GENETIC DIVERSITY AND CHARACTERIZATION OF MUNGBEAN GERMPLASM THROUGH MORPHOLOGICAL CHARACTERS AND MICROSATELLITE MARKERS

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ABSTRACT

Twenty mungbean accessions were characterized and their genetic diversity was studied. Eight morphological characters including, plant height, number of fruiting branches per plant, number of pod per plant, number of pod clusters per plant, pod length, number of grains per pod, 1000 grain weight and total grain yield per plant were measured. Analysis of variance for all traits showed significant differences among genotypes. For cluster analysis UPGMA method was used to determine genetic variation and four main groups were defined. Principal component analysis was done to evaluate diversity, three components explained near 79% of total variation among genotypes. The first principal component (PC1) is related to number of Fruiting Branches/Plant, number of pod /plant, number pod cluster and pod length that, explained 39.4% of total variability. Phenotypic coefficients of variation (PCV) and genotypic coefficients of variation (GCV) were calculated for all characteristics, the highest GCV and PCV were observed for number of pod per plant (39.47%, 38.65%), number of pod cluster (34.28%, 32.15%) and grain yield (31.73%,30.90%) respectively. Higher broad sense heritability was found for the same traits. The results of phenotypic and genotypic correlation analysis indicated that grain yield was positively and significantly associated with pod length and 1000 grain weight. Path analysis based on genotypic correlation coefficients showed high positive direct effect of number of pod per plant (1.874), number of fruits (0.985) and plant height (0.688) on yield per plant. Furthermore, Twenty two microsatellite primer pairs were used for molecular studies. Out of these, thirteen primers were able to amplify the mungbean genome, upon polymerase chain reaction (PCR) result. Those that failed either were unable to amplify product at all, or showed unspecific amplification. Out of these 13

successfully amplified loci, 6 potential polymorphic loci were observed from microsatellite banding profiles on the gel images. These six primer pairs were used to evaluate the genetic variability in six selected mungbean population. The highest expected heterozygosity value count during multi population analysis demonstrated by locus *LR7322B*, ranged from 0.6014 to 0.8743, whilst the highest observed heterozygosity ranged from 0.5000 to 1 and 0.6666 to1 demonstrated by locus *LR7323A* and *LR7319B* respectively. Polymorphism assessments on all populations achieved using these three primers showed that H_O scored were generally higher than H_E. There was no linkage disequilibrium (LD) observed between all primer pairs. All loci, except *LR7319B* conformed to Hardy-Weinberg equilibrium (HWE). The F_{IS} index demonstrated no indication of inbreeding among individuals of each population. Corresponding to UPGMA tree, population NM-1919 and population 40521 were observed to be least similar compared to the other four populations. Population structure analysis of molecular marker data from 6 primer pairs also divided the populations into four distinct groups and corresponding to this analysis, 40521 was observed to be least similar compared to the other populations. Furthermore, result obtained from analysis of molecular variation (AMOVA) showed significant difference within individuals of high and low yield mungbean genotypes, and accordingly, high heterosis effect may be accrued in the previous population.

ABSTRAK

Dua puluh mungbean aksesi telah disifatkan dan kepelbagaian genetik mereka telah dikaji. Lapan ciri-ciri morfologi termasuk ketinggian pokok, bilangan dahan berbuah setiap tumbuhan, bilangan buah per pokok, bilangan kelompok pod per pokok, panjang pod, bilangan biji per pod, berat 1000 benih dan jumlah hasil benih sepokok telah diukur. Analisis varians untuk semua ciri-ciri menunjukkan perbezaan yang signifikan di kalangan genotip. Untuk menentukan variasi kelompok analisis genetik kaedah UPGMA telah digunakan dan 4 kumpulan utama telah dikenalpasti. Analisis komponen utama telah dilakukan untuk menilai kepelbagaian, tiga komponen menjelaskan hampir 79% jumlah variasi antara genotip. Komponen utama pertama (PC1) berkaitan dengan dahan berbuah / pokok, bilangan pod / pokok, bilangan kelompok pod dan panjang pod itu, menjelaskan 39.4% daripada jumlah kepelbagaian. Variasi Pekali Fenotip (PCV) dan Variasi Pekali Genotip (GCV) telah dikira untuk semua ciri-ciri, GCV tertinggi dan PCV telah diperhatikan untuk bilangan pod per pokok (39.47%, 38,65%), bilangan pod kelompok (34.28%, 32.15%) dan hasil biji benih (31.73%, 30.90%) masing-masing. Perwarisan Broad Sense yang tinggi telah dijumpai untuk ciri-ciri yang sama. Keputusan analisis korelasi fenotip dan genotip menunjukkan bahawa hasil benih yang positif dan signifikan dikaitkan dengan panjang pod dan 1000 benih berat. Analisa laluan berdasarkan pekali korelasi genotip menunjukkan kesan positif yang tinggi secara langsung dengan bilangan pod sepokok (1,874), beberapa buah-buahan (0.985) dan ketinggian tumbuhan (0.688) atas hasil per pokok. Tambahan pula, dua puluh dua pasangan primer mikrosatelit telah digunakan untuk kajian molekul. Daripada jumlah ini, 13 primers dapat menggandakan genom mungbeab, dengan hasil tindak belas rantai polymerase (PCR). Primer yang gagal, sama ada tidak dapat untuk menggandakankan produk pada semua, atau menunjukkan penggandaan tidak spesifik. Daripada 13 lokus yang berjaya digandakan, terdapat 6

lokus polimorfik yang berpotensi telah diperhatikan berdasarkan profil banding mikrosatelit pada imej gel. Enam pasangan primer ini telah digunakan untuk menilai kepelbagaian genetik dalam 6 populasi taugeh terpilih. Nilai heterozigositi dijangka tertinggi semasa analisis pelbagai populasi yang ditunjukkan oleh locus *LR7322B*, iaitu 0.6014-0.8743, manakala nilai heterozigositi diperhati tertinggi ditunjukkan oleh locus *LR7323A* dan *LR7319B* masing-masing menunjukkan bacaan antara 0.5000–1.0000 dan 0.6666–1.0000. Penilaian polymorphism pada semua primer yang dicapai menggunakan ketiga-tiga primer menunjukkan bahawa bacaan H_O secara amnya lebih tinggi daripada H_E. Terdapat tiada ketakseimbangan hubungan (LD) diperhatikan antara semua pasangan primer. Semua lokus, kecuali *LR7319B* menepati keseimbangan Hardy-Weinberg (HWE). Indeks FIS menunjukkan tiada tanda-tanda pembiakbakaan di kalangan individu setiap penduduk. Sejajar dengan pokok UPGMA, populasi NM-1919 dan populasi 40521 telah diperhatikan untuk menjadi kurangnya sama berbanding dengan empat penduduk lain. Analisis strukture populasi data penanda molekul daripada 6 pasang primer juga membahagikan populasi kepada empat kumpulan yang berbeza dan sepadan dengan analisis ini, 40521 diperhatikan kurang persamaan berbanding dengan penduduk lain.

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CHAPTER 1

INTRODUCTION

1.1 Background

The genus *Vigna* has been divided to include about 170 species, 120 from Africa, 22 from Asia, and a few from other parts of the world (Ghafoor *et al.*, 2001). Seven species of *Vigna* are cultivated as pulse crops specially in Asia, Africa and some parts of America (Anishetty & Moss, 1988). Two of these cultivated species are of African origin (subgenus *Vigna*) and five are of Asian origin (subgenus *Ceratopropis*). The species from the Asiatic group consists of, adzukibean (*Vigna angularis Willd,* Ohwi and Ohashi), blackgrarn/mash/urd (*Vigna mungo* L. Hepper), mungbean/greengram (*Vigna radiata* L. Wilczek), mothbean (*Vigna aconitofolia* Jack Marechal) and ricebean (*Vigna umbellata Thunb,* Ohwi and Ohashi). Five important Asian pulses include; adzukibea, mothbean, ricebean, blackgram and mungbean. They are from subgenus *Ceratotropis* of the genus *Vigna*. Blackgram and mungbean have been the main crops in Asia since ancient times (Paroda & Thomas, 1987). Presently, mungbean cultivation is widely practiced world wide, because as compared to blackgram it is easily digested (Smartt, 1990). The subgenus *Ceratotropis* is called Asian *Vigna* because it originates from Asia and the subgenus *Ceratotropis* forms about seventeen species largely limited to the Pacific and Asia. It can be subdivided into two groups by the seedling characteristics, i.e., (1) "mungbean group" showing an epigeal germination and (2) "adzuki bean group" showing a hypogeal germination. Today, biochemical markers have been used to show the phylogenetic relationships of *Vigna* species (Yasui *et al.*, 1985) for low molecular weight carbohydrates; (Jaaska & Jaaska,

1990) for isozymes; ((Rao *et al.*, 1992) for grain proteins; (Fatokun *et al.*, 1993) for RFLP; (Vaillancourt *et al.*, 1993) for chloroplast DNA; (Zink *et al.*, 1994) for RFLP of the phytohemagglutinin genes and (Kaga *et al.*, 1996) for RAPD.

The subgenus *Ceratotropis* in the genus *Vigna* contains *Vigna radiata var. sublobata*. This species before was studied by many authors as *Phaseolus sublobatus Roxb*., which was revealed as the common ancestor of both *V. radiata* and *V. mungo* (Verdcourt, 1970). After further investigations, it was defined that the taxon contained two different forms, one related to *V. radiata* and the other to *V. Mungo* (Singh et al., 1974; Lukoki et al., 1980; Jain & Mehra, 1980) agreed the particular difference between the *V. radiata* and the *V. Mungo* and their relations as wild ancestors to the cultivated species. They related two forms as *V. radiata var. sublobata (Roxb.) Verdcourt* and *V. mungo var. silvestris Lukoki*. This was confirmed by Miyazaki and Chandel, based on morphological characters and biochemical studies (Chandel *et al.*, 1984; Miyazaki *et al.*, 1984).

Mungbean (*Vigna radiata L. Wilczek*) is one of the major crops well suited to dry areas, mainly under watered conditions. It is an annual food legume, that cultivated in traditional form by small landholders in every part of tropical, subtropical and mild zones of Asia including India, Bangladesh, Pakistan, Sri Lanka, China, Korea, Japan, Thailand and Nepal. Mungbean is grown under various cropping systems and it has a short maturity period, therefore profitable to landholders as well as sustaining soil productivity (Fernandez & Shanmugasundaram, 1988). In south Asia, it is used to cook dhal, it is the most common food which is made from different kinds of split legumes with spices. In the East and Southeast Asian countries, mungbean is used to make different kinds of bean jam, sweetened bean soup, sweet, bean sprout and vermicelli. In many countries average mungbeens yield procured is much lower than its real potential,

one factor that accounts for low yield is lack of high yielding cultivars with better adaptability.

In the past century world agriculture has been successful in conducting more investigation cereals as compared to legumes. To increase agricultural productivity, it will be essential to use a wide range of the plant genetic diversity, especially of legumes and minor crops genetic resources. Genetic diversity concept is diversity of the sets of genes, that carried by different genotypes of a species. All the plant scientists agree that plant genetic improvement can be achieved by the manipulation of available genetic variability. The value of genetic diversity in plant breeding is well recognised because of, the results achieved in different crops (Rabbani et al., 1998; Ghafoor et al., 2001; Upadhyaya et al., 2002; Upadhyaya, 2003). One of the approaches for making a germplasm stock is to gather germplasm from different geographical origins with a concentration of accessions from known centers of diversity in individual samples. Genetic diversity is a valuable factor in planning experiments and gene-bank management because it assists utilization of germplasm and well-organized sampling either by eliminating and/or identifying duplicates in the gene stock ultimately resulting in the development of core collection philosophy. Representative germplasm from all geographical range of the crop species can assist to make ensure conservation of co-adapted gene complexes (Brown, 1978; Beuselinck & Steiner, 1992; Frankel et al., 1995), since genetically heterogeneous lines generate stable and more yield than genetically homogeneous populations.

In order to utilize, evaluate and maintain germplasm effectively, it is important to consider the extent of genetic diversity available. Smith investigated detailed morphological characters as an important step in classification and description of crop germplasm since a breeding program mostly depends on the amount of genetic

variability (Smith et al., 1991). The multivariate analysis and especially, the cluster analyses and principal component analyses have been utilized for the evaluation of germplasm based on various characters across a large number of accessions (Mardia *et al.*, 1979). Highly heritable, quantitative genetic markers permit assessment of genetic diversity. Sokal supported calculating general variances the determinant of the variance-covariance matrix came from detailed morphological as indices of intra population diversity such as Goodman assessed the comparative intra accession variability of some cotton and maize genotypes (Goodman, 1968; Sokal, 1965).

To estimate the genetic resources maintenance and their utilization, variance can be derived into its components. It ensures the possibility of development for utilization of suitable gene pool in crop improvement for particular plant attributes (Pecetti & Damania, 1996). Various numerical taxonomic techniques (Brown & Weir, 1983; Nei, 1987; Weir, 1996) have been effectively used to measure and classify phenotypic diversity in the relationships of germplasm stocks in a variety of crops by several scientists as in yellow yam (Akoroda, 1983), pea (Amurrio *et al.*, 1992; Amurrio *et al.*, 1995), cole crops (Dias *et al.*, 1993), Indian mustard (Gupta *et al.*, 1991), corn (Revilla & Tracy, 1995), soybean (Perry & McIntosh, 1991), alfalafa (Smith et al., 1991; Warburton & Smith, 1993; Smith et al., 1995), ryegrass (Humphreys, 1991), foxtail millet (Li et al., 1995), cotton (Goodman, 1968; Brown, 1991), blackgram (Shanmugam & Rangasamy, 1982; Dasgupta & Das, 1984; Das Gupta & Das, 1985; Ghafoor et al., 2001), lentil (Ahmad *et al.*, 1997), and mungbean (Ramana & Singh, 1987; S. P. Singh, 1988).

Polygenic morphological characters are often used as genetic markers for taxonomic applications and different plant germplasm management (Stuessy, 2009), but their significant genotype-environment interaction and inferior heritabilities can dramatically raise the expense and complexity of analyzing them, even though computerized imaging systems can help in this attempt. In addition, estimation of obtainable genetic collections to measure the genetic variation for economic important traits is a pre-requisite for joining wanted genes in a particular genotype. The type and amount of genetic variation in a line assists in selection of parents, that after hybridization is possible to produce the superior recombinants for desirable characters for example wider adaptability, resistant to diseases and high yield.

At present, molecular tools have been used for blackgram and mungbean breeding plans, such as, the use of DNA markers and isozymes for evaluation of genetic diversity, relatedness among *Vigna* species subgenus *Ceratotropis*, mapping of disease resistance genes and variety identification (Boonpradub & Chatasiri, 1999; Prammanee et al., 2000; Chaitieng et al., 2002; Seehalak et al., 2006). Some types of DNA markers have been used recently to help in genetic study. These are: Random Amplified Microsatellites (RAM), Amplified Fragment Length Polymorphisms (AFLP), Restriction Fragment Length Polymorphisms (RFLP), Direct Amplification of Length Polymorphisms (DALP), Random Amplified Polymorphic DNA (RAPD) and Variable Number of Tandem Repeats (VNTR) such as microsatellites and mini satellites. Despite the several types of markers accessible, the most well-organized and helpful marker method is microsatellites that is also identified as simple sequence repeats (SSR). Microsatellite markers have the properties of reproducibility, multiallelic nature, codominant inheritance, relative abundance and good genome coverage. These properties make microsatellites markers useful for a variety of applications in plant genetics and breeding (Powell *et al.*, 1996). Development of microsatellite markers

from genomic libraries is rarely informative regarding their function; they can belong to either transcribed or non-transcribed region of the genome. By contrast, genic-microsatellite markers often have known or 'putative' functions. As a result, genic-microsatellite markers are gene targeted markers and have the potential of representing functional markers in those cases where polymorphisms in the repeat motifs affect the function of the gene in which they reside (Andersen & Lubberstedt, 2003). However, due to greater DNA sequence conservation in transcribed regions, genic microsatellite primers have been reported to be less polymorphic compared with genomic microsatellites in crop plants (Rungis *et al.*, 2004; Scott *et al.*, 2000). For assessment of functional diversity, the genic microsatellites are useful; however, genomic microsatellites are more useful for fingerprinting or varietal identification study due to higher polymorphism (Varshney *et al.*, 2005).

Unfortunately, microsatellite markers have not been used in mungbean due to the complexity of their isolation. Therefore, other types of markers for example RAPD, RAM, RFLP, proteinase inhibitors and isozymes have been developed for taxonomic and phylogenetic aims in mungbean with varying degrees of achievement. Germplasm stocks are of minimal value unless they are used by the breeders for crop improvement. Accessibility of valuable variation in germplasm is necessary for organized breeding. Keeping in view the value of mungbean as a pulse crop.

1.2 Hypothesis

a. The mungbean genotypes show heterosis and also genetic diversity among twenty mungbean germplasm.

b. Molecular markers are able to show significant differentiation between high grain yield genotypes and low grain yield genotypes.

1.3 Objectives of study

The objectives of this study are summarized as:

1. To determine the genetic diversity of mungbean germplasm on the basis of morphological and agronomic characteristics.

2. To evaluate the genetic structuring six populations of mungbean with high and low yield performance using microsatellite markers.

CHAPTER 2

Literature review

2.1 Mungbean background

The genus *Vigna* composed of more than 150 species that are of considerable economic importance in many developing countries (Polhill & Van der Maesen, 1985). Mungbean [*V. radiate* (L.) Wilczek], urd bean [*V.mungo* (L.) Hepper] and cowpea [*V. unguiculata* (L.) Walp.], are key dietary staples for millions of people. Additionally, adzuki beans [*V. angularis* (Willd.) Ohwi & Ohashi], bambara groundnuts [*V. subterranea* (L.) Verdn.], mat bean [*V. aconitifolia* (Jacq.) Marechal], and rice bean [V. *umbellata* (Thunb.) Ohwi & Ohashi] are also consumed in many countries.

Mungbean is an important grain legume, particularly in Asia. It has been estimated that annual production is 2.5–3 million tons per year (Poehlman, 1991). It is a warm season crop that can grow during hot, wet seasons and be cultivated in the arid and semi-arid tropics (Kulkarni & Pandey, 1988; Pannu & Singh, 1988). Traditional indeterminate mungbean varieties have long growth duration (90 to 110 days) and required multiple harvests (suitable for home gardening, but unsuitable for commercial production due to high labor costs). The traditional varieties were susceptible to key diseases such as cercospora leaf spot, powdery mildew, and mungbean yellow mosaic virus (MYMV); insect pests such as beanfly (*Melanagromyza sojae, M. dolichostigma, and Ophiomyia centrosematis*), lima bean pod borer (*Etiella zinckenella*), and mungbean weevil (*Callosobruchus chinensis*), and did not respond to inputs (Asian

Vegetable & Development, 1976). In the semi-wild state, mungbean is of little value; AVRDC saw an opportunity to domesticate the crop and make substantial improvements. To achieve this objective, it was essential to have a broad, deep pool of genetic diversity for breeders to use.

India is the biggest producer of mungbean where about 2.99 million ha are cultivated. The major constraints for achieving higher yield are inherently low yielding potential of the varieties from lack of genetic variability, absence of suitable ideotypes for different cropping systems, poor harvest index and susceptibility to abiotic stresses (that is, drought, calcareous or saline soil) or biotic stresses (diseases and insects) (Souframanien & Gopalakrishna, 2004). Mungbean is often included in rice or wheat-based cropping systems in the tropics and subtropics. Growing mungbean after rice is best. Planting mungbean immediately after mungbean or cabbages should be avoided because toxic residues and disease organisms from the previous mungbean or cabbage crops may affect the following mungbean crop adversely.

2.2 Uses of mungbean

The crops are utilized in several ways, where grains, sprouts and young pods are consumed as sources of protein, amino acids, vitamins and minerals (Table 2.1).It is regarded as quality pulse due to its excellent digestibility and rich protein (25-28%), especially when combined with cereals (Thirumaran & Seralathan, 1988). It is an important source of readily available proteins in cereal-based diet of the people of South Asia and Southeast Asian countries. It is also consumed as boiled dry beans. Moreover, mungbean is regarded as fodder for livestock and also incorporated in soil for enriching organic matter. Mungbeans adapt well to various cropping systems owing to their ability to fix atmospheric nitrogen (N2) in symbiosis with soil bacteria of *Rhizobium*

spp. rapid growth, and early maturity.

