R_f value) and molecular weight of protein bands exhibited variation ranging from 0.003 to 0.990 and 14 to 97 kDa respectively. Quantitative variation of seed protein among different varieties of lentil studied in the present

experiment was also found and is represented by bar diagram in Fig. 6. The highest amount of protein content i.e. 148.166 ± 0.763763 mg/g of seed was obtained in WBL 58. However, in other varieties such as WBL 81, WBL 256, WBL 77 and B 77 seed protein contents were 121.5 ± 1.322 , 136.7167 ± 1.1026 , 126.666 ± 1.5275 and 137.666 ± 1.6441 mg/g of seed respectively.

3.3. Analysis of seed proteins cluster

Similarity indices among the 5 lentil varieties based on protein analysis is given in Table 3. The similarity relationships ranged from 0.690 to 0.594. The highest similarity index (0.690) was found between WBL 77 (Moitree) and B 77 (Asha) followed

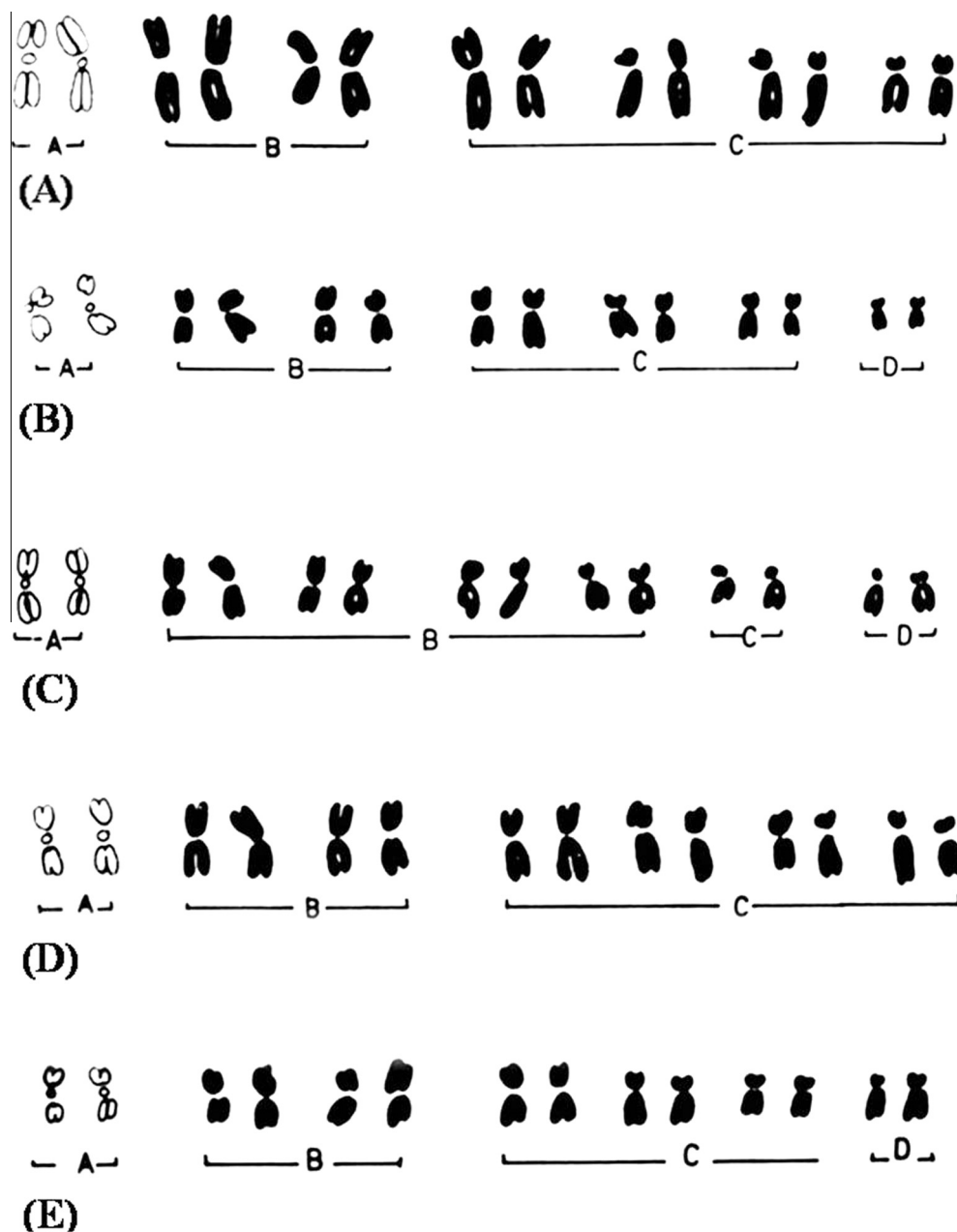


Figure 3 Showing Comparative karyotypes of different varieties of lentil (A) Variety Suwendu (WBL 81), (B) Ranjan (WBL 256), (C) Variety Subrata (WBL 58), (D) Variety Moitree (WBL 77), (E) Variety Asha (WBL 77).

by in descending order between WBL 58 (Subrata) and B 77 (0.683) then WBL 256 and B 77 (0.683) next between WBL 81 (Suwendu) and WBL 77 (0.662), WBL 58 and WBL 256 (0.662), WBL 256 and WBL 77 (0.655) then between WBL 81 and B 77 (0.647), WBL 58 and WBL 81 (0.640) lastly between WBL 256 and WBL 81 (0.594).