Table 2.1: Boiled mungbean nutritional value per 100g

Energy 110 Kcal 440KJ

Carbohydrates	19.15 g
- Sugars	2.00 g
- Dietary fiber	7.6 g
Fat	0.38 g
Protein	7.02 g
Vitamin C 1.0 mg	2%
Calcium 27 mg	3%
Magnesium 0.298 mg	0%
Phosphorus 99 mg	14%
Potassium 266 mg	6%
Sodium 2 mg	0%

Source: USDA Nutrient database

Mungbeans are commonly used in Chinese cuisine, as well as in Japan, Korea, India, Thailand and Southeast Asia. They are generally eaten either whole (with or without skins) or as bean sprouts, or used to make the dessert "green bean soup". The starch of mungbeans is also separated from the ground beans to make jellies and "transparent/cellophane" noodles.

2.3 Effect of mungbean in enriching soil

Mungbean has the capacity for organic nitrogen fixation. Planting mungbean is reported to help the succeeding cereal crop. Incorporation of mungbean rests give a rice

yield raise the same as to 25 kg N manure for each hectare (Meelu & Morris, 1988). Some studies in Punjab have revealed that the extra-short period SML668 mungbean, which fits well between rice and wheat as a catch crop, leaves a residual of 33–37 kg N per ha for the succeeding crop after meeting its own requirement. Sekhon reported that this extra nitrogen gives 25% of the N requirement of the cropping system (Sekhon *et al.*, 2006).

Stable prices for cereals and subsidized inputs give slight motivation for farmers to spread the rice-wheat cropping system. Hobbs reported that exhaustive crop diversification has provided in answer to a decline in partial part productivity of nitrogen fertilizer (Hobbs & Morris, 1996). Cereal cropping is the solution to reversing decreasing crop yield (Pingali & Shah, 1999). Two hundred farmers in four areas in Punjab was survived, with several adopting variety SML668, in 2002.

2.4 Botany of mungbean

Mungbean belongs to the order Leguminosae and Papilionoideae family and is botanically recognized as *Vigna radiata* (L.)Wilczek syn. *PhaseolusradiatusL.*, *P. aureus* Roxb (Verdcourt, 1970; Wilczek, 1954). The taxonomic status of the species is given in Table 2.2. The genus *Vigna* has been broadened to include about 150 species; twenty-two species are native to India and sixteen to Southeast Asia, but the largest number of species are found in Africa (Polhill & Van der Maesen, 1985).

Table 2.2: Taxonomic status of mungbean

Kingdom	Plant kingdom
Division	Spermatophyta
Subdivision	Angiospermae
Class	Dicotyledoneae
Order	Leguminosae
Family	Papilionoideae
Tribe	Phaseoleae
Genus	*Vigna*
Subgenus	*Ceratotropis*
Species	*Radiate*
Variety	*Radiate*

Source:(H. K. Jain & Mehra, 1980; Rochie & Roberts, 1974)

2.5 Mungbean genomics

Mungbean is diploid in nature with 2n=2x=22 (Karpechenko, 1925). Mungbean has small genome size estimated to be 0.60 pg/1C (579 Mbp), which is similar to those of other *Vigna* species (Somta *et al.*, 2007; Somta & Srinives, 2007).

2.6 Morphological traits

Mungbean is an annual plant, sometimes a bit twining at the tips, Sen reported that twining growth is dominant to erect habit (Sen & Ghosh, 1959). Mungbean is 0.3 to 1.5 m tall, erect or suberect plant with deep-rooted, long petioles and much branched. The leaves are dark or light green, trifoliolate, alternate, ovate and vary from 2 to 10 cm long and 5 to 12 cm wide. Heterozygous genotypes was reported have intermediately

lobed leaflets (Sen & Ghosh, 1959). Mungbean inflorescence is with a peduncle 2 to 13 cm long and an axillary raceme. Rochie & Roberts (1974) reported that the flowers are light yellow and mungbean keel petal is spirally coiled with a horn-like. Pods are slender, short, hairy and 6 to 10 cm long. The pod color can be buff, grey or dark brown with fine hair covering. Bose (1939) reported that the genes for flower color are known to affect the unripe pod color. grains are mostly green but sometimes black, mottled, yellow or tawny brown. Variations in seed coat colors were discovered to be due to number of chloroplasts, to the color of chloroplasts and sap-soluble pigments (Sen & Ghosh, 1959). Van Rheenen (1965) recognized dominant genes conditioning green seed coat and spotted seed coat colors. Grain weight is 15 to 85 mg and shape is mostly globose. Verma (1969) reported that seed shattering at maturity is due to a single dominant gene and in mungbean photoperiod insensitivity is dominant over photoperiod sensitivity.

2.7 Genetic diversity based on morphological characters

Estimates of genetic diversity and relationships between germplasm collections are very important for facilitating efficient germplasm collection evaluation and utilization. Many tools are now available for identifying desirable variation in the germplasm including total grain protein, isozymes and various types of molecular markers. However, morphological characterization is the first step in the description and classification of germplasm (Singh & Tripathi, 1985; Smith & Smith, 1989), thoroughly discussed the importance of a hierarchical approach to quantitatively define the variance in the centre of genetic diversity over a range of micro environments. Subdividing the variance into its components may assist in genetic resources conservation and utilization by determining the relative contribution of the different

levels of variability to the total diversity available in any one area. This would enable planning of future germplasm sampling, establishment of *in-situ* gene conservation, or use of appropriate gene pools in crop improvement for specific plant attributes (Bekele, 1984; Pecetti *et al.*, 1992). Germplasm evaluation must be considered as the first step in plant breeding program and it is commonly based on a simultaneous examination of a large number of populations for several characters of both agronomic and physiological interest (Pezzotti *et al.*, 1994).

Virmani categorised mungbean germplasm in various groups for different traits (Virmani *et al.*, 1983). The genetic diversity between *V. radiata* and *V. mungo* was reported by Egawa (Egawa, 1988). Singh categorized pea germplasm into various groups (Singh & Tripathi, 1985). Ghafoor classified blackgram germplasm and selected eleven pure-lines for further exploitation (Ghafoor *et al.*, 1989). In a study on mungbean, Ghafoor selected twenty eight genotypes on the basis of high yield potential and resistance to diseases (Ghafoor *et al.*, 1992).

Falcinelli showed multivariate analyses to be a valid system to deal with germplasm collection (Falcinelli *et al.*, 1988; Veronesi & Falcinelli, 1988). Nevertheless, qualitative traits must often be used for separating varieties when a limited range of quantitative traits is found in certain groups (Sneddon, 1970). Principal component analysis (PCA) was considered a useful data reduction technique which worked by removing inter-relationship among variables. By using PCA, not only the number of comparisons between treatment means is reduced, but the meaningfulness of these comparisons is also enhanced. Interactions among two or more variables may be highlighted by such analysis. In taxonomy, it can be used to express multidimensional inter-OTU (Operational Taxonomic Unit) distances in 3 or fewer dimensions which can readily be conceptualized. Additional applications of this technique will certainly

be found as its use becomes more widespread in fields of biological sciences, where it has been used extensively for more than two decades (Broschat, 1979).

Seventy two landraces of pea (*Pisum sativum*) evaluated for 19 morphological characters exhibited broad genetic diversity. Seven landraces were selected for special attention for having promising breeding value (Amurrio *et al.*, 1992). Amurrio reported a wide genetic diversity in 105 pea landraces at the intra specific level based on 19 quantitative characters (Amurrio *et al.*, 1995). Taxonomically useful results were provided and six groups were established but the grouping pattern of these landraces did not reflect any association with geographic origin. Smith studied principal components and average cluster analyses in alfalfa and established six geographically distinct groups. Significant regional variation was observed within the germplasm evaluated but ecotypes from neighboring countries were generally closely associated. All elite germplasm accessions fel1 in one group and this revealed that only a small portion of genetic diversity has been used in formal breeding (Smith et al., 1991). Yimram evaluated 9 qualitative and 21 quantitative traits in 340 diverse cultivated mungbean accessions collected at AVRDC to assess the extent and pattern of their diversity (Yimram *et al.*, 2009). The germplasms represented a wide range of diversity for most of the traits evaluated. High genetic variability were found in yield components .Penology traits such as plant height, days to flowering, and days to maturity also showed high genetic variability. Cluster analysis grouped the germplasms into 5 major and 1 minor cluster. In general, germplasms from India and West Asia were present in all major clusters, while those from Southeast Asia and other origins were mainly grouped into one cluster. They recommended that the germplasms from West Asia be exploited more in cultivar development to enrich the breeding gene pool. Multivariate analyses have been used successfully to classify and order variation observed in both qualitative and quantitative traits in collection of crop germplasm (Caradus *et al.*, 1989;

Peeters & Martinelli, 1989; S. P. Singh, 1988). Rumbaugh used discriminant analysis of morphological and agronomic characters to place 146 accessions of alfalfa from Morocco into five geographical groupings that were defined initially based on the area of collection (Rumbaugh *et al.*, 1988).

One approach for building gene pool is to collect material from diverse geographical origins with a concentration of accessions from proposed centre of diversity. This should capture inherent and unexploited diversity in the individual samples. Representative samples from the complete geographical range of the crop species can help to ensure that co-adapted gene complexes (or correlated adaptations) are conserved (Frankel, 1984; Frankel & Soule, 1981). Brown advocated that the maximum genetic conservation would be achieved by sampling populations from as many distinct environments as possible (Brown, 1978).

Malhotra while working on genetic divergence in blackgram reported narrow range of variability for 100-grain weight and pod length (Malhotra & Singh, 1971) whereas, Shanrnugam while analyzing 45 genotypes of blackgram reported that yield per plant contributed most to the genetic diversity (Shanmugam & Rangasamy, 1982). Malik studied genetic divergence in 12 indigenous varieties of mungbean for six quantitative characters. The study indicated the presence of ample genetic variation among the cultivars irrespective of their origin. They suggested that plant height, days to flowering and grain yield should be considered for selecting genetically divergent lines in mungbean (Malik et al., 1985).

Clements investigated the pattern of morphological diversity in relation to geographical origins of 157 accessions of wild *Lupinus angustifolius* using multivariate technique. Genetic diversity was extremely large for most of the morphological traits, with significant variation detected among localities in Greece and within and

between collection sites for same trait. Thirteen groups were identified by hierarchical clusters analysis. Accessions from northern Greece grouped together as late flowering, shorter, and smaller grain size, but some accessions from southern Greek Islands were grouped with the northern mainland types (Clements & Cowling, 1994). Multivariate analyses provide a good evaluation of landraces by identifying those that should be further evaluated at the genetic level (Rouamba *et al.*, 1996).

A research team reported phylogenetic relationships of 15 genotypes of genus *Lens* and seven of their interspecific hybrids were determined by morphological (quantitative and qualitative) characters (Ahmad *et al.*, 1997). They observed that cluster analyses on the basis of quantitative characters were phenotypically more distinct and exhibited more breeding value. Though cluster analyses grouped together accessions with greater morphological similarity, the cluster did not necessarily include all the accessions/genotypes from the same or nearby sites.

The extent of diversity and relationships among *Brassica juncea* germplasm from Pakistan was determined for 35 morphological characters in 52 accessions using cluster and principal component analyses (Rabbani *et al.*, 1998). The germplasm was categorized into six groups. Landrace group was primarily associated with morphological differences among the accessions and secondarily with the breeding objectives and horticultural uses. The germplasm showed a comparatively low level of phenotypic variation which revealed that the evaluated germplasm appears to have a narrow genetic base and undergoes a high level of genetic erosion. Though cluster analyses grouped together accessions with greater morphological similarity, the clusters did not necessarily include all the accessions from the same or nearby sites.

Upadhyaya et al. (2002) studied phenotypic diversity for morphological and agronomic characteristics in 1956 accessions of chickpea core collection, comprising

desi, kabuli and intermediate types. The kabuli and intermediate types were not significantly different for growth habit and grain color, while they differed significantly from desi types for both traits. Principal component analysis showed that days to 50% flowering, flowering duration, apical secondary branches, tertiary branches,100-grain weight, grain color and grain testa texture were important traits in explaining multivariate polymorphism (Upadhyaya *et al.*, 2002).

33 mungbean genotypes derived from ten crosses was analyzed to determine genetic diversity using multivariate analysis (Manivannan, 2002). The genotypes were grouped into seven clusters. Among the characters studied, 100-grain weight and powdery mildew reaction contributed the most towards the total divergence. Based on the performance, they suggested seven genotypes to be included in the hybridization program.

Thirty seven diverse genotypes of blackgram and three of mungbean resembling blackgram, were studied to determine the extent of genetic variation based on morphological characters (Ghafoor *et al.*, 2002). High variance was observed for plant height, days to maturity, branches per plant, pods per plant, pod length, grains per pod, biological yield per plant, grain yield per plant and harvest index (%). The first four components of PCA with eigenvalue >1 contributed 78.7% and 79.1% of the total variance amongst 40 genotypes during two consecutive years.

Twenty two blackgram genotypes representing a broad based germplasm were analyzed using multivariate analyses for two consecutive years (Ghafoor *et al.*, 2003). High genetic variance was observed for plant height, maturity, pods, grain weight, biomass, grain yield and harvest index. The first four PCs contributed 80.0% of the variation during 1998, and 80.9% during 1999. Five yield contributing traits, i.e. branches, pods, pod length, biomass and grain yield were observed to be important for

first component during both the years. PC2 was more related to maturity rather than reproductive traits. First two PCs which exhibited about 60% of the variance were plotted to observe the relationship between the cultivars. Five genotypes were separated from others during both the years.

2.8 Mungbean yield and yield influencing characters

One of main parts of breeding studies is related to the relationships between yield and its components for increasing of yield. Study of direct and indirect effects of yield components can provide the basis for its successful breeding program and hence the problem of yield increase can be more effectively tackled on the basis of performance of yield components and selection for closely related characters (Aycicek & Yildirim, 2006), so selection for high yield genotypes can be done through yield components. Correlation analyses provide a good measure of the association between characters and facilitate identification of important characters for effective selection for increasing yield. The important yield components in mungbean are: harvested plant number per unit area, number of pods per plant, grains per pod, and weight of grain (Kuo, 1998), that many research projects have been done to study the relationships among important characteristic affecting grain yield in mungbean (Ghafoor *et al.*, 1990; Khattak *et al.*, 2001; V. V. Malhotra *et al.*, 1974; Yucel, 2004).

In two separate studies positive association of pods per plant and grains per pod with grain yield in mungbean genotypes of diverse origin were observed (Ajmal & Hassan, 2002; Aslam *et al.*, 2002). In mungbean, positive correlation of yield with yield components was observed (Tomar et al., 1973; Khalid et al., 1984), whereas, Malik reported negative correlation of yield with maturity, pod length and grain weight. He also investigated maximum relative selection efficiency for branches per plant in

mungbean (Malik et al., 1983; Malik et al., 1987). In previous studies positive association of yield with days to maturity, plant height, pods and pod length, whereas negative association with grain weight were observed (Malhotra et al., 1974). Many researchers in different studies gave emphasis for the selection of legume genotypes on the basis of high harvest index (Singh & Patel, 1977; Patel & Shah, 1982; Malik et al., 1986; Khan & Malik, 1989; Ghafoor et al., 1993). Positive correlation among yield and its components has been reported in blackgram (Rani & Rao, 1981).

Cluster analysis was considered for nine quantitative traits in mungbean (Ghafoor *et al.*, 2000). They observed significant negative correlation of days to maturity with all the characters except branches per plant and suggested that short to medium maturity mungbean cultivars were to be selected for high yield. They identified 44 pure-lines on the basis of important agronomic traits that were recommended for testing under a wide range of agro-ecological condition in pursuit of best mungbean cultivars.

2.9 Genetic diversity based on molecular markers

Recently, molecular tools have also been applied for mungbean and blackgram breeding programs, for instance, the use of isozymes and DNA markers for variety identification, assessment of genetic diversity and relatedness among *Vigna* species subgenus *Ceratotropis* and mapping of disease resistance genes (Chaitieng *et al.*, 2002; Prammanee *et al.*, 2000; Seehalak *et al.*, 2006). Knowledge of genetic diversity of the genetic resources is crucial for breeders to better understand the evolutionary and genetic relationships among accessions, to select germplasm in a more systemic and effective fashion, and to develop strategies to incorporate useful diversity in their breeding programs (Z. Li & Nelson, 2001; Paterson *et al.*, 1991). These genetic

diversity measures can be used to maximize the level of variation present in segregating populations by crossing genotypes with greater genetic distance. Evaluation of morphological traits, pedigrees, geographic origins, isozymes and DNA markers have been used for the assessments of *Vigna spp*. genetic diversity (Bisht *et al.*, 2005; Ehlers & Hall, 1997; Fatokun *et al.*, 1993; Kaga *et al.*, 1996; Tosti & Negri, 2002).

One of the molecular markers that has become popular more recently for major crop plants, is known as simple sequence repeats (SSR) or microsatellite markers and this marker system is predicted to lead to even more rapid advances in marker development and implementation in breeding programs due to high abundance and its high polymorphism of microsatellites in plant genome (Korzun, 2002).

Microsatellites or short sequence repeats (SSRs) are relatively small, that is about 1-6 base-pair (bp) tandem repeats that are found in the genomic DNA of prokaryotes and eukaryotes (Kupper *et al.*, 2008). Microsatellites could be located either in non-coding sequences or located in functional regions. The majority of microsatellites is located in non-coding sequences (Metzgar *et al.*, 2000), whereas the minority are located in functional regions. Microsatellites located within noncoding region are considered to be selectively neutral, whereas microsatellites located in functional regions are involved in chromatin organization, regulation of gene activity and metabolic processes such as DNA replication and recombination (Li et al., 2002). The importance of microsatellites in genetic studies has been greatly acknowledged over the years (Chambers & MacAvoy, 2000). This is due to microsatellite markers being a co dominant marker system which is more informative than dominant markers such as Random Amplified Polymorphic (RAPD), Amplified Fragment Length Polymorphisms (AFLP) and Direct Amplification of Length Polymorphisms (DALP). Another added advantage of microsatellite markers is that they amplify regions of

repetitive elements with simple repeat motifs of one to six nucleotides which show high levels of allelic variations in the number of repeat units. All these make microsatellites a popular and effective marker system that is useful for various genetic studies such as population, linkage and phylogenetic studies and also for quantitative traits loci (QTL) studies. However the conventional method used for microsatellite detection is far from being cost effective as it is a laborious and time-consuming process. In recent years, researchers around the world especially in developing countries have developed different methods for detecting microsatellites. The main objectives were targeted on saving cost and time, increasing effectiveness and productivity.

Microsatellite markers are useful and popular for population genetics studies and conservation management of biological resources. Although microsatellite markers for mungbean have been developed, all of them are genomic microsatellites (Kumar et al., 2002a,2002b; Gwag et al., 2006; Somta et al., 2008; Seehalak et al., 2009). For the first time Somta et al. (2009) reported the development of 33 polymorphic genic microsatellite markers for mungbean by database mining, and their cross-species amplification in 19 Asian *Vigna* species. The genic microsatellites will be useful for studying genetic diversity and population structure, which will contribute to the understanding and conservation of the Asian *Vigna.*

Although SSRs are highly polymorphic, the labor intensive and high cost associated with SSR cloning and their single-locus nature still limit their uses for genetic diversity study in several plant species. In *Vigna spp*. SSRs have been cloned from *Vigna unguiculata* and *Vigna angularis* (Li & Nelson, 2001; Wang et al., 2004). No report has been found for SSR cloning in blackgram and only a few SSRs have been cloned in mungbean, therefore, the development and usage of SSR markers in these species are rather limited (Li & Nelson, 2001; Chaitieng et al., 2006; Miyagi et al.,

2004).

Due to the limitation of SSRs, a new method called inter-simple sequence repeat (ISSR) analysis was developed based on DNA amplification with a single 15 to 20-bp primer homologous to a microsatellite repeat and has a short (1 - 4 bp) random degenerated sequence (an anchor) at the 3' or 5' end. ISSR primers allow DNA amplification of regions located between two closely spaced, oppositely oriented SSRs, yielding a reproducible pattern of genomic fragments, which is similar to a RAPD pattern but usually includes more bands and is more reproducible. Therefore, ISSRs have a high capacity to reveal polymorphism and offer great potential to determine genetic diversity at inter and intra-specific levels as compared to other arbitrary primers including RAPDs, particularly in many cultivated species exhibiting relatively low genetic diversity (Ajibade *et al.*, 2000; Pradeep Reddy *et al.*, 2002; Souframanien & Gopalakrishna, 2004; Zietkiewicz *et al.*, 1994). Previous reports have showen that ISSRs are useful for the intra-specific or inter-specific classification of genetic diversity and identification of varieties in various crops including tomato, potato, rice, grapevine and soybean (Blair *et al.*, 1999; Kochieva *et al.*, 2002; Moreno *et al.*, 1998; Prevost & Wilkinson, 1999; G. Wang *et al.*, 1998). In blackgram were identified a set of ISSR primers with high polymorphism information content (PIC) scores which would be useful in surveying genetic diversity among accessions of blackgram and perhaps other *Vigna spp* (Souframanien & Gopalakrishna, 2004).

2.10 Pest and disease and pre-harvest sprouting

Mungbean growth and production is affected by several pathogens. These pathogens consist of nematodes, bacteria, fungi and viruses. There are some diseases which could directly affect mungbean, for example, powdery mildew, mungbean yellow

mosaic virus, cercospora leaf spot, bacterial leaf spots and bruchid. Resistance to bacterial leaf spot and cercospora leaf spot, are conditioned by single dominant genes (Singh, 1977; Thakur et al., 1978), also resistance to the mungbean yellow mosaic virus (MYMV) is governed by a dominant gene and complementary recessive genes (Poehlman, 1991). Studies by Young have indicated that resistance to powdery mildew, is controlled by quantitative genes and confirmed that resistance to bruchid is governed by a single dominant gene (Young et al., 1992; Young et al., 1993). It is seen that high degree of resistance is not available in germplasm lines (Kaur, 2006). Several studies summarize performance of accessions for resistance. However a few researches have reported on the inheritance of resistance.

Mungbean is an important rainy season pulse crop of India. The average productivity of this crop is low and uncertain due to neglected management and poor adoption of the production technology due to the risk of preharvest sprouting. Preharvest sprouting (PHS) is the premature germination of mungbean grains or in other words starting of embryo growth while still attached to the mother plant in the field. In preharvest sprouting prone mungbean, once mungbean grain reaches harvest maturity, it begins to germinate if it is exposed to adequate moisture and suitable temperature. Therefore, preharvest sprouting depends on duration and severity of moist condition prior to harvest, temperature. During such wet weather, growth stage of ripening grain and the inherent dormancy level attributable to a variety's genetics. Mungbean genes interact with environment to predispose a variety to prehavest sprouting. Therefore depending on the environment and weather conditions to which the plants are exposed.

Sometimes losses due to preharvest sprouting will be as high as 60-70%. Preharvest sprouting negatively affects the grain quality by loosing the grain weight, viability, seedling vigor. High yielding varieties developed/identified in recent years,

despite their high yield potential, could not increase/stabilize the yields of this crop due to lack of resistance to preharvest sprouting. Therefore it is essential to develop resistant or tolerant varieties to preharvest sprouting by understanding the mechanism/genetics of resistance. Information on the genetics of preharvest sprouting and the traits responsible for preharvest sprouting are not available. Genetic analysis indicated the predominance of additive gene action for pod beak length, pod wall thickness and pod wall epicuticular wax, while hard grain percent and preharvest sprouting were under the control of non-additive gene action. Both additive and non-additive gene actions were found to operate for moisture absorption rate through the pod wall (Cheralu *et al.*, 1999).

CHAPTER 3

Methodology

3.1 Materials and methods

The research project comprising of two experiments was conducted under field and laboratory conditions. The materials and methods of each experiment are given separately for each experiment. A summary of the experiments is given (Table 3.1).

Table 3.1: Summary of the experiments

Experiment	Number of accessions	Number of sample for each accession	Experimental condition
To determine genetic diversity based on morphological characters	20	10	Field
To evaluate the differences of genetic makeup between mungbean genotypes (3 best and 3 lowest yield performing genotypes)	6	20	Laboratory

3.2 Genetic diversity based on morphological characters

3.2.1 Germplasm collection

Mungbean originally comes from Southeast Asia and India. It later moved out to the rest of Asia, Africa and America. Pakistan is one of the main origin of mungbean germplasm, the area under cultivation of the bean in Pakistan is 225.4 hectares and the total annual production is about 130 tons. The local germplasm/land-races are valuable source for agricultural prosperity due to high adaptability, good in quality and resistance to biotic and abiotic stresses. Initially 69 mungbean germplasms (Table 3.2) were collected from different agro-ecological zone in Pakistan. After primary screening at a research field in University of Malaya (UM), based on the yield performance 20 mungbean germplasms were selected for further investigation. In the present study these 20 germplasms were used to determine genetic diversity of mungbean genotypes and evaluate the difference of genetic makeup between mungbean genotypes.

Table 3.2: Sixty nine mungbean genotypes and their origin

Genotype	Origin
NM-92	Pak NIAB Mung
Chakwal Mung-97	Pak
NM-06	Pak NIAB Mung
NM-98	Pak NIAB Mung
VC1560D x NM 92	Pak (Parental Taiwan)
6601	Pak
AEM-96	Pak (NIFA)
Ramzan	Pak (NIFA)
NM-1919	Pak
N-F-M-14-6	Pak (NIFA)
Pak-22	Pak
Karak Mung-1	Pak
NM-93	Pak NIAB Mung
Chakwal	Pak
N-F-M-12-7	Pak (NIFA)
NM-51	Pak NIAB Mung
N-F-M-8-1	Pak (NIFA)
VC1971 x NM-92	Pak (NIFA), (Parental Taiwan)
NM-28	Pak NIAB Mung
AZRI-06	Arid Zone Research Institute, Quetta
M-1	IABGR, NARC, Collection
VC-20-10	IABGR, NARC, Collection
40426	IABGR, NARC, Collection
M-6	IABGR, NARC, Collection
SML-267	IABGR, NARC, Collection
40995	IABGR, NARC, Collection
NCM252-7	IABGR, NARC, Collection
40618	IABGR, NARC, Collection
41006	IABGR, NARC, Collection
40521	IABGR, NARC, Collection
41046	IABGR, NARC, Collection
40217	IABGR, NARC, Collection
C1-94-4-19	IABGR, NARC, Collection
PDM-11	IABGR, NARC, Collection
40998	IABGR, NARC, Collection

Table 3.2, continued'

Genotype	Origin
41003	**IABGR, NARC, Collection**
41038	**IABGR, NARC, Collection**
40999	**IABGR, NARC, Collection**
NM 20-11	**IABGR, NARC, Collection**
40536	**IABGR, NARC, Collection**
40432	**IABGR, NARC, Collection**
40457	**IABGR, NARC, Collection**
41031	**IABGR, NARC, Collection**
40222	**IABGR, NARC, Collection**
40593	**IABGR, NARC, Collection**
40714	**IABGR, NARC, Collection**
41018	**IABGR, NARC, Collection**
40434	**IABGR, NARC, Collection**
40934	**IABGR, NARC, Collection**
41009	**IABGR, NARC, Collection**
41000	**IABGR, NARC, Collection**
40504	**IABGR, NARC, Collection**
40591	**IABGR, NARC, Collection**
41013	**IABGR, NARC, Collection**
5197A	**IABGR, NARC, Collection**
NM-15-11	**IABGR, NARC, Collection**
NCM 252-5	**IABGR, NARC, Collection**
SWAT MUNG-1	**IABGR, NARC, Collection**
NM 6173 (36-13-13)	**IABGR, NARC, Collection**
BASANTHI	**IABGR, NARC, Collection**
VC 3960A88	**IABGR, NARC, Collection**
NM 45-10	**IABGR, NARC, Collection**
NCM 254-1	**IABGR, NARC, Collection**
NM 38-20-3	**IABGR, NARC, Collection**
NCM 225-2	**IABGR, NARC, Collection**
NCM 255-4	**IABGR, NARC, Collection**
NCM 253-1	**IABGR, NARC, Collection**
BARI-M-2	**IABGR, NARC, Collection**
NM 9800	**IABGR, NARC, Collection**

3.2.2 Experimental material

Twenty mungbean germplasm accessions/genotypes (Table 3.3) were evaluated for various agronomical traits. The experiment was laid out on Randomized Complete Block Design (RCBD) with two replications at a research field at the Institute of Biological Science University of Malaya (UM), Malaysia. The experiment was started on 28 April, 2010 for morphological characterization and agronomic evaluation. grains were planted in rows with 1 m in length. Distances between rows (genotypes) and between plants were 50 cm and 10 cm respectively. Basal fertilizer dose of N P (@ 25 kg N + 60 kg P2O5 per hectare) was applied, and during the crop growth period, agronomic practices were used as recommended for mungbean crop. Pesticide (Karate 2.5EC @ 750 ml/ha) was sprayed to save the crop from infestation of pests especially white fly, a vector for MYMV. For plant and agronomic characters, data were recorded following descriptors for *Vigna* spp. (IBPGR, 1985). The data regarding days to maturity were recorded when about 90% pods turned brown/black after planting. Quantitative data including plant height, number of fruiting branches per plant, number of pod per plant, number of pod clusters per plant, pod length, number of grains per pod, 1000 grain weight and total grain yield per plant were recorded on ten guarded plants selected randomly and then averaged to per plant basis. Pod length (cm) and number of grains were recorded on ten pods selected at random within each accession and then averaged to per plant basis. The grain weight for each accession/genotype was recorded after counting 1000 grains and weighed in grams.

Table 3.3: Twenty selected mungbean genotypes and their origin

Genotype	Origin
NM-92	Pak NIAB Mung
Chakwal Mung-97	Pak
NM-98	Pak NIAB Mung
VC1560DxNM-92	Pak(parental Taiwan)
Pak-22	Pak
6601	Pak
NM-1919	Pak
AZRI-06	Arid Zone Research Institute, Quetta
M-6	IABGR,NARC Collection
SML-267	IABGR,NARC Collection
40995	IABGR,NARC Collection
40521	IABGR,NARC Collection
40998	IABGR,NARC Collection
41031	IABGR,NARC Collection
40593	IABGR,NARC Collection
40714	IABGR,NARC Collection
41018	IABGR,NARC Collection
40934	IABGR,NARC Collection
5197A	IABGR,NARC Collection
NM 45-10	IABGR,NARC Collection

3.2.3 Statistical analysis

The data recorded were averaged and analyzed for simple statistics (mean, standard deviation, variance), frequency distribution and phenotypic correlation coefficients following the methods of Steel & Torrie (1980). Eight quantitative traits (plant height, number of fruiting branches per plant, number of pod per plant, number of pod clusters per plant, pod length, number of grains per pod, 1000 grain weight and total grain yield per plant), were also analyzed by numerical taxonomic techniques following the procedure of Principal Component (PC) Analyses (Sneath & Sokal, 1973).

Analysis of variance based on the RCBD was done to show the differences among genotypes for all characteristics. Before doing ANOVA, normality test (Kolomogorove –Smirnove) was applied. All data showed a normal distribution so ANOVA was done using SAS9.1. Means were compared by DMRT (Duncan multiple range test). The multivariate analysis, especially the principal component and cluster analysis was done for the study of germplasm based on various traits (Cruz & Regazzi, 1994; Mardia *et al.*, 1979). Cluster analysis using UPGMA (between group linkages) was used to investigate distance, similarity and relatedness of genotypes or populations, so that similar genotypes can be classified into one group and dissimilar ones into distinct groups. Principal component analysis (PCA) was done to understand variable independence and balanced weighting of traits, which leads to an effective contribution of different characters on the basis of respective variation. PCA utilized to derive a 2 dimensional scatter plot of individuals, such that the geometrical distances among individuals in the plot reflect the genetic distances among them with minimal distortion.

In order to maintain, evaluate and utilize germplasm effectively, it is important

to investigate the extent of genetic diversity available. Smith considered morphological characterization as an important step in description and classification of crop germplasm because a breeding program mainly depends upon the magnitude of genetic variability (Smith & Smith, 1989; Smith et al., 1991).

The data analyzed based on RCBD (Randomized Complete Block Design), was used to partition the gross (phenotypic) variability into the components due to genetic and non-genetic factors and to estimate the magnitude of these. Variance components (genotypic, phenotypic and error variance) were estimated using the formula as follows: (Prasad *et al.*, 1981; Wricke & Weber, 1986).

$$V_g = \frac{MSG - MSE}{r}$$

$$V_p = \left[MSG + MSE\right]$$

$$V_e = MSE$$

MSG, MSE and r are the mean squares of genotypes, mean squares of error and number of replications, respectively. Phenotypic (PCV) and genotypic (GCV) coefficient of variation were computed according to following:

$$PCV = \left[\frac{\sqrt{V_p}}{\overline{X}}\right] \times 100$$

$$GCV = \left[\frac{\sqrt{V_g}}{\overline{X}}\right] \times 100$$

Where Vp, Vg and X are the phenotypic variances, genotypic variances and grand mean, Broad sense heritability (Hbs) is the ratio of the genotypic variance (Vg) to

the phenotypic variance (Vp) as percent and it was estimated on genotypic mean basis (Allard, 1999). Genetic advance (GA) expected and GA as percent of the mean assuming selection of the superior 5% of the genotypes were estimated in accordance with the methods in the following:

$$GA = K(S_P) \cdot Hbs$$

GA (as % of the mean) = (GA / x) x 100

(Fehr *et al.*, 1987)

Where k is a constant (which varies depending upon the selection intensity and, if the latter is 5%, it stands at 2.06). Sp is the phenotypic standard deviation (√vp), Hbs is the heritability ratio and x refers to the season mean of the character (Y. Ali *et al.*, 2008; Sunday *et al.*).

Broad sense heritability (Hbs) can be estimated based on the procedure described by Poehlman (1994), as follows:

$$Hbs = \frac{\sigma_g^2}{\sigma_{ph}^2} \times \frac{100}{1}$$

Genotypic correlation was calculated using the following equation for all traits as follows:

$$r_G = \frac{\sigma_{G(X,Y)}}{\sqrt{\sigma_{G(X)}^2 \cdot \sigma_{G(Y)}^2}}$$

(Becker, 1992).

Where r_G is genetic correlation between the traits X and Y, $\sigma_{G(X,Y)}$ is genotypic covariance between the traits X and Y, $\sigma^2_{G(X)}$ is genotypic variance of the trait X, and $\sigma^2_{G(Y)}$ is genotypic variance of the trait Y.

3.3 Genetic diversity base on molecular markers

3.4 Experimental material

After morphological screening for 20 mungbean accessions, six mungbean germplasm were chosen (Table 3.4). Three genotypes with the highest grain yield and three genotypes with the lowest grain yield were selected based on grain yield diagram from Duncan`s Multiple Range Test (DMRT). Then these six selected mungbean genotypes were planted in different pots. After 2-3 weeks plant leaves were collected, avoiding the hard parts of leaves such as petiole. Young leaves (about 1cm long) of each plant were plucked and placed on aluminum foil. Then, the aluminum foil was labeled and stored in ice for temporary preservation. After harvesting, leaves were wiped with distilled water to remove the dirt on the surface of the leaves. Finally, samples in labeled aluminum foil were transferred into liquid nitrogen as soon as possible. The samples were then stored in -80˚C freezer. To accumulate sufficient samples for DNA extraction (ie, 1.5 gram), sampling for 2 to 3 times was needed.

Table 3.4: Six selected mungbean genotypes and their origin

Genotype	Origin
NM-1919	Pak
40995	IABGR,NARC Collection
40521	IABGR,NARC Collection
41031	IABGR,NARC Collection
40593	IABGR,NARC Collection
40714	IABGR,NARC Collection

3.4.1 DNA extraction

Five samples were extracted for each round of DNA extraction. Firstly, five 2ml microcentrifuge tubes were labeled. Then, 5µl of β-mercaptoethanol and 500µl of CTAB solution were added into each tube and pre-heated at 65˚C the in water bath.

The samples were taken out from the -80˚C freezer and stored in liquid nitrogen. Five set of clean pestles and mortar were prepared. Next, liquid nitrogen was poured into mortar until approximately could cover the leaves sample. Then the sample was taken out from the liquid nitrogen and transferred into the mortar. Leaves must be ground thoroughly before thawed in room temperature using pestle until it became powdery. After grinding, a small size spatula (which was immersed in the liquid nitrogen for a few seconds before use) was used to scrap all the leaves powder in mortar and transferred into the pre-heated CTAB buffer solution and mixed well. The same procedure was carried out for the rest of leaves samples.

Then, the samples were incubated at 65˚C in water bath for 30 minutes. Tubes were shaken for every 5 minutes interval. After 30 minutes, the tubes were taken out

from the water bath and allowed to cool to room temperature. Then, 500ml of chloroform: isoamyl (in 24:1 ratio) was added into each tube and mixed gently for 15 minutes. Next, the mixtures were centrifuged at 503 × g at 4˚C for 10 minutes. After centrifugation, 3 layers appeared in each tube, only the top phase was transferred to a new labeled 2ml microcentrifuge tube.

Two thirds of the total volume of the top phase of cold (-20˚C) isopropanol was added into the tubes and mixed gently to precipitate DNA. Then, the tubes were left in a -20˚C freezer overnight. The next day, the tubes were centrifuged at 503 × g at 4˚C for 10 minutes. After centrifugation, the DNA pellet precipitated at the bottom of the tubes, the supernatant was discarded carefully. Then, 1.5ml of wash buffer was added into the tubes and centrifuged again at 8050 × g at 4˚C for 10 minutes.

After centrifugation, supernatant was discarded. Micropipette was used to withdraw the remaining supernatant without disturbing the pellet. The tubes were inverted on a piece of tissue paper to allow the pellets to dry. Finally, after the pellet was dried, 500µl of TE buffer was added to each tube to resuspend the pellet. To ensure the pellets was completely dissolved, the samples was incubated at 55˚C to aid resuspension.

3.4.2 Evaluation of DNA quality and quantity

DNA is quantified by using nanodrop (NANODROP Spectrophotometer, ND-2000). Concentration range then can be measured by ND-2000 is from 2ng/µl to 15,000 ng/µl dsDNA without dilution. Sample volumes used for measurement is only 1µℓ. Nucleotides, RNA, ssDNA, and dsDNA will all absorb at 260nm and contribute to the total absorbance. The ratio of absorbance at 260nm and 280nm is used to assess the

purity of DNA and RNA. A ratio of around 1.8 is generally accepted as ‘pure’for DNA. The ratio of absorbance at 260/230 is used as a secondary measure of nucleic acid purity. The 260/230 values for ‘pure’ nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2. If the value is appreciable lower than expected, it may indicate the presence of contaminants which absorb at 230nm.

DNA quality is also evaluated by running the DNA on gel electrophoresis with 0.8% agarose gel. Three microlitre of DNA samples is mixed with 3µl of 6X loading buffer on a parafilm paper and then loaded in the well of Agarose gel. The electrophoresis is operated at 70 V, 150 mA, 40 minutes in 1X TBE buffer and Hind III ladder is used.

3.4.3 Primer design

Bioinformatics analysis and primer design were done for the microsatellites in the DNA sequence. Mungbean data previously obtained from transcriptome sequencing were screened for microsatellites (or short tandem repeats (SSR)) using the IQDD program (http://primer3.sourceforge.net/, (Meglecz *et al.*, 2009). The analysis implemented in IQDD involves three successive stages: sequence cleaning and detection of microsatellites, sequence similarity detection, and microsatellite selection and primer design (Meglecz *et al.*, 2009).

In this study, only perfect microsatellites were targeted, and identification of microsatellites was limited to the detection of strings of repeats sequences that contained a minimum of four motif repeats for all di-, tri-, tetra-, penta-, and hexanucleotide motifs, primer selection conditions are as shown in (Table 3.5).

Table 3.5: Primer selection condition

Value Condition	Min	Optimum	Max
Primer Size (bp)	16	20	24
Primer Tm (˚C)	45	55	65
Primer GC%	40	50	60

3.4.4 Validation of microsatellite loci

Primers were synthesized for 22 microsatellite loci and then initial validation was performed to confirm that the microsatellite regions could be amplified from genomic DNA. Validation included optimization of annealing temperature via temperature gradient PCRs; and amplificability of targeted products. This step was carried out using several individuals selected from each studied population. Successful PCR amplification was determined by agarose gel electrophoresis, and primers with no significant amplification (i.e., visual product of expected size) were then discarded from further data collection.