The dendrogram, which represents the genetic relationships among the tested lentil varieties, is presented in Fig. 7. The dendrogram indicates that variety B 77 (Asha) is separated as outgroup and the remaining four varieties are included into one ingroup or one cluster i.e. cluster I with two sister/sub-groups. The outgroup variety is known to be less closely related to the rest of varieties than they are to each other. Therefore, B 77 variety is plesiomorphic and rest varieties are apomorphic. The values on each branch are actually

branch value and the values at each node are divergence values. Among the 5 lentil varieties, the first sister group comprising of WBL 77 and WBL 58 under cluster I are a comparatively and phylogenetically advanced group due to their high branch value (0.312) and low divergence value (0.015). However, the second sister group consisting of WBL 256 and WBL 81 is a relatively less advanced group due to their comparatively low branch value (0.297) and high divergence value (0.030).

4. Discussion

Karyotype analysis in 5 varieties of lentil shows gross uniformity in morphology and the chromosomes with graded

Table 2 Detail of comparative karyotype of five varieties of lentil.

Variety	2n	Karyotype formula*	No. of SC	Mean of total TF%**	Total chromosome length (μ)***	Range of length (μ)	DI%
WBL 81 (SUVENDU)	14	A ₂ + B ₄ + C ₆ + D ₂	2	34.19	70.90	6.32–3.98	22.71
WBL 256 (RANJAN)	14	A ₂ + B ₄ + C ₆ + D ₂	2	33.46	72.64	6.04–4.26	17.18
WBL 58 (SUBRATA)	14	A ₂ + B ₄ + C ₈	2	33.69	83.00	7.26–4.13	27.48
WBL 77 (MOITREE)	14	A ₂ + B ₄ + C ₆ + D ₂	2	33.23	79.04	6.56–4.21	21.81
B 77 (ASHA)	14	A ₂ + B ₄ + C ₈	2	35.19	71.38	5.91–4.00	19.27

* WBL 58 and B 77 in one hand and rest three varieties in other hand are identical in karyotypic formulae.