3.4.5 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) amplification for each primer set was performed in a C1000 Thermal Cycler (Bio-Rad) in a total volume of 10µl reaction solution consisting of 1.4µl of DNA extracted from tissues, 0.9 µl $MgCl_2$ (25 mM), 3.0µl of 1X PCR Buffer (Promega), 0.2µl of each dNTPs (10mM), 0.3µl of *Taq* Polymerase, and 0.5µl of each primer (10mM). The PCR reactions were carried out as follows: initial denaturation at 95 C for 5min, 35 cycles of denaturation at 94°C for 30s, annealing temperature for 30s, and 1 min of extension step at 72°C. The program was

then completed with a final extension at 72°C for 10 min. Initial PCR reactions were performed across an annealing temperature gradient (50-60°C) to determine the best annealing temperature for each primer pair, with subsequent PCR reactions conducted at this optimal temperatures (Table 3.6). Magnesium chloride optimization was also carried out to determine the optimum Magnesium chloride concentration of the microsatellite primer to anneal to the DNA template. Following amplification, the presence of PCR products were verified via electrophoresis. 1.0% agarose gel was used in electrophoresis of PCR products for optimization, whilst 4.0% Metaphor® agarose gel was used in electrophoresis of microsatellite PCR products in polymorphism screening. The gel electrophoresis for agarose and Metaphor® were carried out at 70-75V, 150mA using 1 x TBE Running Buffer for 45 min and 1.5-2 hours respectively. The gels were stained with ethidium bromide (10mg/ml) before being visualized under ultraviolet light (Alpha Imager Gel Documentation System, Siber Hegner, Germany). The primers (Table 3.6) were selected from Kumar published primers (Kumar et al., 2002a, 2002b).

Table 3.6: List of primers with optimized annealing temperature (C)

Locus	Primer sequences 5'-3' (Forward and reverse)	Annealing temperature(C)	Expected product size(bp)	Repeat motif
VJ31122A	TGGTTGGTTGGTTCACAAGA CACGGGTTCTGTCTCCAATA	57.7	205-220	$(TGGT)_3$
VJ31122B	TCACAAAGGGAGGGAAGAGA CCCCAGGTTGGTTGGTTGGA	52.6	209-220	$(CCAA)_3$
LR7319B	CTGCTTTTTGGGGATTTCAG CACGCAAACAGAAAGCAGAG	54.1	257	$(TG)_5…(CT)_7$
LR7322B	TCAGTCAGTGTCGATAGCATAGC GACACAGAGAGAGAGAGAGAG	60.0	171	$(TC)_{10}$
LR7323A	TGACGGAGAGAGAGAGAGAGAG TGCTTCCTTTTGTCTGAGTTAGAA	59.6	201	$(GA)_{13}$
LR7323B	GCTATGCTATCGACACTGACTGA GCGCAAAGAGAGAGAGAGAGA	60.0	285	$(CT)_{10}$

3.4.6 Fragment analysis

Mungbean primers that exhibited potential polymorphism as identified from initial screening (Section 3.4.4) were used to screen 20 samples randomly selected from among 6 sampled populations for size polymorphism. One primer in each set was labeled with FAM fluorophore, PCR reactions were performed following the protocol detailed above (Section 3.4.5), and product was run on an ABI PRISM ® 3130xl Genetic Analyzer (Applied Biosystem, USA).

In preparation for fragment analysis labeled PCR products were diluted 1:9 or 1:19 in ddH2O depending on the amount of amplicon present as revealed by gel electrophoresis, and then 1 µl of the diluted mixture was transferred into a new PCR tube and 10 µl of Hidi Formamide loading dye (Analisa Resources (M) Sdn. Bhd.) and 0.2 µl of Genescan 500 LIZ ladder were added into the tube. The mixture was thoroughly mixed by brief vortexing, followed by a brief centrifugation step. Next, the tubes were heated for 5min at 95°C to denature double stranded PCR product, and then kept in ice for exactly 5min, before all samples were transferred into 96-well plate and subjected to fragment analysis using ABI 3130 Genetic Analyzer.

Results of the fragment analysis were interpreted, evaluated and allele sizes were scored using the software packages GeneMapper 4.0 (Applied Biosystems, Foster City, CA, USA) and Peak Scanner v1.0 (Applied Biosystem, USA). Genotyping of each individual at each locus was accomplished by scoring peaks in electropherogram which represent exact alleles sizes in base pairs (bp) of amplified loci.

3.4.7 Data analysis

Data analysis was undertaken to examine levels of diversity at each locus, and also to determine the extent of population genetic structure present among sampled populations. Prior to data analysis, all raw genotypic data obtained from GeneMapper 4.0 (Applied Biosystems, Foster City, CA, USA) software was collated in Microsoft Excel, and then the data files for specific population genetics software were generated using the program CONVERT software version 1.31 (Glaubitz, 2004).

3.4.8 Identification And Checking For Scoring Errors

The data set was checked for any genotyping errors that could potentially bias population genetic analysis. These genotyping errors include incorrectly scoring individuals as homozygotes because mutations in the priming site result in non-amplification of specific alleles (null alleles), or because PCR may preferentially amplify shorter alleles in heterozygote individuals (short allele dominance / large allele dropout). Other errors include mis-scoring stutter peaks as true alleles, resulting in an artificial excess of heterozygote genotypes with only one motif repeat difference between alleles. Data was checked for errors using Micro-Checker software (van Oosterhout *et al.*, 2004). Where evidence was found for the presence of null alleles the frequency of null alleles was estimated (Brookfield, 1996; Chakraborty *et al.*, 1992), using Micro-checker, and the allele and genotype frequencies of the amplified alleles were recalculated and corrected based on new equation to account for the downward bias resulting from the null alleles, thus permitting their use for further population genetic analysis (van Oosterhout *et al.*, 2004).

3.4.9 Tests For Conformation To Equilibrium Expectations

Conformation to Hardy-Weinberg Equilibrium (HWE) was investigated to find out if the samples constitute collections of randomly mating individuals and/or to see if the EST microsatellites showed evidence for non-neutral evolution (i.e., selection). The principle of HWE states that both allelic and genotypic frequencies in a population will not change over generations in the absence of disturbing factors (selection, genetic drift, gene flow, and mutation) with the condition those individuals in the population exhibit random mating. Statistical test for HWE aims to determine whether the observed data for each particular locus "sufficiently fits" the expected assumption i.e., the population is in the expected HWE proportions (Hedrick, 2000). Hardy-Weinberg (HWE) Principles can be represented by binomial (with two alleles) or multinomial (with multiple alleles) functions of allelic frequencies.

HWE tests were performed based on Wright model (1951) with the incorporation of more sophisticated model as implemented in Arlequin (Excoffier *et al.*, 2005), with significance recalculated following the False Discover Rate procedure (Benjamini & Hochberg, 1995). When any evidence of deviations from HWE was found, subsequent analyses were performed including and excluding data from the deviating locus. Number of steps in Markov chain were set as 100000 and number of dememorisation Steps were set as 1000.

Linkage disequilibrium analysis was performed to check for the presence of any non-random associations of alleles among different loci. Associations between polymorphisms at different loci are measured by the degree of linkage disequilibrium (D). D was first proposed by Lewontin (Lewontin & Kojima, 1960). Numerically, it is the difference between observed and expected allelic frequencies (assuming random distributions). If the alleles at a pair of loci are not randomly associated with one

another, then there will be a deviation (D) in the expected frequencies, in which case the loci are said to be in linkage disequilibrium (i.e., linked).

Pairs of loci may deviate from linkage equilibrium (i.e be in linkage disequilibrium, LD), due to physical or demographic reasons. For example, LD may indicate that the observed loci might be physically linked to each other by occurring in close proximity on the same chromosome. In this case, recombination is unlikely to lead to independent assortment during meiosis, and alleles at different loci that are closely linked physically will tend to be inherited together. In contrast, LD may also be observed when two divergent populations are sampled together, as divergent alleles at multiple loci in each population are likely to occur together in single individuals, while divergent alleles drawn from different populations are unlikely to occur together. Thus, the occurrence of LD can provide information about the loci or about the populations sampled. Linkage analysis was performed in GENEPOP software (Markov chain parameters set as follows: dememorisation-10000; batches-20; iterations per batch-5000).

3.4.10 Estimating Genetic Diversity

To investigate the level of genetic diversity present at each locus with each population sample, several measures of genetic variation were calculated. Allelic frequencies, observed number of alleles (A_t), effective number of alleles (A_e), observed and expected heterozygosity (H_O, H_E) were obtained using software POPGENE version 1.32 (Yeh *et al.*, 1997), and allelic richness was calculated using FSTAT software Ver 2.9.3.2 ((Goudet, 1995)

http://www2.unil.ch/popgen/softwares/fstat.htm).

Allele frequency is one metric used to quantify genetic variation. It is sometimes synonymously used with gene frequency to measure the commonness of a given allele in a population, that is, the proportion of all alleles of particular gene in the population. On the other hand, genotypic frequency can be defined as the proportion of particular genotype relative to all genotypes at a specific locus in a population.

In a sample of N individuals, N_{ii} and N_{ij} are the numbers of A_iA_i and A_iA_j genotypes observed respectively; whereby $A_{i,}$ A_j are alleles at the particular locus in the sample. To estimate genotype frequencies, the formula is:

$$P(A_i A_j) = \frac{N_{ij}}{N}$$

Therefore, the estimated allelic frequencies of allele A_i for codominant, multiple-alleles system, can be calculated from the sample as:

$$P_i = \frac{N_{ii} + \frac{1}{2}\sum_{j=1}^{n} N_{ij}}{N}$$

Where $j \neq i$.

Several other measures have been used to describe the genetic variation in a population, but heterozygosity remains as the most widespread measure of variation. It is defined as the probability that a random individual chosen from the population is heterozygous at a locus (Shete *et al.*, 2000), and its value ranges from zero to one. Based on known allele frequency, the expected heterozygosity of a randomly mating population for a particular locus with *n* alleles can be calculated as:

$$H_E = 1 - \sum_{i=1}^{n} P_i^2$$

which is one minus the Hardy-Weinberg homozygosity (Hedrick, 2000).

Effective number of allele, n_e basically the inverse of expected heterozygosity and the formula is given as: $N_E = 1 / (1-H_E)$

Meanwhile, the observed heterozygosity (H_0) for a locus can be estimated using formula:

$H_0 = n(A_iA_j)/N$

where, $n(A_iA_j)$ = number of individuals with genotype A_iA_j, $i \neq j$

N = total number of individuals in sample

A_iA_j = alleles at the locus

In most outbreeding populations, the observed heterozygosity is quite close to the theoretical heterozygosity. Deviation of the observed from the expected can be used as an indicator of important population AZ

Assessing allelic richness (R_s) in a set of populations was achieved by estimating the number of alleles expected in samples of specified size using rarefaction approach. This approach uses the frequency distribution of alleles at a locus to estimate the number of alleles that would occur in smaller samples of individuals, and R_s is standardized to the smallest samples size (N) in a comparison (Leberg, 2008). Allelic richness may be useful as an indication of a decrease in population size or of past bottleneck (Nei *et al.*, 1975).

3.4.11 Measuring Sub-Population Differentiation

F_{IS} is measure of the deviation from Hardy-Weinberg proportions within subpopulations and in the total population, respectively, where (I) represent individuals.

The F_{IS} fixation index relates to an approximation of the deviation of the observed heterozygosity, *Ho* from the expected, H_E, and is calculated based on Wright model (1951) with the incorporation of more sophisticated model as implemented in Arlequin (Excoffier *et al.*, 2005), with 1023 permutations. If the H_E is higher than H_O, then the value of F_{IS} will be large (positive value), suggesting inbreeding has resulted in a reduction in heterozygotes at a particular location.

Population tree was constructed through Unweighed Pair-Group Method of Arithmetic (UPGMA) clustering based on Nei's 1978 unbiased genetic distance using genetic data analysis (GDA)version 1.1 (Lewis & Zaykin, 2001) illustrate the magnitude of differentiation among population and subsequently describe the relationship between population.

3.4.12 Inferring Population Structure

Model based Bayesian clustering of genotypic data was carried out to assign individuals into theoretical populations, conformation to HWE and LD was maintained. This analysis was performed in the software STRUCTURE version 2.3 (Pritchard *et al.*, 2000). Individuals in the studied populations were clustered into *K* new populations regardless of their geographical locations, and probabilities of assignment to each cluster were assigned to each new population. Apart from demonstrating the presence of population structure, this software is also widely applied to identify distinct genetic populations, assigning individuals to populations, and identifying migrants and admixed

individuals (Pritchard *et al.*, 2000). The preliminary K values was set from 1 (no structured population) to 4 (each population is structured accordingly), with burning period of 500,000 and No. of MCMC Reps after burning: 500,000.

CHAPTER 4

Results

4.1 Quantitative characters

Basic statistics for measured quantitative traits, viz, plant height, number of fruiting branches per plant, number of pod per plant, number of pod clusters per plant, pod length, number of grains per pod, 1000 grain weight and total grain yield per plant are presented in Table 4.1 which showed variability in genotypes. The important traits, grain yield, plant height and 1000 grain weight exhibited high variation which, in general revealed that the selection for these economic traits is effective in developing high yielding varieties of mungbean.

Table 4.1: Basic statistics for quantitative traits in mungbean genotypes

Traits	Mean±SE	Minimm	Maximum
PH	37.38 ± 1.08	26.9	57.00
NFB	1.71 ± 0.10	1.00	3.50
NP	14.40±0.92	6.33	32.25
NPC	6.49 ± 0.94	3.20	13.50
PL	6.42 ± 0.16	3.50	8.34
NSP	9.36 ± 0.22	6.89	12.12
SW	37.50 ± 1.09	24.00	50.00
SY	33.64 ± 1.76	18.00	60.00

PH: Plant height, NFB: Number of fruiting branches per plant, NP: Number of pod per plant, NPC: Number of pod clusters per plant, PL: Pod length, NSP: Number of grains per pod, SW: 1000 grain weight SY: Total grain yield per plant

4.2 Analysis of variance

The analysis of variance showed significant difference among all genotypes at 0.01 level except for 1000 grain weight which was significant at 0.05 level. (Table 4.2)

Table 4.2: The Mean square values (MS) from ANOVA of yield and yield components of mungbean genotypes.

	PH	NFB	NP	NPC	PL	NSP	SW	SY
Mean Square	82.67	0.63	63.27	9.32	1.05	2.22	86.96	221.27
F Value	14.75**	3.41**	47.67**	15.59**	5.22 **	2.88*	9.94**	37.54**
CV (%)	6.34	25.11	8	11.90	6.97	9.4	7.88	7.22

PH: Plant height, NFB: Number of fruiting branches per plant, NP: Number of pod per plant, NPC: Number of pod clusters per plant, PL: Pod length, NSP: Number of grains per pod, SW: 1000 grain weight SY: Total grain yield per plant,* significant at 0.05 level,** significant at 0.01 level

To show the differences between the genotypes after ANOVA, Duncan`s Multiple Range Test (DMRT) was applied. Figure 4.1 to 4.8 showed Mean comparison of all traits among all genotypes. Mean comparison among all genotypes showed that the most grain yield belong to genotypes number 40521, 40714 and NM-1919 which were significantly different from other genotypes. The lowest grain yield was for genotypes 40593 (Figure 4.1).

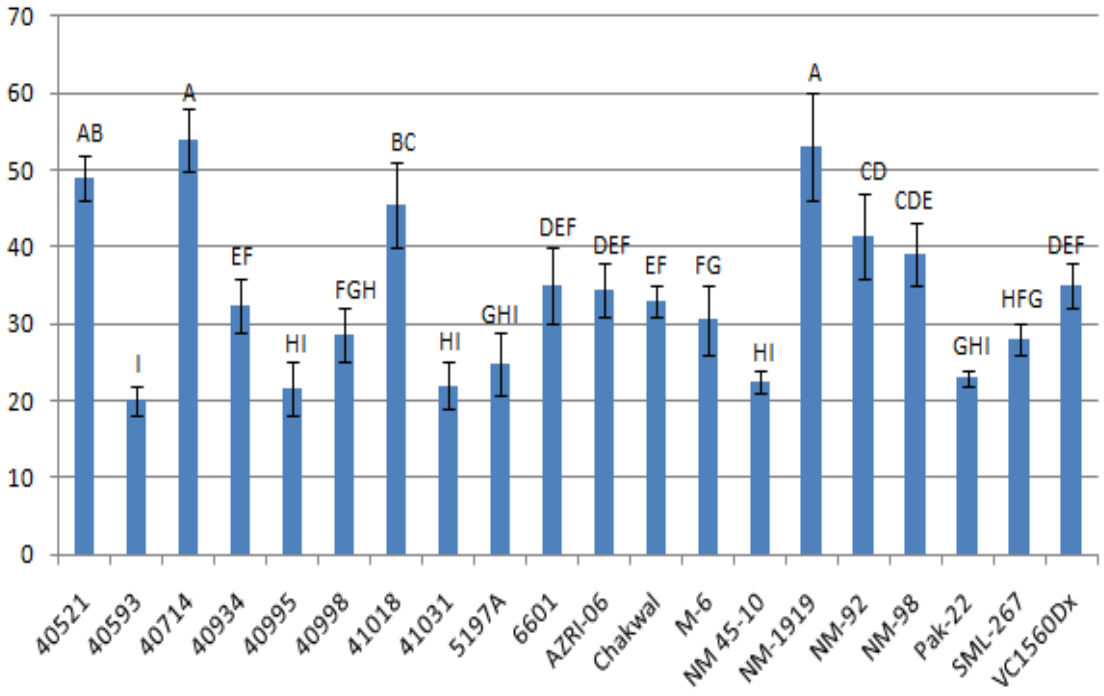

Figure 4.1: Mean comparison of grain yield in mungbean genotypes

(Genotypes with the same letter are not significantly different)

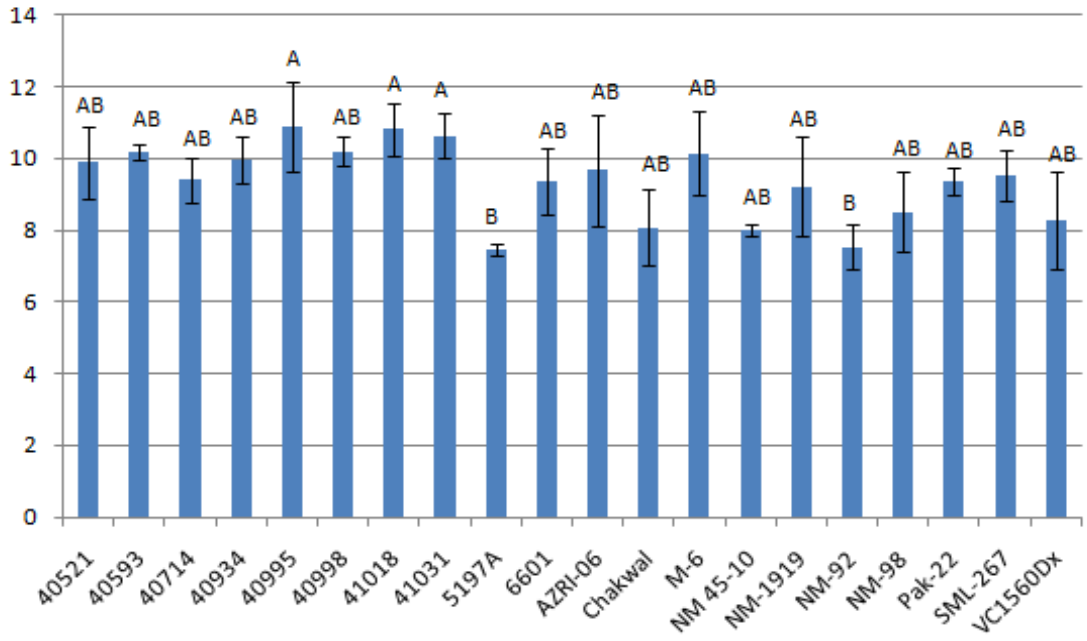

Figure 4.2: Mean comparison of grain/pod in mungbean genotypes

(Genotypes with the same letter are not significantly different)