** Mean of TF% is nearly same in all varieties.

*** Total chromosome length differs to some extent among the varieties.

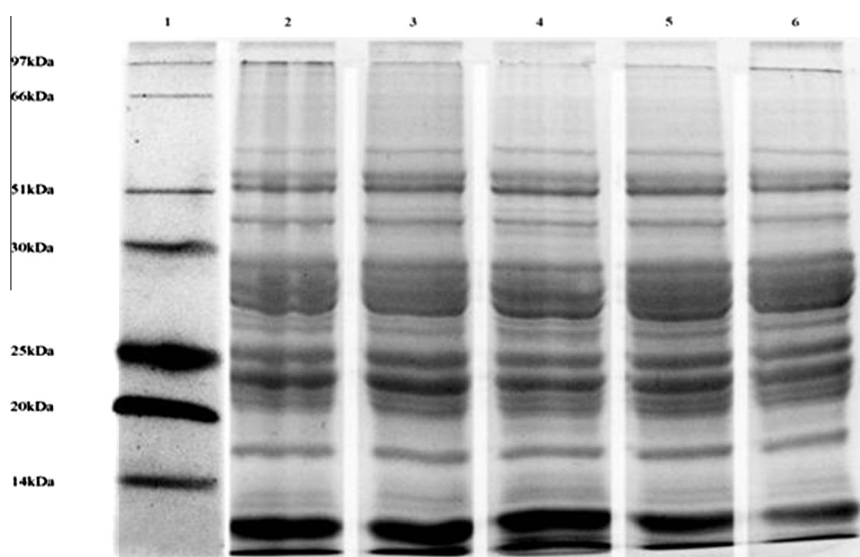


Figure 4 Showing seed storage protein profiles of five varieties of lentil on 10% SDS polyacrylamide gel (six lanes are marked by 1, 2, 3, 4, 5 and 6 at the top. Lane 1 contains marker protein showing seven bands with molecular weights 97, 66, 51, 30, 25, 20, 14 kDa (A) Variety Suvendu (WBL 81), (B) Variety Ranjan (WBL 256), (C) Variety Subrata (WBL 58), (D) Variety Moitree (WBL 77), (E) Variety Asha (B 77).

symmetrical karyotype. Somatic chromosome number $2n = 14$ is constant for all the varieties. Identical chromosome numbers have been recorded earlier by different workers [33,34,35,34,36,37,29,5].

Chromosomes are mostly long to medium in length with secondary constrictions in only pair of chromosome of all 5 varieties of lentil. While studying with 15 varieties of lentil Sinha and Acharia [37] reported the presence of one pair chromosome in some varieties but absence of this pair in rest varieties. It was also noted [37,38] that there is a variation in the distance between the 2 constrictions (primary and secondary). From their study they also concluded that there might have been a gradual reduction in the distance between the 2 constrictions due to translocation and hybridization which might have led to their total loss and thus giving rise to the varieties without the presence of chromosome with

secondary constriction. But no such absence of chromosomes with secondary constriction and variation in the distance between the 2 constrictions was found in the present study.

Notwithstanding this gross homogeneity, karyotype analysis reveals minute differences in details. Each variety is thus characterized by its own karyotype, serving as one of the identifying criteria. Based on this parameter, the varieties WBL 58 (Subrata) and B 77 (Asha) can be distinguished from other 3 varieties by the absence of one pair of chromosome with nearly subterminal primary constrictions. Despite the fact that the same karyotype formula A₂ + B₄ + C₆ + D₂ is represented in WBL 77 (Moitree), WBL 81 (Subhendu) and WBL 256 (Ranjan), the varieties can be distinguished from one another by their range of chromosome length, namely 4.21–6.56 μ in WBL 77, 3.98–6.32 μ in WBL 81 and 4.26–6.04 μ in WBL 256. All the features of the chromosome

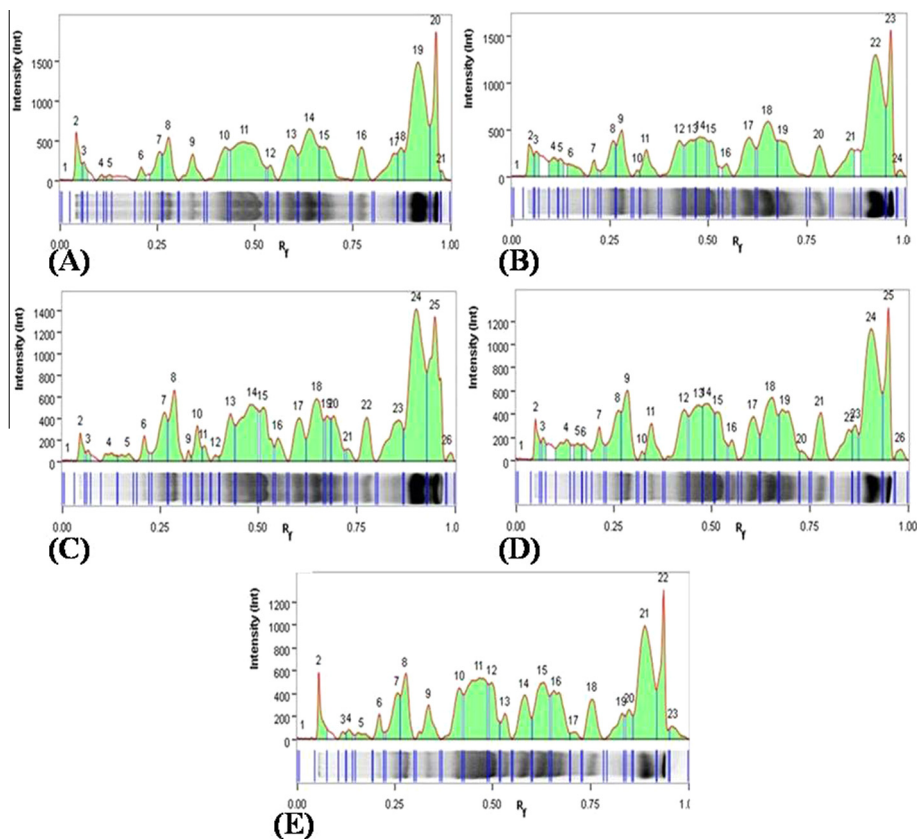


Figure 5 Showing Gel Doc data of individual lane profile showing the number of peaks of different heights obtained on the basis of the intensity of bands against their respective relative mobility (R_f value) of bands of (A) Variety Suvendu (WBL 81), (B) Variety Ranjan (WBL 256) (C) Variety Subrata (WBL 58), (D) Variety Moitree (WBL 77), (E) Variety Asha (WBL 77).