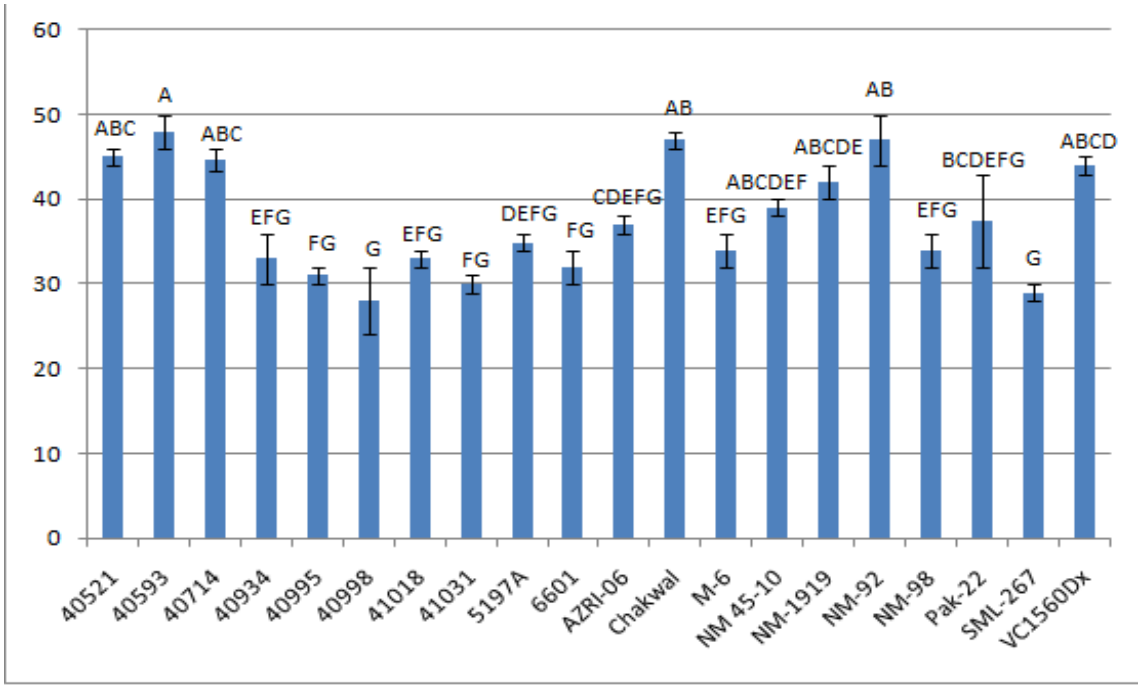

Figure 4.3: Mean comparison of 1000 grain weight (gr) in mungbean genotypes

(Genotypes with the same letter are not significantly different)

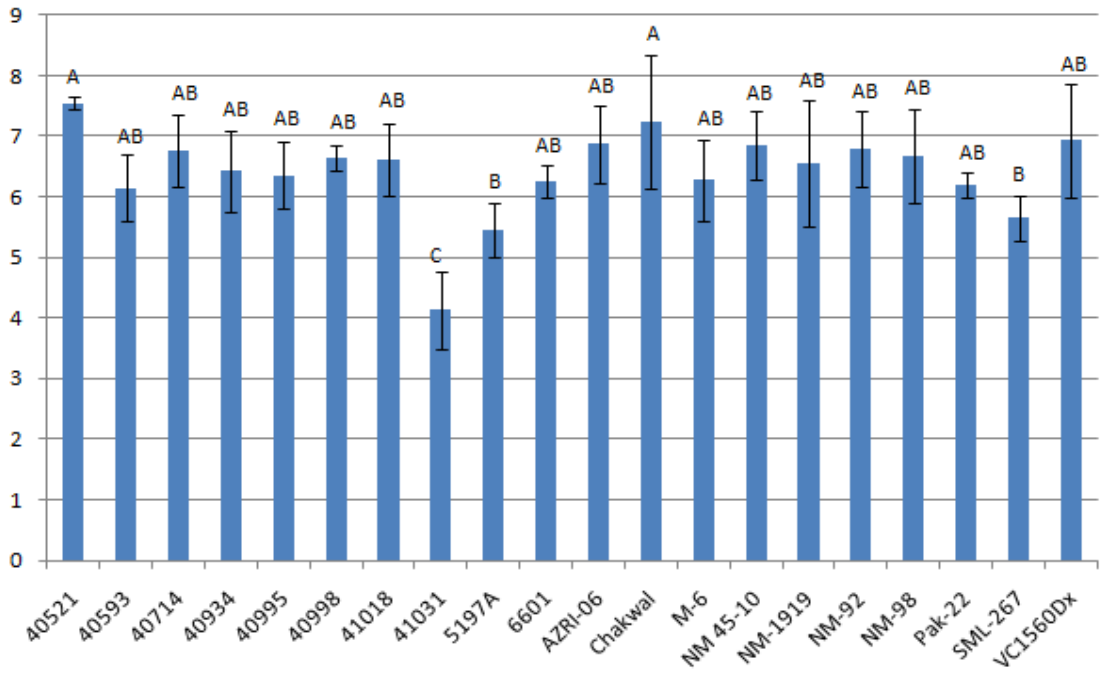

Figure 4.4: Mean comparison of pod length in mungbean genotypes

(Genotypes with the same letter are not significantly different)

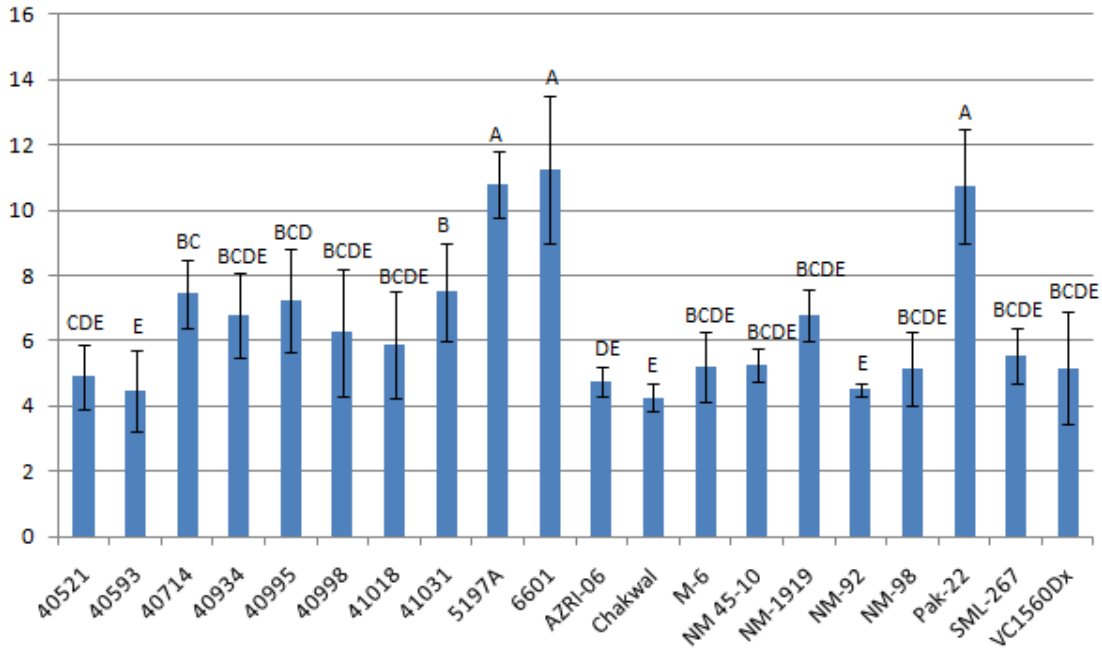

Figure 4.5: Mean comparison of pod cluster/plant in mungbean genotypes

(Genotypes with the same letter are not significantly different)

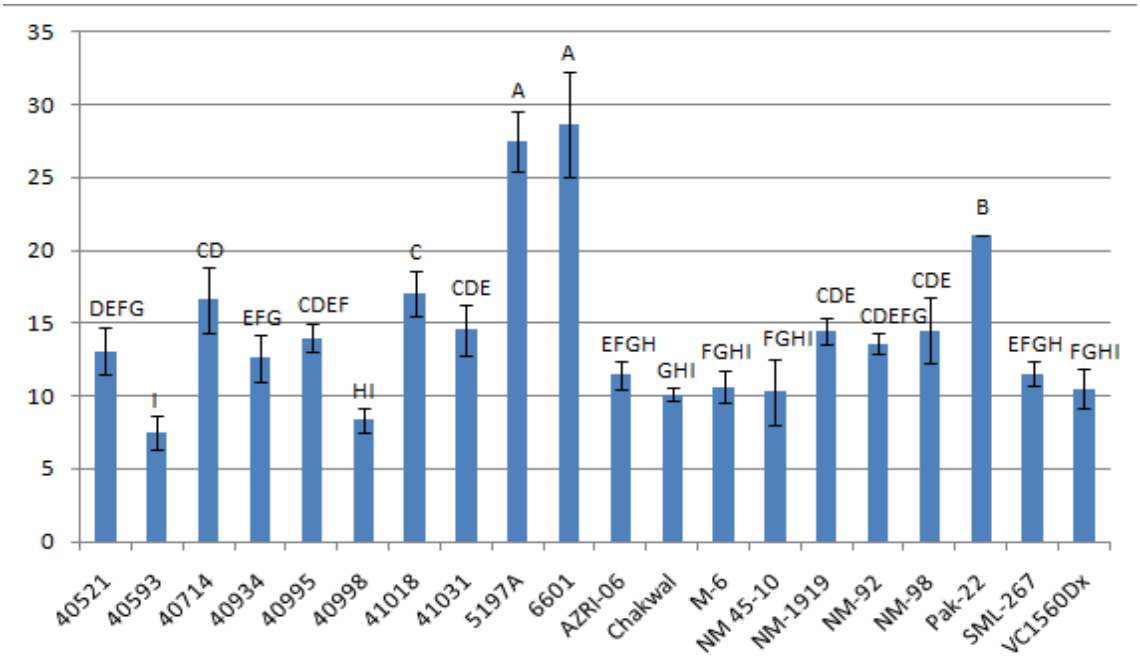

Figure 4.6: Mean comparison of pod /plant in mungbean genotypes

(Genotypes with the same letter are not significantly different)

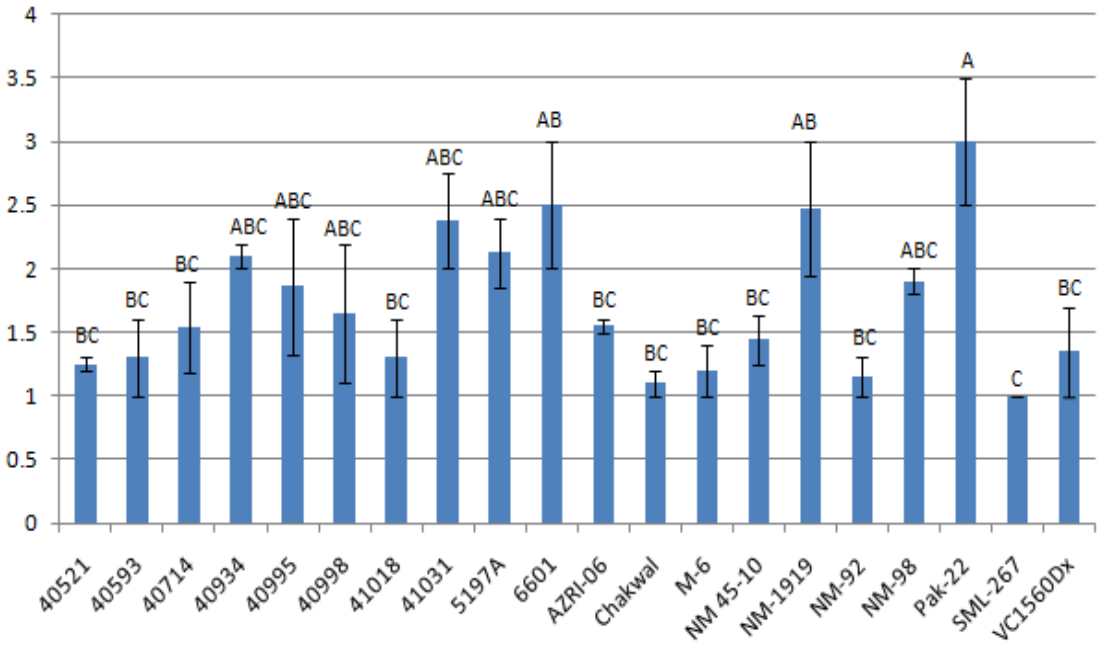

Figure 4.7: Mean comparison of number of fruiting branches/plant in mungbean genotypes

(Genotypes with the same letter are not significantly different)

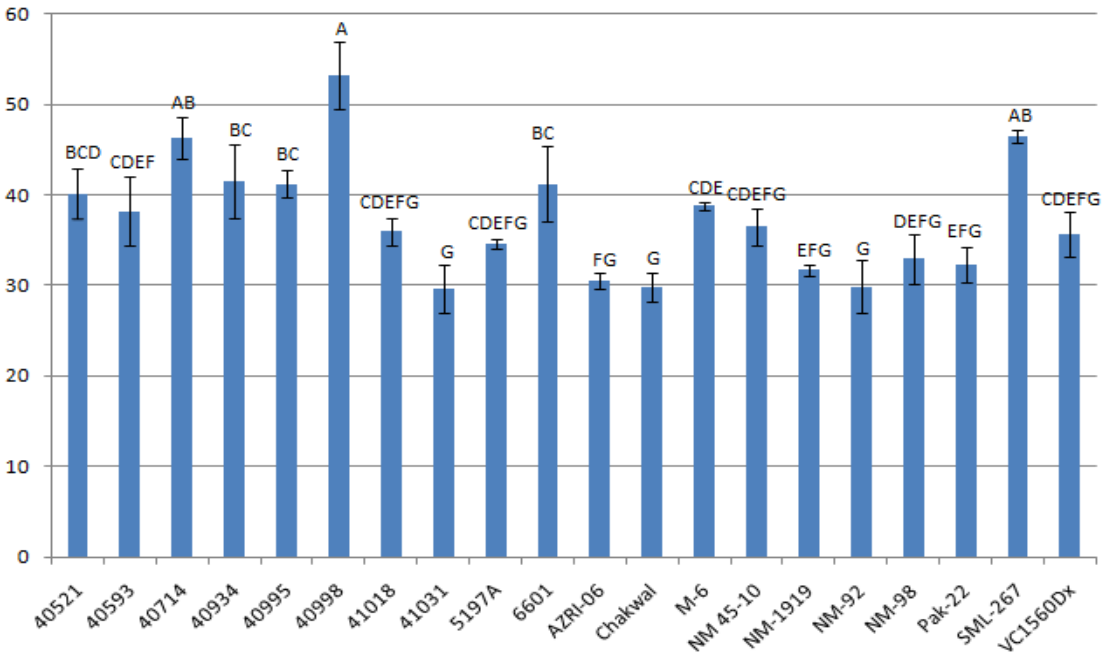

Figure 4.8: Mean comparison of plant height (cm) in mungbean genotypes

(Genotypes with the same letter are not significantly different)

4.3 Cluster analysis

Using cluster analysis by unweighted pair group method with arithmetic mean (UPGMA) method all genotypes were grouped into 3 main groups and 1 minor group. A dendogram based on average linkage distance for 20 mungbean genotypes was also calculated and presented in Figure 4.9. Members of each cluster are shown in Table 4.3 Cluster I consisted of 9 genotypes, cluster II of 7, cluster III of 1 and cluster IV of 3 genotypes.

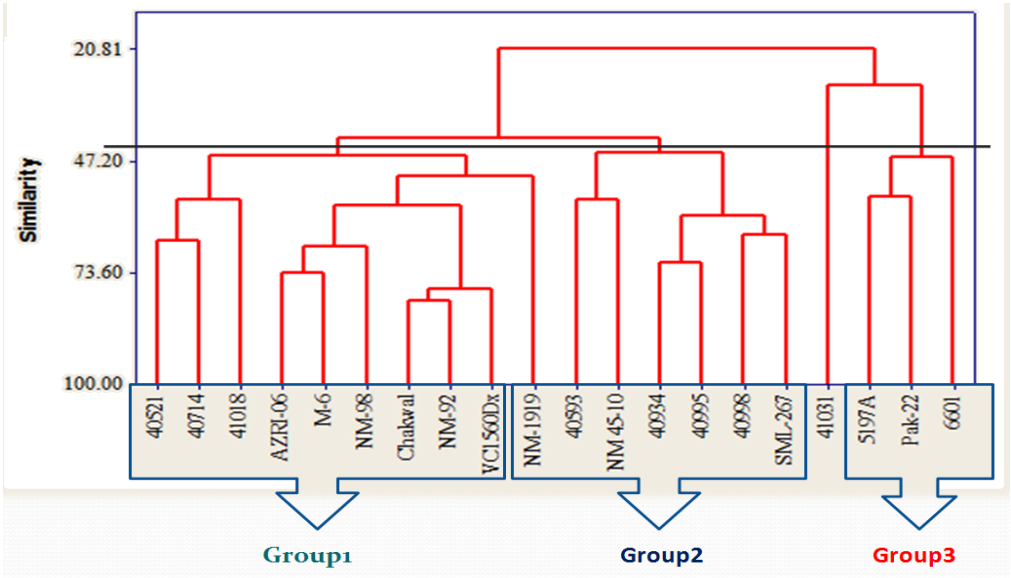

Figure 4.9: Dendogram based on 8 morphological traits in mungbean genotypes

The mean of all traits were calculated for each groups (Table 4.3). The results shows that for plant height cluster II has the highest mean and cluster III has the lowest mean of plant height, that were 41.27 and 29.63 respectively. For 1000 grain weight and grain yield cluster I showed high value while for number of fruiting branches per plant and number of pod clusters per plant had the lowest.

So depending on breeding objective the results of cluster analysis can be applied for crossing program for mungbean improvement.

Table 4.3: Mean for four clusters based on quantitative characteristic

Group	Number of genotypes	PH	NFB	NP	NPC	PL	NSP	SW	SY
1	9	35.46	1.37	13.06	5.25	6.86	9.15	40.63	40.16
2	7	41.27	1.69	11.24	6.05	6.37	9.71	35.71	29.43
3	1	29.63	2.38	14.53	7.5	4.14	10.63	30	22
4	3	36.03	2.54	25.71	10.92	5.97	8.73	34.8	27.64

PH: Plant height, NFB: Number of fruiting branches per plant, NP: Number of pod per plant, NPC: Number of pod clusters per plant, PL: Pod length, NSP: Number of grains per pod, SW: 1000 grain weight SY: Total grain yield per plant

4.4 Principal component analysis

In this project the results showed that three principal components and factors with Eigen values more than one explained 78 % of total variability. The first principal component (PC1) is related to number of fruiting branches per plant, number of pod per plant and number of pod cluster per plant that explained 39.4% of total variability (Table 4.4). The characters with greatest positive weight on PC2 were 1000 grain weight, grain yield and number of pod per plant. Findings revealed that these first two components are related to yield components of mungbean.

Table 4.4: Principal components (PCs) for 8 morphological traits in mungbean genotypes

Traits	1st component	2nd component	3rd component
PH	0.0277	-0.4488	0.5467
NFB	0.4583	0.2100	0.0501
NP	0.4134	0.3817	0.2496
NPC	0.5077	0.2072	0.2049
PL	-0.4036	0.1792	0.3986
NSP	0.1153	-0.5353	0.2215
SW	-0.3691	0.4112	-0.0681
SY	-0.2188	0.2788	0.6176
Eigenvalue	3.15	1.88	1.22
Proportion σ^2	0.3946	0.2354	0.1534
Commulative σ^2	0.3946	0.6299	0.7834

PH: Plant height, NFB: Number of fruiting branches per plant, NP: Number of pod per plant, NPC: Number of pod clusters per plant, PL: Pod length, NSP: Number of grain s per pod, SW: 1000 grain weight SY: Total grain yield per plant

The first two components contributing 63% of the variance were plotted to observe the relationships between the clusters (Figure 4.10).

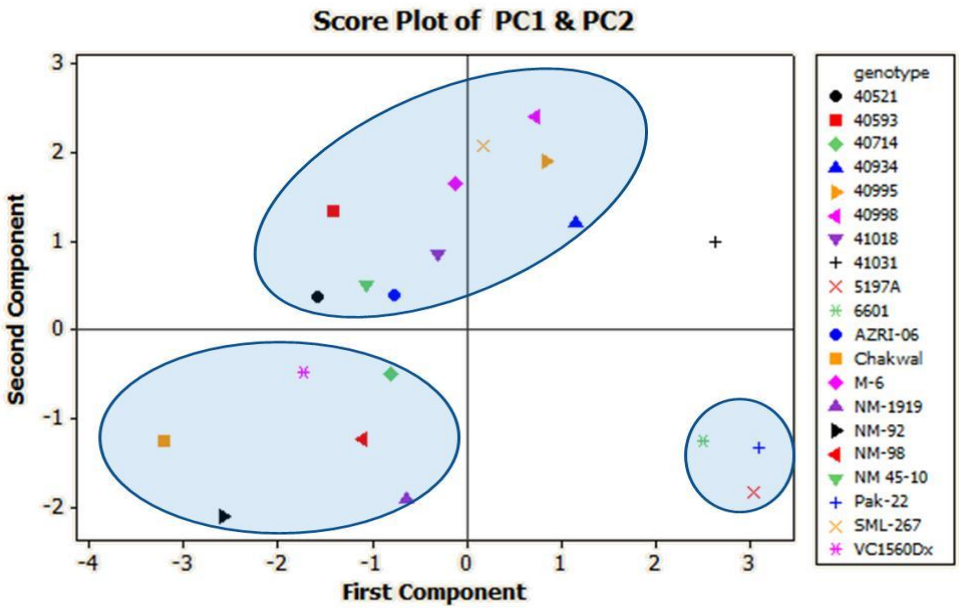

Figure 4.10: Scatter diagram of mungbean genotypes for first two PCs score.

The result of this analysis confirmed the grouping pattern which found by cluster analysis. All clusters are clearly separated from each other (Table 4.5).

Table 4.5: Comparison of grouping genotypes in cluster analysis with principal component analysis

Group / Analysis	G_1	G_2	G_3	G_4
Cluster Analysis	40521 40714 41018 AZRI-06 M-6 NM-98 Chakwal NM-92 VC1560DxNM-92	NM-1919 40993 NM-4510 40934 40995 40998 SML-267	5197A PAK-22 6601	41031
Principal Component Analysis	40521 40714 41018 AZRI-06 M-6 NM-98 Chakwal NM-92 VC1560DxNM-92 SML-267	NM-1919 40993 NM-4510 40934 40995 40998	5197A PAK-22 6601	41031

4.5 Genetic parameters of yield and yield components of mungbean

Genotypic and phenotypic variances are presented in Table 4.5. There is a difference among genotypic and phenotypic variances for all the traits which indicate the influence of environment on these characters. Genotypic coefficient of variability was highest for number of pod per plant (38.65%), number of pod cluster (32.15%) and yield (30.90%), the phenotypic coefficient of variability also was high for these characters. These results were in agreement with previous studies (Siddique *et al.*, 2006; Tabasum *et al.*, 2010), who reported high genotypic and phenotypic variances for the same characters. Almost all traits showed high heritability except number of grain per pod and number of fruits. Maximum heritability belonged to number of pod/plant (95. 9%) followed by grain yield per plant (94.8%), and number of pod cluster (87.9%). Maximum genetic advance was also observed for number of pod per plant, number of pod cluster and grain yield per plant. A higher heritability estimate correlated with good

estimates of genetic advance expected in the next generation for number of pod per plant, number of pod cluster and grain yield indicated that these characters are supported by additive gene affects. Rahim reported a high heritability (broad) along with high genetic advance in percent of mean was observed for plant height, number of pods per plant, number of grains per pod, 1000-grain weight and grain yield per plant and suggested these characters would be best for phenotypic selection (Rahim *et al.*, 2010).

Table 4.6: Genetic parameters of yield and yield components of mungbean

Characters	Genotypic Variance	Phenotypic variance	Phenotypic Coefficient of variation %	Genotypic Coefficient of variation %	Heritability in broad sense %	Genetic advance	Genetic advance Expressed as percent of mean
Height	38.531	44.136	17.793	16.625	87.3	11.948	31.999
Number of fruits	0.222	0.406	37.27	27.543	54.6	0.716	41.93
Number of pod/plant	30.97	32.297	39.474	38.654	95.9	11.226	77.974
Number of pod cluster	4.359	4.957	34.289	32.156	87.9	4.033	62.12
Pod length	0.423	0.624	12.304	10.133	67.8	1.104	17.192
Number of grain per pod	0.725	1.496	13.073	9.101	48.5	1.221	13.052
1000 grain weight	39.104	47.856	18.447	16.675	81.7	11.644	31.05
Grain yield	108.054	113.969	31.734	30.90	94.8	20.851	61.98

4.6 Estimates of genotypic and phenotypic correlation coefficients among different characters

The correlation coefficients (Table 4.6) represented that, in general, the genotypic and the phenotypic correlation coefficients showed similar direction but genotypic correlation coefficients were higher than the corresponding phenotypic correlation coefficients, which might be due to the effect of environment. Grain yield showed a significant and positive correlation with pod length and 1000 grain weight. Number of fruits per plant had negative significant genotypic correlation with grain yield per plant. Negative but non significant genotypic correlations were observed by yield per plant with plant height. It seems logical to select for short genotypes for lodging resistance associated with high yield. Negative significant association was observed between 1000 grain weight and number of grain per pod.

Table 4.7: Estimates of genotypic (rg) and phenotypic (rp) correlation coefficients among different characters

Characters		Height	Number of fruits	Number of pod/plant	Number of pod luster	Pod length	Number of grain per pod	1000 grain weight
Number of fruits	Rg	-0.249						
	Rp	-0.074						
Number of pod/plant	Rg	-0.138	0.753**					
	Rp	-0.017	0.554**					
Number of pod cluster	Rg	0.071	0.991**	0.91**				
	Rp	0.207	0.693**	0.847**				
Pod length	Rg	0.098	-0.677**	-0.316	-0.468*			
	Rp	0.225	-0.063	-0.054	-0.006			
Number of grain per pod	Rg	0.55**	0.013	-0.193	0.085	-0.535*		
	Rp	0.368*	0.219	-0.043	0.187	0.257		
1000 grain weight	Rg	-0.451	-0.474	-0.263	-0.419	0.595**	-0.601*	
	Rp	-0.251	-0.197	-0.172	-0.252	0.447**	-0.254	
Grain yield	Rg	-0.017	-0.203	0.07	-0.183	0.55**	-0.115	0.353*
	Rp	0.094	-0.023	0.153	0.007	0.562**	0.108	0.397*

** significant at 1 % level ,* significant at 5% level

4.7 DNA extraction

Total genomic DNA of each plant was extracted from 100 mg of fresh leaf tissue using a DNeasy Plant Mini Kit. Average DNA concentration was recorded as 0.6 µg/µℓ with purity of $A^{260/280}$ 1.80. Diluted DNA samples with average concentration of 60 ng/µℓ were further utilized for PCR amplification (Figure 4.11).

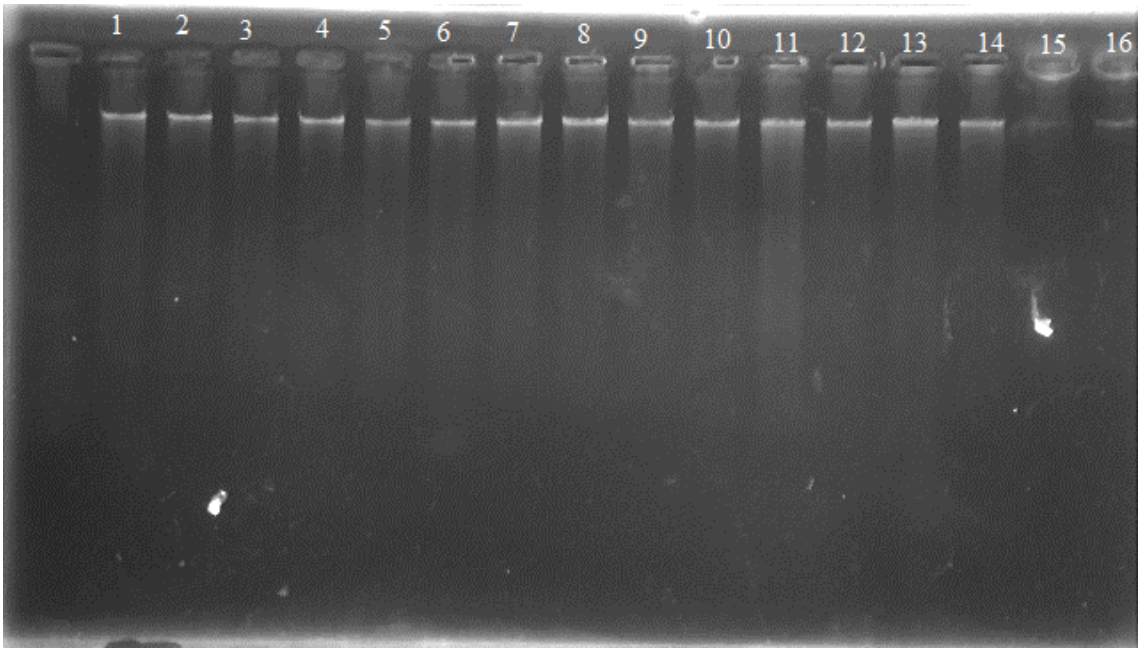

Figure 4.11: DNA Extraction using DNeasy Plant Mini Kit

4.8 Microstaellite primers and preliminary polymorphism testing

Out of all 22 loci initially tested, PCR validation showed that 13 SSR loci were able to be amplified successfully, while the rest of the primers were discarded due to various reasons such as failed amplification, amplification with larger PCR product size, inconsistent amplification or significant stuttering. Out of these 13 successfully amplified loci, 6 potential polymorphic loci were observed from microsatellite banding profiles on the gel images Figure 4.12 and 4.13.

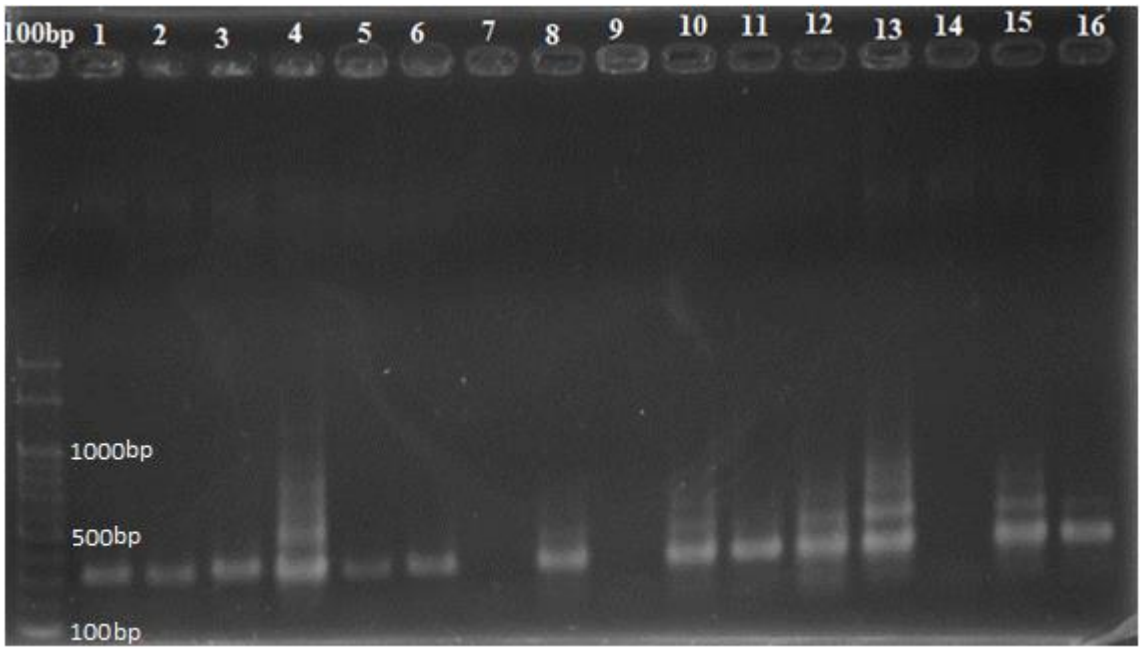

Figure 4.12: Example of gel image showing validation of *LR7323B* on 16 individuals

These 16 individuals randomly selected from four populations. polymorphism criteria was revealed with the presence of possibile heterozygous and homozygous individuals.

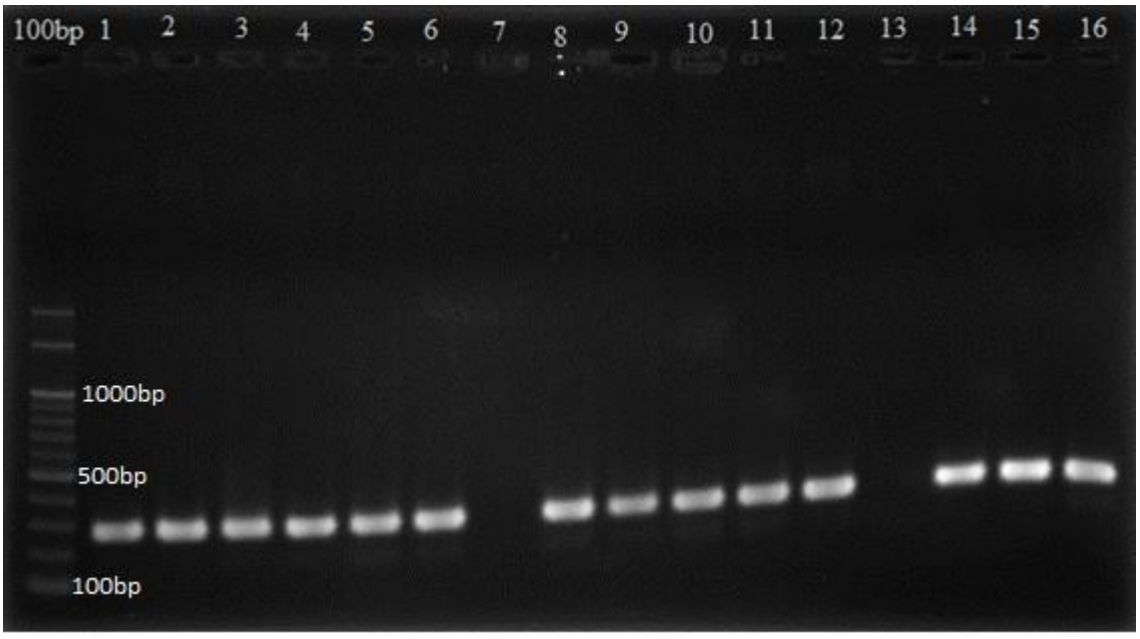

Figure 4.13: Example of gel image from *LR7319B* on 16 individuals

4.9 Determination of microsatellite allele sizes

Full panel of individuals screened by all six loci was found to have a different multilocus genotypes. However, some individuals show no amplification products when PCR was carried out. All alleles fell within the expected size range. Homozygotes and heterozygotes were observed at all loci. The following pictures (Figures 4.14, 4.15, 4.16, 4.17, 4.18) display images of peaks observed in electropherogram as displayed by GeneMapper and Peak Scanner.

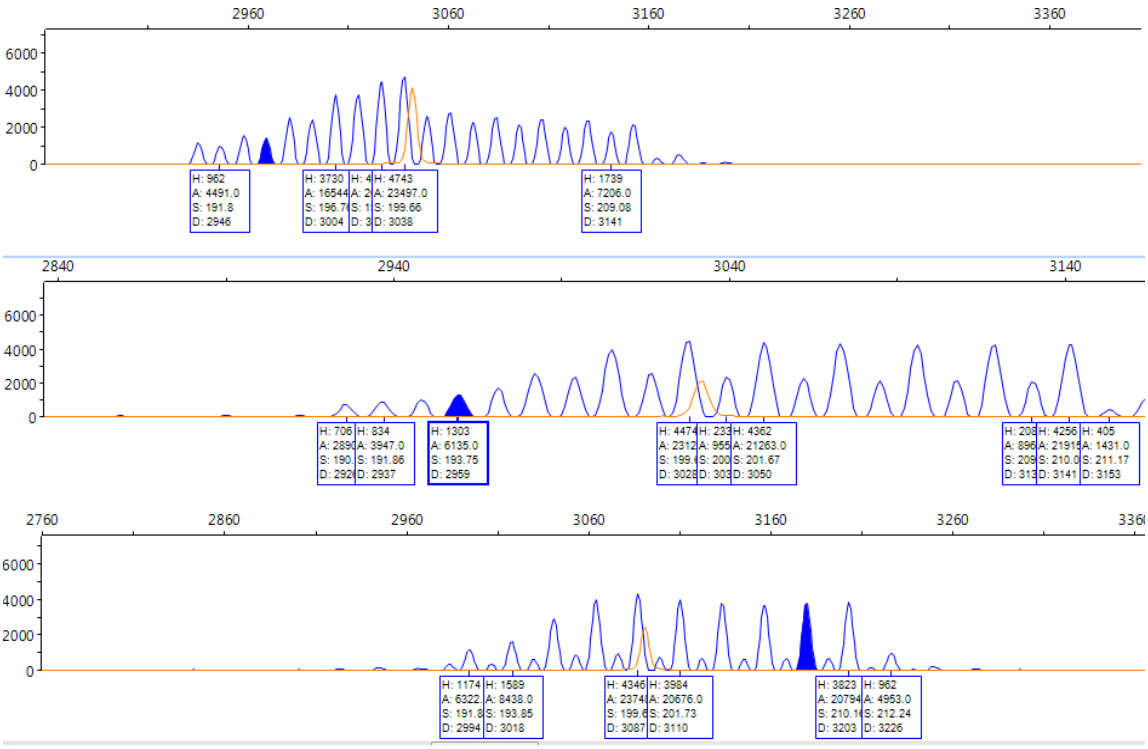

Figure 4.14: Electropherogram of LR7315A, alleles from three individuals.

All graphs illustrate the significant stuttering exhibited at the LR7315A locus. This primer set was omitted from further evaluation. Significant stuttering hinder the correct determination of allele size, thus would have biased the final result of subsequent analysis had the locus been retained (Figure4.14).

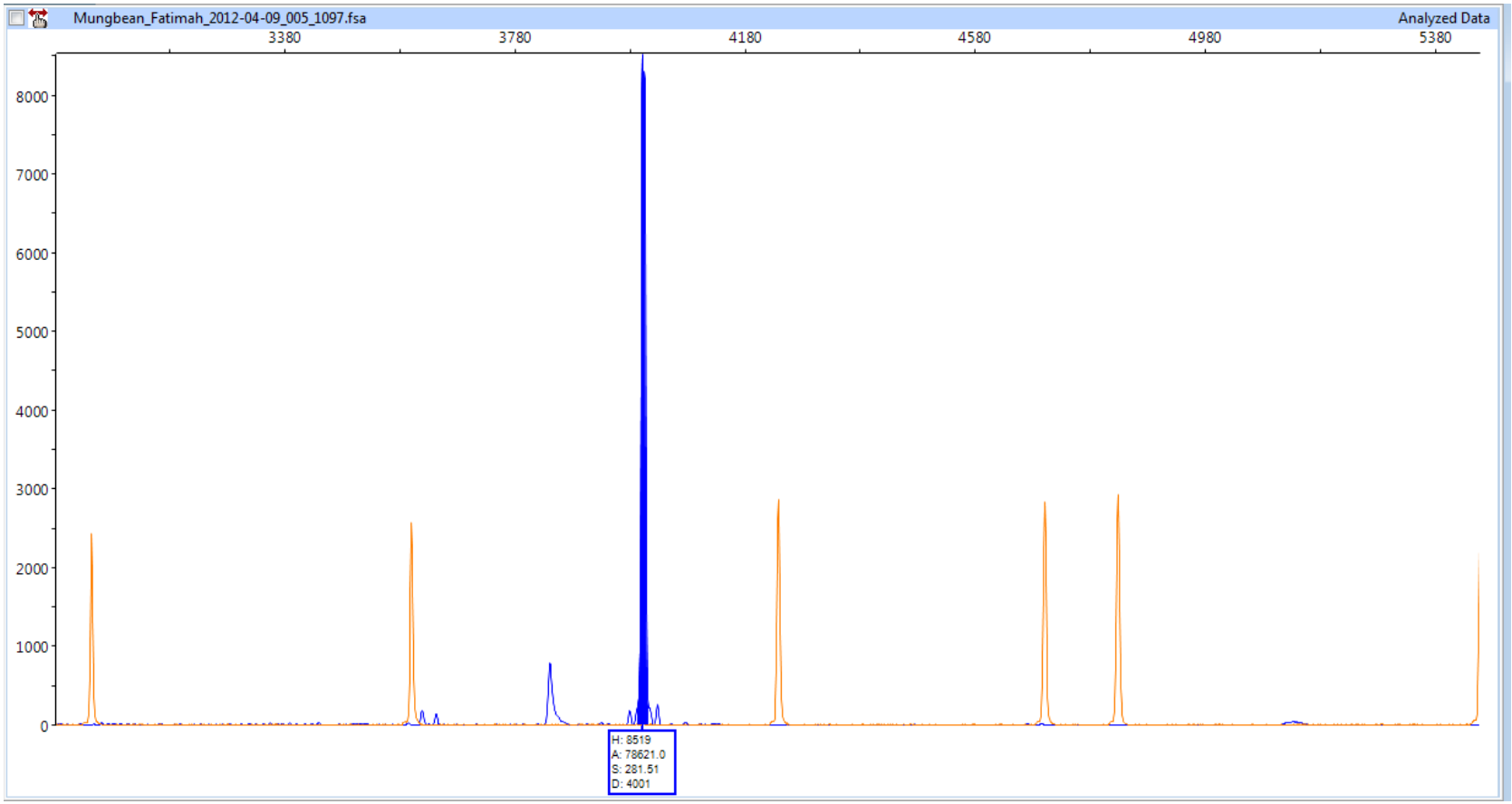

Figure 4.15: Electropherogram of *LR7323B* alleles for an individual from NM19-19.