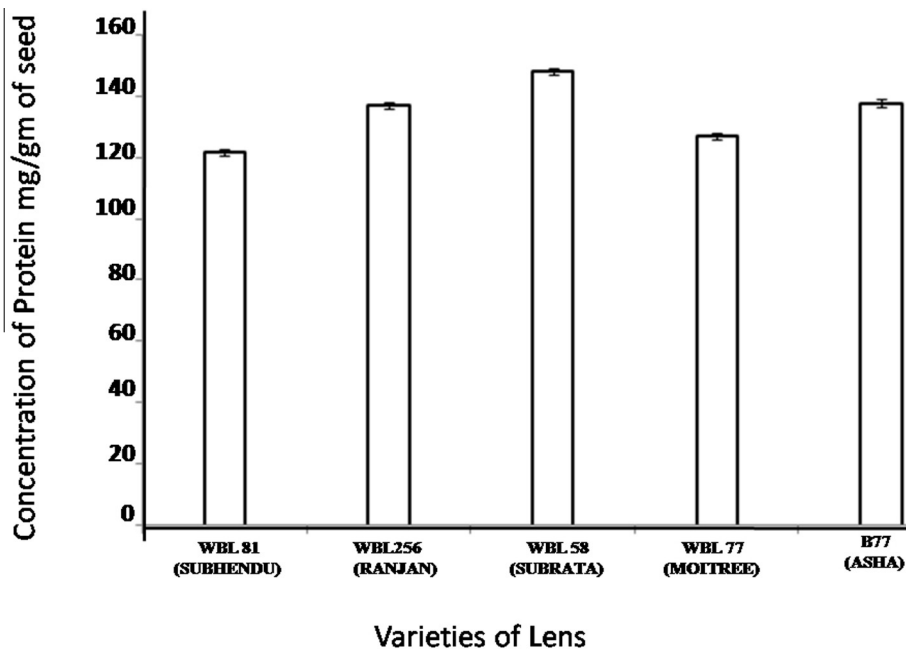
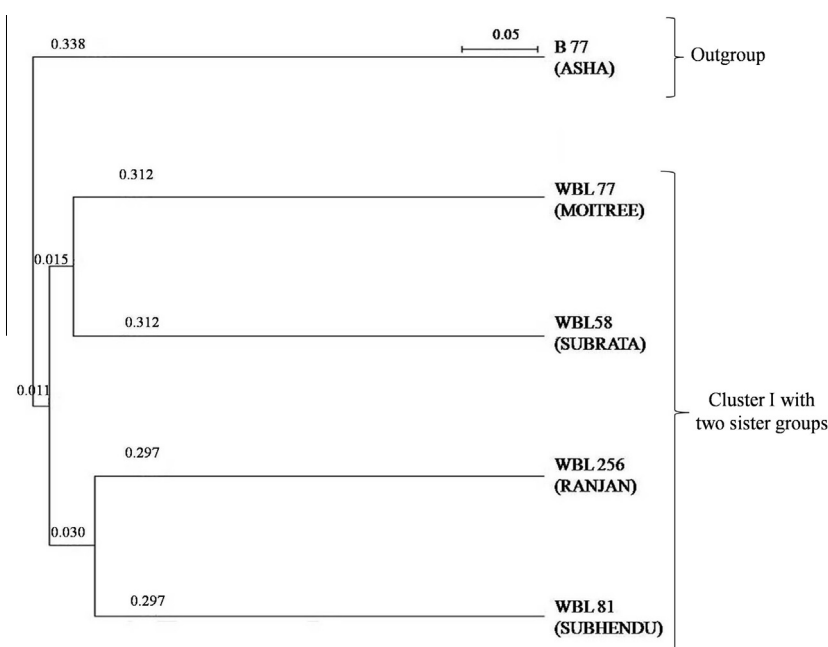


Figure 6 Bar graph with error bars indicating standard deviation around the mean of concentration of protein (mg/gm) of seed protein of different varieties of lentil.

Table 3 Similarity coefficient of R_f value of electrophoretically separated seed protein bands of five varieties of lentil calculated using SEAVIEW version 2.6 Software.

	WBL 81 (SUBHENDU)	WBL 256 (RANJAN)	WBL 58 (SUBRATA)	WBL 77 (MOITREE)	B 77 (ASHA)
WBL 81 (SUBHENDU)	0				
WBL 256 (RANJAN)	0.594	0			
WBL 58 (SUBRATA)	0.640	0.662	0		
WBL 77 (MOITREE)	0.662	0.655	0.625	0	
B 77 (ASHA)	0.647	0.683	0.683	0.690	0

**Figure 7** Showing dendrogram based on R_f value of electrophoretically separated seed protein bands of five varieties of lentil using SEAVIEW version 2.6 Software.

morphology clearly indicate that in the karyotype, the parameters tested, may to a great extent serve as identifying criteria of different varieties.

The total chromosome length does not show marked variation except in one variety, WBL 58 where it is 83.00 μ as compared to WBL 81, WBL 256, WBL 77 and B 77, where the values are 70.90, 72.64, 79.04 and 71.38 μ respectively. Variations in chromosome in length within the same complement have also been observed among the varieties. A decrease in chromosome length is one of the factors responsible for evolution of higher plant.

However, among the varieties of lentil, the size ranges show a remarkable constancy. It may thus be inferred that rather than deletion or duplication, structural rearrangements involving certain chromosomes have been of principal importance in bringing about changes. Accumulation of such changes can sometimes lead to genetic diversity during the process of evolution.