Primer *LR7323B* is a di-repeat locus. The graph shows only one prominent peak, indicating that this individual is homozygous at the *LR7323B* locus, with both copies of alleles 281bp in length.

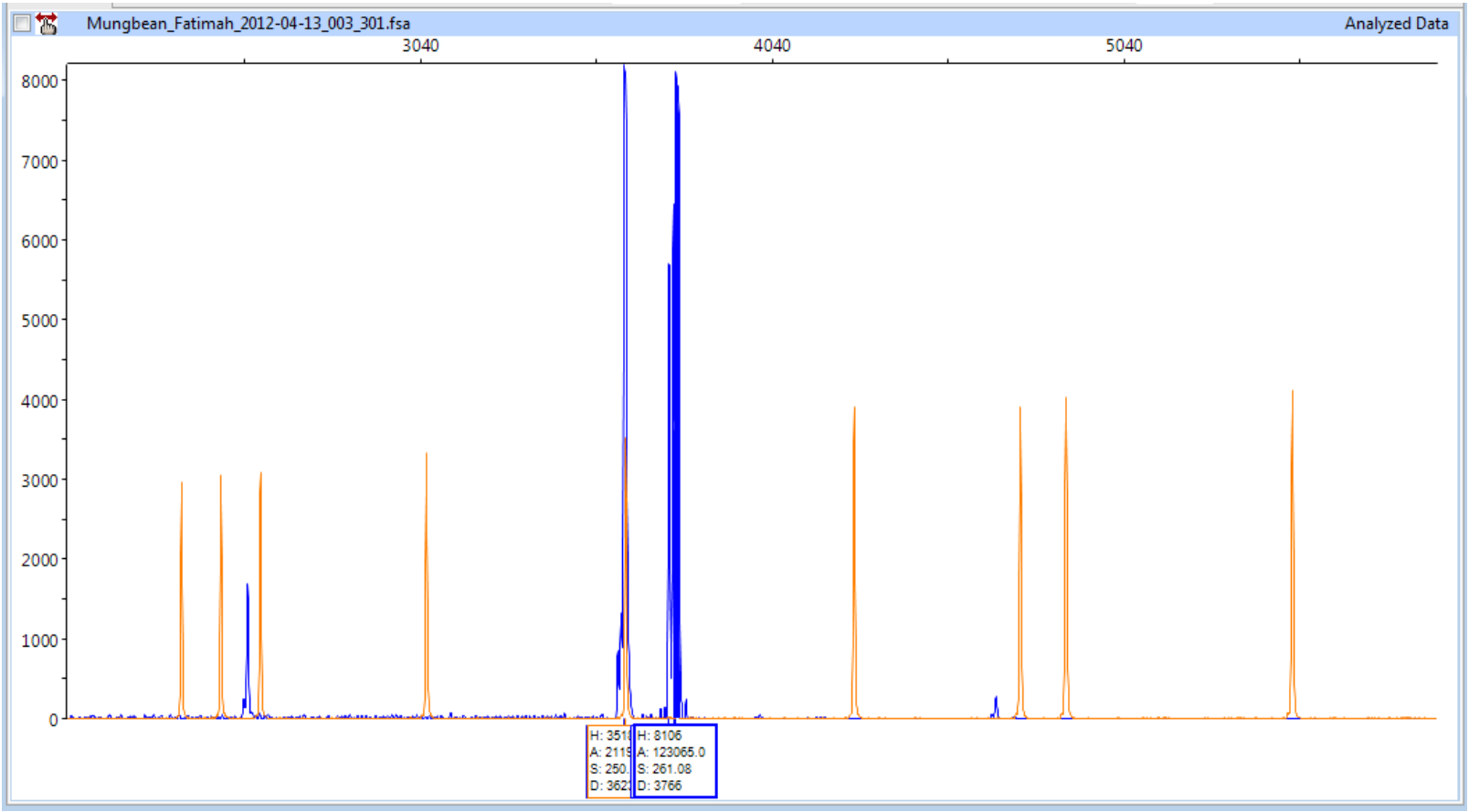

Figure 4.16: Electropherogram of *LR7319B* alleles for an individual from 40521.

LR7319B is a di-repeat locus. The graph shows two prominent blue peak (alleles) which were sized as 249bp and 261bp respectively, indicating that the individual is hetrozygous at this locus. Picture is taken using peak scanner v1.0 software.

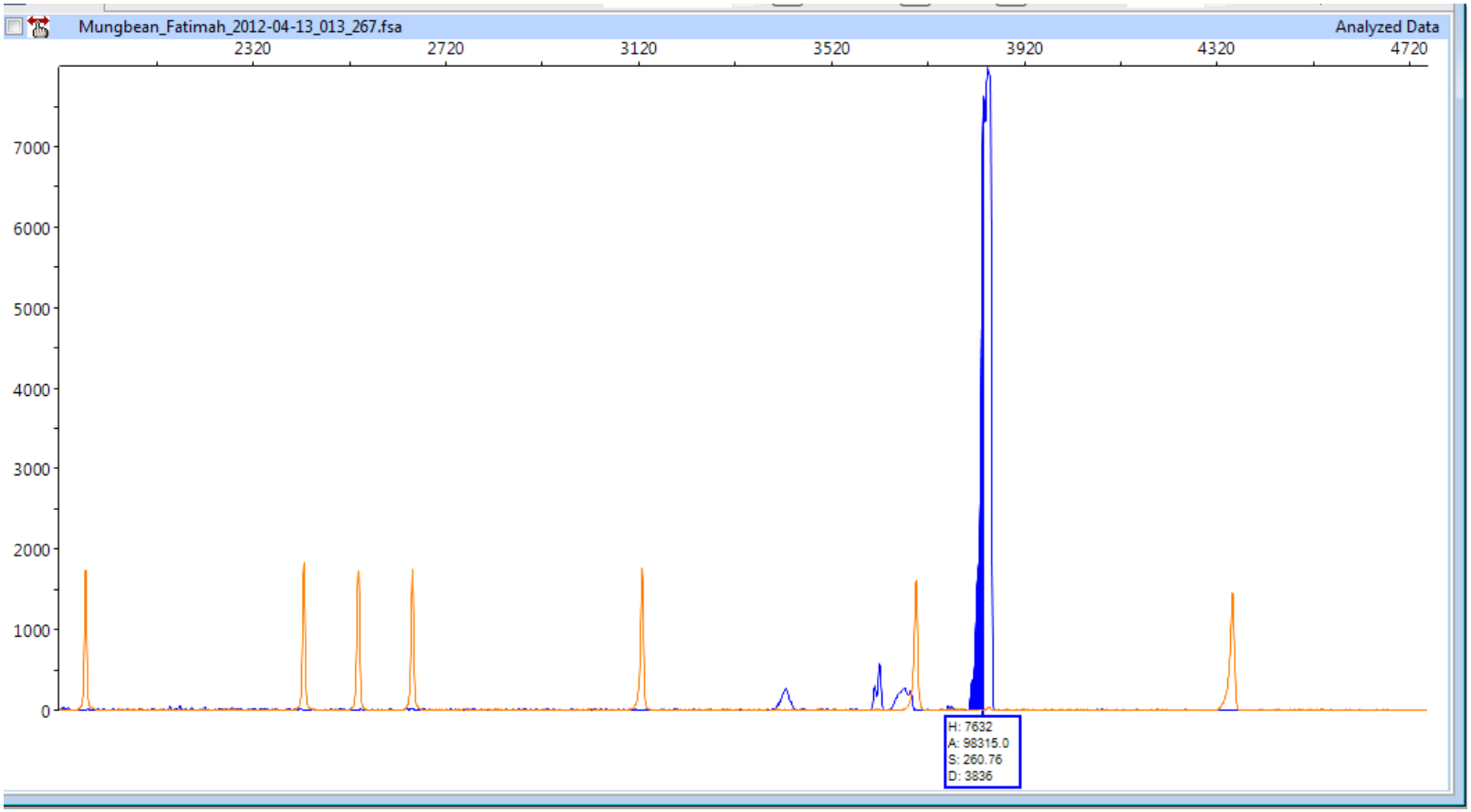

Figure 4.17: Electropherogram of *LR7319B* alleles for an individual from 40995.

LR7319B is a di-repeat locus. The graph shows only one prominent peak that indicating, this individual is homozygous at the *LR7319B* locus, with both copies of alleles 261bp in length. Picture is taken using peak scanner v1.0 software.

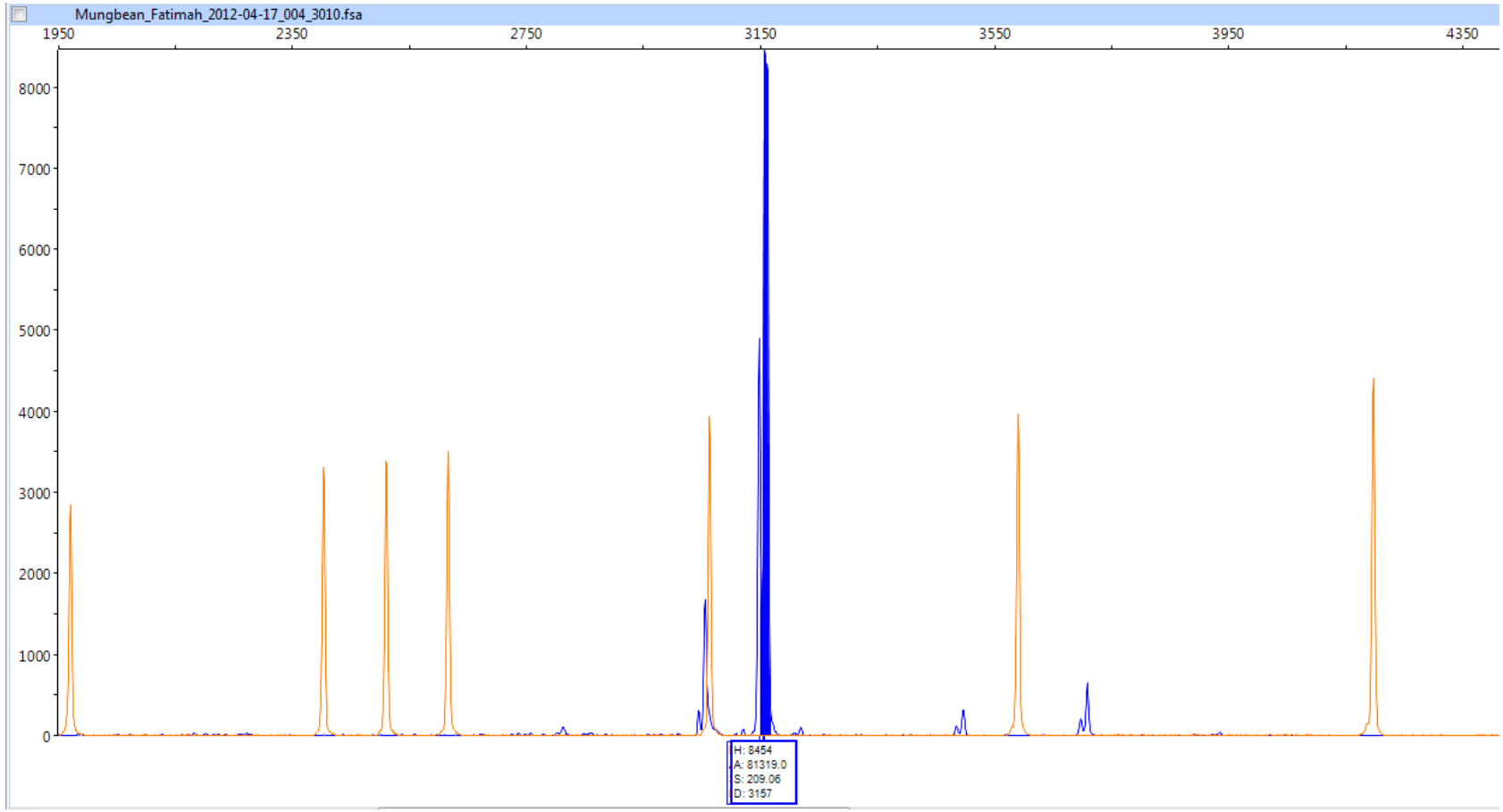

Figure 4.18: Electropherogram of *VJ31122B* alleles for an individual from 40521.

VJ31122B is a tetra-repeat locus. The graph shows only one prominent peak that indicates, this individual is homozygous at the *VJ31122B* locus, with both copies of alleles 209bp in length. Picture is taken using peak scanner v1.0 software.

4.10 Statistical data analysis

4.10.1 Error checking

All individuals genotypes scored in GeneScan were screened for any anomalies. Results obtained from Micro-checker version 2.2.3 analysis (van Oosterhout *et al.*, 2004) demonstrated no evidence of null allele occurrence, no large allele drop outs, or presence of stutter bands. This indicated that the data was suitable for subsequent genetic analysis.

4.10.2 Hardy-Weinberg Equilibrium and Linkage Disequilibrium Tests

The exact probability test for deviations from Hardy-Weinberg Equilibrium, HWE, was performed on all loci for each population. Among the six loci that were analyzed, *LR7319B* showed significant deviations ($p < 0.05$) from HWE for four out of six sampling sites. This implies that one or a combination of factors contribute to the violation of HWE, such as selection, might be significantly impacting on the proportion of genotypes that occurred in that particular locus. Since *LR7319B* was found to have not conformed to HWE, data collected for this locus may potentially bias subsequent analyses of population differentiation that assume conformation to HWE. After FDR correction, most p-values were found to be statistically significant, shown in the Table 4.7.

Table 4.8: Probability values of HWE for each locus per each studied populations

Pop	VJ31122A	VJ31122B	LR7319B	LR7322B	LR7323A	LR7323B
NM19-19	0.1089	0.0108**	0.00005**	0.0207**	0**	0.0152**
40995	1.000	0.1373	0.0024**	0.6293	1.000	0.2549
40521	0.2786	0.1000	0.0001**	0**	0**	0.2762
40031	1.000	1.000	0.6418	0.1741	1.000	0.4215
40593	1.000	0.2862	0.1900	0.0301*	0.3347	0.9217
40714	0.2733	0.0638	0.0135*	0.3107	0.1986	0.0219*

(*P value< 0.05; ** P value significant after FDR correction where α=0.05)

4.10.3 Heterozygosity

Based on Table 4.8 calculation of expected heterozygosity and observed heterozygosity were illustrated for all loci, within and among all populations.

Generally, by looking at H_E and H_O values among loci, *LR7322B* demonstrated the highest values for H_E in comparison to other loci, with value ranging from 0.6014 to 0.8743, also *LR7323A* and *LR7319B* demonstrated the highest values for H_O with value ranging from 0.5000 to 1 for *LR7323A* and 0.6666 to 1 for *LR7319B* respectively. Polymorphism assessments on all populations achieved using these three primers showed that H_O scored were generally higher than H_E.

Table 4.9: Summary of observed and expected heterozygosity (H_E and H_O) for each locus across six populations. Expected heterozygosity (H_E), Observed heterozygosity (H_O) , p-value (P)

Populations	**NM1919**			**40995**			**40521**		
Type of repeat/locus	H_E	H_O	**P**	H_E	H_O	**P**	H_E	H_O	**P**
Di-repeat									
LR7319B	0.5142	1	0.00005**	0.5448	0.9333	0.0024**	0.5487	0.8000	0.0001**
LR7322B	0.8222	0.9444	0.0207**	0.7217	0.8750	0.6293	0.8743	0.9500	0**
LR7323A	0.6079	1	0**	0.4896	0.6000	1.0000	0.6859	0.9500	0**
LR7323B	0.4807	0.7500	0.0152**	0.4173	0.5625	0.2549	0.4794	0.6500	0.2762
Tetra-repeat									
VJ31122A	0.4438	0.6315	0.1089	0.2258	0.2500	1.0000	0.3846	0.5000	0.2786
VJ31122B	0.5222	0.8333	0.01085**	0.4827	0.5333	0.1373	0.3102	0.3000	0.1000

Table 4.9, continued'

Populations	NM1919			40995			40521		
Type of repeat/locus	H_E	H_O	**P**	H_E	H_O	**P**	H_E	H_O	**P**
Di-repeat									
LR7319B	0.4891	0.6666	0.6418	0.4848	0.7272	0.1900	0.4979	0.8125	0.0135*
LR7322B	0.7391	0.6666	0.1741	0.6014	0.5000	0.0301*	0.8698	0.8333	0.3107
LR7323A	0.4202	0.5000	1.000	0.6732	0.6666	0.3347	0.7593	0.8235	0.1986
LR7323B	0.5671	0.4545	0.4215	0.6406	0.7272	0.9217	0.7278	0.6875	0.0219
Tetra-repeat									
VJ31122A	0.4372	0.5454	1.0000	0.3679	0.4545	1.0000	0.4010	0.5294	0.2733
VJ31122B	0.3246	0.3636	1.000	0.3246	0.1818	0.2862	0.5705	0.5625	0.0638

Conversely, the lowest H_O values recorded were shown to be associated with *VJ31122B*, with value ranging from 0.1818 to 0.8333 (Table 4.8).

4.10.4 SSR loci for characterizing populations genetics of six mungbean germplasm

4.10.4.1 Genetic diversity

The level of genetic diversity estimated based on six microsatellite loci are summarized in Table 4.9. All assayed primers detected polymorphism in each of the studied populations, with a minimum of two alleles and a maximum of ten alleles present at each locus in each population. The number of alleles per locus (*At*) observed for NM19-19 ranged from 2 to 7, from 2 to 6 for 40995, 2 to 10 for 40521, 3 to 6 for 40031, 2 to 6 for 40593 and 2 to10 for 40714.

The value of A_t, very likely contributed to the higher value of allelic richness (R_s) for population number 40714 in comparisons with others. NM19-19 scored the lowest for this parameter at 2.959 whilst populations number 40995, 40521, 40031 and 40593 were in between of those two with 3.207, 3.854, 3.291 and 3.608 respectively. Generally, however, average allelic richness was very similar among all sample sites except population number 40714.

On the other hand, the mean effective number of alleles (A_e) was observed to be highest in population number 40714 with 3.269 (Std. dev = 1.7760), whilst the lowest was achieved by population number 40995 (mean = 2.0283, Std. dev = 0.6943).