The disparity index (DI) value corresponds to the homogeneity or heterogenous assemblage of chromosomes.

Normally a low disparity index value corresponds to the homogeneity of chromosome whereas a high disparity index value points towards the general heterogeneity of chromosomes. In the present study, the range of lower values of DI found in lentil is 17.18–27.48% which corresponds to the homogeneity of chromosomes among the 5 varieties. In addition to that, the mean centromeric index (TF%) value undoubtedly confirms the status of a taxon with respect to chromosome study. In lentil, the lower TF% exhibited among different varieties shows that it represents the climax of evolution i.e. its advanced status. The abundance of submetacentric chromosomes in the karyotype of 5 varieties of lentil is also advanced karyomorphological features. Thus individuals with same chromosome number but with minute differences in karyomorphological details reflect the ongoing evolutionary processes at a microlevel.

The SDS-PAGE for water-soluble seed protein electrophoresis was used to investigate the genetic differences among the varieties. The band patterns indicate differences among the

varieties in number of bands, position of the bands and molecular weight of the bands etc. The present investigation revealed that protein profiling is one of the basic methods to detect inter varietal genetic diversity and study phylogenetic relationship among 5 selected experimental lentil varieties. During the present study on 5 varieties of lentil, the similarity index ranged from 0.690 to 0.594. The highest similarity index (0.690) was found between WBL 77 (Moitree) and B 77 (Asha) followed by in descending order between WBL 58 (Subrata) and B 77 (0.683) then WBL 256 and B 77 (0.683) next between WBL 81 (Suvendu) and WBL 77 (0.662), WBL 58 and WBL 256 (0.662), WBL 256 and WBL 77 (0.655) then between WBL 81 and B 77 (0.647), WBL 58 and WBL 81 (0.640) lastly between WBL 256 and WBL 81 (0.594) showing lowest similarity index and they also genetically related to each other. The higher the similarity coefficient between two genotypes, the more the similarity between two genotypes based on protein bands [1]. With regard to seed protein banding patterns, slight polymorphism has been identified among the 5 varieties under study. Binary data obtained for absence (0) and presence (1) from protein gel electrophoresis among 5 lentil varieties showed 14.285% polymorphism. It means that the level of protein polymorphism is very low and it is correlated with low genetic diversity. The low level of protein polymorphism could result from conservative nature of the seed protein [6]. Moderately high similarity index values ranging from 0.690 to 0.594 found among the lentils genotypes tested indicate that the genetic diversity between them is narrowed due to their more or less common origin in the breeding program. Similar results were reported by Hamdi and Omar [17], Hamadi and Elemery [16], and Hamadi et al. [15] who found that the highest similarity index was between Giza 370 and Family 29 followed by Giza 9 and Giza 370 then between FLIP95-67L and 81-17, the most promising lentil genotypes in Egypt. This result indicated the strong genetic relations among the Egyptian genotype Giza 370 and Family 29 and Giza 9 and Giza 370 that is logical since these genotypes originated from similar Egyptian landraces and hence they have a similar genetic base. Sharma et al. [35] also obtained similar results using AFLP and RAPD marker techniques to evaluate and study the genetic diversity and phylogeny of 54 lentil accessions. The study on genetic diversity in ex-situ conserved lentils for botanical descriptors, biochemical and molecular markers and identification of landraces from indigenous genetic resources of Pakistan also gave a low level of genetic diversity of seed protein [41]. While studying the genetic characterization of ninety-six genotypes of lentil germplasm using SSR markers, Kushwaha et al. [25] obtained a wide range of genetic variability among the lentil genotypes due to their different centres of origin, different genetic constitution and different cluster forming group. Therefore, it is concluded that all the experimental varieties of lentil studied here show low genetic diversity due to their similar genetic base, indigenous genetic resources and conservative nature of the seed protein and should be diversified using modern breeding techniques. The genetic relatedness detected in this study may constitute the foundation for future systematic lentil breeding programmes.

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