Table 4.10: Summary of genetic diversity measures based on six microsatellite loci in six populations of NM19-19, 40995, 40521, 40031, 40593, and 40714: total number of alleles (A_t), effective number of alleles per locus (A_e), allelic richness (R_s) and effective sample size (N)

Population	**NM1919**				**40995**				**40521**			
Type of Repeat/locus	A_t	*Ae*	R_s	*N*	A_t	*Ae*	R_s	*N*	A_t	*Ae*	R_s	*N*
Di-repeat												
LR7319B	2	2.0000	2.000	36	3	2.1127	2.600	30	3	2.1505	2.704	40
LR7322B	7	4.9846	5.871	36	6	3.3247	5.185	32	10	6.7797	7.962	40
LR7323A	4	2.4453	3.386	36	4	1.8987	3.691	30	6	3.0189	4.702	40
LR7323B	2	1.8824	2.000	40	2	1.6787	2.000	32	4	1.8779	2.900	40
Tetra-repeat												
VJ31122A	2	1.7610	2.000	38	2	1.2800	1.972	32	2	1.6000	1.999	40
VJ31122B	3	2.0313	2.500	36	5	1.8750	3.798	30	4	1.4337	2.860	40
Total	20	-	-	222	22	-	-	186	29	-	-	200
Average (St dev.)	3.3333 1.966	2.5174 1.230	2.959	37	3.6667 1.633	2.0283 0.694	3.207	31	4.8333 2.857	2.8101 2.022	3.854	40

Table 4.10, continued'

Population	**40031**				**40593**				**40714**			
Type of Repeat/locus	A_t	Ae	R_s	N	A_t	Ae	R_s	N	A_t	Ae	R_s	N
Di-repeat												
LR7319B	3	1.8824	2.750	24	2	1.8615	2.000	22	3	2.0729	2.562	32
LR7322B	6	3.4286	5.444	24	6	2.3607	5.381	24	10	6.4800	7.960	36
LR7323A	3	1.6744	2.944	24	5	2.7458	5.000	18	7	3.8026	5.914	34
LR7323B	3	2.1802	2.974	22	5	2.5745	4.455	22	6	3.3907	4.938	32
Tetra-repeat												
VJ31122A	3	1.7163	2.816	22	2	1.5414	2.000	22	2	1.6374	2.000	34
VJ31122B	3	1.4491	2.816	22	3	1.4491	2.816	22	5	2.2358	4.195	32
Total	21	-	-	138	23	-	-	108	33	-	-	200
Average	3.500	2.0552	3.291	23	3.8333	2.0888	3.608	22	5.5000	3.2699	4.594	33
(St dev.)	1.224	0.715			1.722	0.548			2.881	1.776		

732The maximum number of alleles were observed in any one site was 33 alleles (found in 40714 population, see (Table 4.9), followed by 4052S (29), and the lowest recorded in NM19-19 (20). Generally allelic frequencies were observed in low frequencies at each locus in all population based on genotypic and allelic frequencies, refer to Appendix B.

4.10.4.2 Heterozygosity and inbreeding

Based on Table 4.10, calculation of expected heterozygosity and observed heterozygosity were illustrated for all loci, within and among all populations. H_E estimates ranged from 0.4803 (40995) to 0.6377 (40714), whereas the highest recorded for H_O was 0.8598 (NM19-19) and the lowest was 0.5327 (40031). This implies that there are large differences in genetic variability among the six populations.

The studied populations of mungbean can be ranked based on the H_O (mean value) as follows: NM19-19 > 40714 > 40521 > 40995 > 40593 > 40031. The H_O value displayed in the table was recorded to be higher compared to H_E for all populations.

As displayed in the same Table of 4.10, all populations exhibited negative F_{IS} values (NM19-19: -0.31234; 40995: -0.12908; 40521: -0.29768; 40031: -0.20962; 40593: -0.04613; and 40714: -0.65377). The negative values of F_{IS} are associated with the excess of observed heterozygosity over the expected heterozygosity, and were treated as zero, indicating no inbreeding. Based on the table, F_{IS} value indicating that for all populations, the study probably suggests no inbreeding occurs in the studied population as denoted by the negative value of the coefficient.

Table 4.11: Summary of observed and expected heterozygosity (H_E and H_O) for each locus across six populations

POPULATION (MARKERS)	H_E	H_O	F_{IS}
NM1919			
VJ31122A	0.4438	0.6315	
VJ31122B	0.5222	0.8333	
LR7319B	0.5142	1	-0.31234
LR7322B	0.8222	0.9444	
LR7323A	0.6079	1	
LR7323B	0.4807	0.7500	
MEAN	0.5651	0.8598	
40995			
VJ31122A	0.2258	0.2500	
VJ31122B	0.4827	0.5333	
LR7319B	0.5448	0.9333	-0.12908
LR7322B	0.7217	0.8750	
LR7323A	0.4896	0.6000	
LR7323B	0.4173	0.5625	
MEAN	0.4803	0.6256	
40521			
VJ31122A	0.3846	0.5000	
VJ31122B	0.3102	0.3000	
LR7319B	0.5487	0.8000	-0.29768
LR7322B	0.8743	0.9500	
LR7323A	0.6859	0.9500	
LR7323B	0.4794	0.6500	
MEAN	0.5471	0.6916	

Table 4.11, continued'

POPULATION (MARKERS)	H_E	H_O	F_{IS}
40031			
VJ31122A	0.4372	0.5454	
VJ31122B	0.3246	0.3636	
LR7319B	0.4891	0.6666	-0.20962
LR7322B	0.7391	0.6666	
LR7323A	0.4202	0.5000	
LR7323B	0.5671	0.4545	
MEAN	0.4962	0.5327	
40593			
VJ31122A	0.3679	0.4545	
VJ31122B	0.3246	0.1818	
LR7319B	0.4848	0.7272	-0.04613
LR7322B	0.6014	0.5000	
LR7323A	0.6732	0.6666	
LR7323B	0.6406	0.7272	
MEAN	0.5154	0.5428	
40714			
VJ31122A	0.4010	0.5294	
VJ31122B	0.5705	0.5625	
LR7319B	0.4979	0.8125	-0.65377
LR7322B	0.8698	0.8333	
LR7323A	0.7593	0.8235	
LR7323B	0.7278	0.6875	
MEAN	0.6377	0.7081	

4.10.4.3 Genetic differentiation using UPGMA tree

Population tree phenogram in Figure 4.19 above was constructed to illustrate the relationships among all six populations. Results obtained in the GDA provides further evidence. Corresponding to UPGMA tree, population 40714 with 40031, 40995 with 40593 and NM-1919 with population 40521 were found to be less genetically differentiated, however population NM-1919 and population 40521 were observed to be least similar compared to the other four populations.

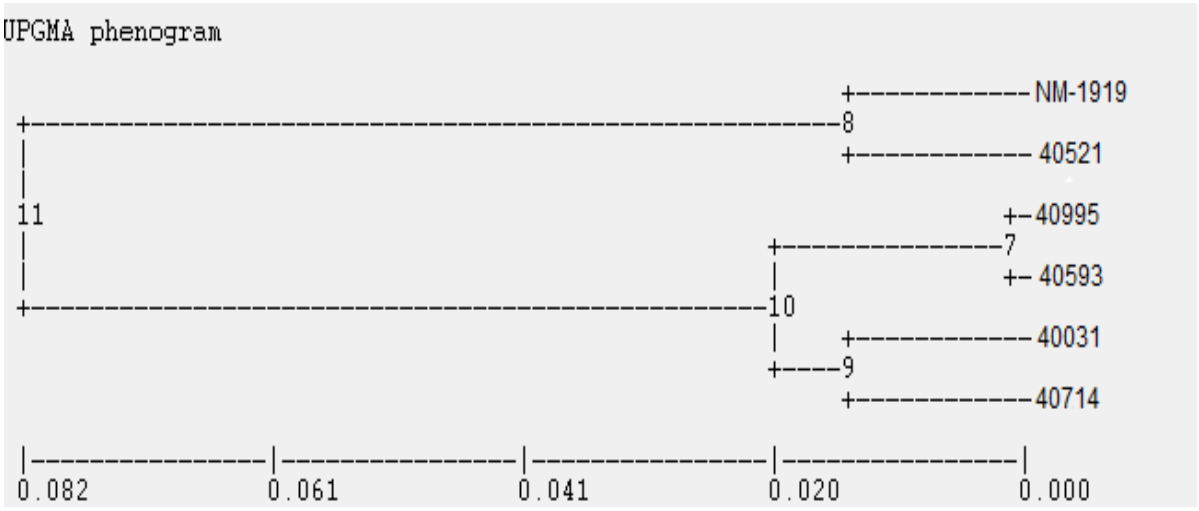

Figure 4.19: Population tree phenogram using GDA software

4.10.4.4 Population structure

Marker data was analyzed using the program Structure (Pritchard *et al.*, 2000), which identifies clusters of related individuals from multilocus genotypes. The full data set was analyzed for all models from K=1 through 6. The highest log probability value (Ln P(D)) (closest to zero) was recovered when k=3 (all individuals assigned to the same population). This analysis of molecular marker data from 6 primer pairs also

divided the populations into distinct groups (Figure 4.20). Populations 40995(2) and 40031(4) composed one group, while 40593(5) and 40714(6) comprised a second cluster. Corresponding to Population structure 40521(3) was observed to be least similar compared to the other populations.

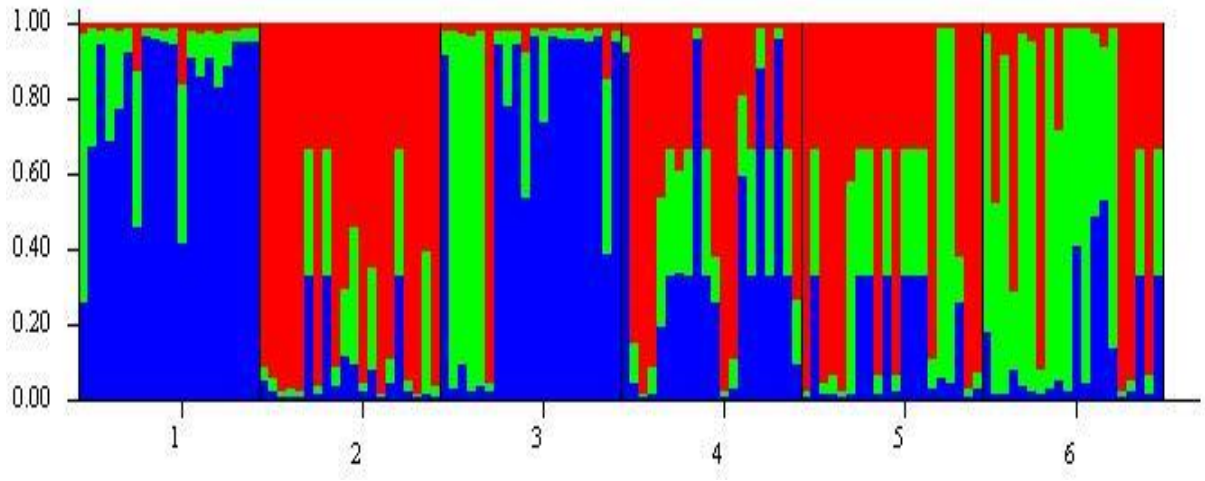

Figure 4.20: Structure clustering, using structure Ver 2.2.

Pop 1: NM-1919, Pop 2: 40995, Pop 3: 40521, Pop 4: 40031, Pop 5: 40593, Pop 6: 40714

4.10.4.5 Genetic differentiation between mungbean germplasms using Analysis of molecular variance (AMOVA)

To evaluate the genetic difference of high and low yield mungbean genotypes, the marker data from 6 microsatellite primers were subjected to analysis of molecular variation (AMOVA) using Arlequin 3.1software (Excoffier et al., 2005). The percentage of variation among populations was (9.15) and it was (-19.99) and (110.85) to among

individuals within populations and within individuals respectively.

Results obtained from AMOVA illustrated that, the percentage of variation was much higher within individuals than both among population and among individuals within populations (Table 4.11). It demonstrated that significant difference exist within individuals of mungbean genotypes, and accordingly, high heterosis effect may be accrued in the previous population.

Table 4.12: Analysis of molecular variance (AMOVA) from two populations of mungbean using six microsatellite primers

Source of variation	Sum of squares	variance components	Percentage variation
Among populations	16.503	0.16805	9.14707
Among individuals within populations	119.076	-0.36731	-19.99282
Within individuals	189.500	2.03645	110.84576
Total	325.078	1.83719	

CHAPTER 5

Discussion

5.1 Selection of suitable parents based on morphological traits

In order to maintain, evaluate and utilize germplasm effectively, it is important to investigate the extent of genetic diversity available. Smith considered morphological characterization as an important step in description and classification of crop germplasm because a breeding program mainly depends upon the magnitude of genetic variability (Smith & Smith, 1989; Smith et al., 1991). In a breeding program choosing, of parents play a very important role, parents with more genetic distance can create higher variation which can increase genetic gain of selection. The selection on the basis of best performance has been suggested by many researchers (Donald, 1962; Lal, 1967; Singh, 1977; Singh et al., 1980; Khan & Malik, 1989; Ghafoor et al., 2000). Subdividing the variance into its components assists the genetic resources conservation and utilization and it enables planning for use of appropriate gene pools in crop improvement for specific plant attributes (Bekele, 1984,1985; Pecetti et al., 1992; Pecetti & Damania, 1996). For other traits such as number of pod per plant, number of pod cluster per plant, moderate variances was observed and for the number of fruiting branches per plant, pod length and number of grains per pod, low variance was observed and hence low genetic variability seemed to restrict the scope of selection for these traits in the present germplasm collection. The genes for these important economic traits should be investigated and exploited from other sources i.e. inter-specific hybridization or mutation. Large scale testing of broad base germplasm needs to be built up by making extensive local collection and obtaining germplasm from abroad to develop a sound

breeding program (Ghafoor et al., 1992; S. K. Jain et al., 1975). Some studies advocated that maximum genetic conservation would be achieved by sampling populations from as many environments as possible (Brown, 1978; Laghetti et al., 1998).Yimram et al. (2009) evaluated 9 qualitative and 21 quantitative traits in 340 diverse cultivated mungbean accessions collected at Asian Vegetable Research and Development Centre (AVRDC) to assess the extent and pattern of their diversity. The germplasm represented a wide range of diversity for most of the traits evaluated. High genetic variability was found in yield components. Penology traits such as plant height, days to flowering, and days to maturity also showed high genetic variability (Yimram *et al.*, 2009). They observed 5 major and 1 minor group when they clustered several mungbean germplasm. They described that germplasm from India and West Asia were in all major clusters, while those from Southeast Asia and other origins were mainly grouped into one cluster. They recommend that the germplasm from West Asia be exploited more in cultivar development to enrich the breeding gene pool. We also observed that the germplasms grouped in 3 major groups and 1 minor group, where those collected from different agro-ecological zones of Pakistan have wider genetic base. The grouping of accessions by multivariate methods in this study is of practical value to mungbean breeders. Representative accessions may be chosen from particular groups for hybrid program with other approved varieties. Several potentially important agronomic types have been identified and these may be exploited for genetic potential to transfer the desirable genes and this facilitates the assembly of a core collection of accessions from the large genetic resources collection (Clements & Cowling, 1994; Frankel, 1984; S. P. Singh, 1988; Tolbert *et al.*, 1979). Tawar et al. (1988) conducted genetic divergence in 34 diverse genotypes of mungbean which were grouped in five clusters, and they observed that variability in the parents was related to genetic diversity. Inclusion of such genotypes from distinct clusters and their implication in mungbean breeding program

was suggested (Tawar *et al.*, 1988).

Ghafoor et al. (2001) studied genetic diversity in 484 blackgram germplasm accessions. Quantitative traits were analyzed for cluster and principal component analyses. The first four PCs with eigenvalues >1 contributed 79.5% of the total variability amongst accessions. The germplasm was categorized in five clusters based on average linkage. The first two principal components were plotted to observe relationship between the clusters. Clusters II, III and IV showed more clear separation than clusters I and V (Ghafoor *et al.*, 2001). Elizabeth et al. (200l) investigated nineteen *Sesbania* accessions to characterize them on morphological and agronomic data using multivariate methods. Principal component analysis indicated that variance accumulated by the first two components for morphological and agronomic data was 74.4% and 77.0%, respectively. In this study the cluster analysis performed with the eight selected characters classified the accessions into five groups. The result of this analysis confirmed the grouping pattern which was found by Elizabeth *et al.* (2001). In this study all clusters were clearly separated from each other. Selection of proper parents is playing a vital role for a successful plant breeding program. So depend on breeding objective the result of cluster analysis and principal component analysis (PCA) can be applied for crossing program for mungbean improvement. Based on these analyses, choosing parents with more genetic distance to generate higher variation can enhance genetic achievement in selection.

5.2 Correlation analysis

Knowledge of the relationship among plant characters is useful for selecting traits to combine for yield improvement. The correlation coefficients were computed among all the measured quantitative traits and are shown in Table 4.6.

The correlation is a measure of the degree to which variables vary together or a measure of intensity of association (Steel & Torrie, 1980). Generally, a high magnitude of correlation with positive signs was observed between different traits. In mungbean, some researchers observed positive correlation of yield with yield components (Tomar et al., 1973; Khalid et al., 1984), whereas, Malik et al. (1987) reported negative correlation of yield with maturity, pod length and grain weight (Malik et al., 1987). Significant positive correlation of grain yield with other yield contributing characters has been reported in blackgram (Rani & Rao, 1981). Maximum relative selection efficiency for branches per plant has been investigated in mungbean (Malik et al., 1983), and Malhorta observed positive association of yield with days to maturity, plant height, pods and pod length, whereas, negative with grain weight (Malhotra et al., 1974).

The genotypic and the phenotypic correlation coefficients in this study showed similar direction but genotypic correlation coefficients were higher than the corresponding phenotypic correlation coefficients. This might be due to effect of the environment (Kole *et al.*). Grain yield showed a significant and positive correlation with pod length and 1000 grain weight. Number of fruits per plant had negative significant genotypic correlation with grain yield per plant. Negative but non significant genotypic correlations were observed by yield per plant with plant height. It seems logical to select for short genotypes for lodging resistance associated with high yield. A previous study confirmed these results (Tabasum *et al.*, 2010). In the current research negative significant association was observed between 1000 grain weight and number of grain per pod. Similar results were observed in many studies by different researchers (Ali & Shaikh, 1987; Ahmad et al., 1997; Rohman et al., 2003).

5.3 Microsatellite loci and preliminary polymorphism testing

The primers used in this study were designed to amplify microsatellite regions, the success ratio of mungbean SSR amplification was more than 50%, with 13 primers able to be amplified out of the initial 22 that were tested. Those that failed either were unable to amplify product at all, or showed unspecific amplification. Failure of amplification can be attributed to a variety of reasons, such as location of primer spanning across introns and/or mutations and indels (insertions or deletions) at the primer annealing sites. These issues are especially important when considering microsatellite loci, as there are potentially fundamental problems when applying microsatellite loci to genomic DNA regions.

In this study, the number of di-nucleotide SSRs found in successful amplified products is slightly higher compared to tetra-nucleotide repeats. From the 13 SSRs loci tested, about 55% of the di-repeat motifs were successfully amplified from genomic DNA, while 45% of tetra-repeats were able to be amplified. The prevalence of SSR motif type obtained in this current study is incongruent with other studies on plants, which showed that tri-nucleotide repeats are more common (Li et al., 2002; Morgante et al., 2002; Varshney et al., 2005).

As shown in the results (Figure 4.14), image taken from GeneMapper displayed a characteristic feature namely "stutter bands"- that is, minor products that differ in size from the main product by multiples of the length of the repeat unit (Ellegren, 2004; Hauge & Litt, 1993; Murray *et al.*, 1993). This results from replication slippage of Taq polymerase that occurs during PCR amplification of microsatellite sequences in vitro (Ellegren, 2004). The stutter peaks are observed as multiple artifact peaks preceding the true allele peak, and this could lead to incorrect scoring and hinder

the genotyping of individuals, thus complicating data interpretation. The presence of stutter artifacts are more commonly found in di-nucleotide loci compared to other SSR type motifs (Bakker *et al.*, 2005; Ellegren, 2004). Despite the fact that some stuttering was observed in the present study, as the stutter bands were able to be distinguished from the real alleles their presence did not compromise the data collection process.

5.4 Linkage Disequilibrium (LD) and Hardy-Weinberg Equilibrium in mungbean

Linkage Disequilibrium tests revealed that none of the loci showed significant linkage disequilibrium for all pairs of loci. This indicates that there is probably no physical linkage between any of the loci used in this study. Furthermore, it means that the individuals that were collected at each population are not likely to constitute a mix of two or more breeding populations with very different gene frequencies.

However, significant departure from HWE was observed in locus *LR7319B* suggesting that this locus is not assorting randomly. One possible explanation for violation of HWE is the presence of null alleles. Null alleles can occur as a result of priming site mutations or large or small allele dropout, thus leading to the failure of PCR amplification of one or both microsatellite alleles, resulting in a lack of visible amplicons for one or both alleles in a diallelic genotype (Rodrigues *et al.*, 2009). If null alleles are caused by a mutation in the priming site then individuals that are heterozygous and possess one null allele and one non-null will appear to be homozygous for the visible allele, whereas there are no visible alleles in the case of null-null homozygotes. Likewise, long or short allele dropout will result in scoring only one allele per genotype. Hence, falsely recording homozygote genotypes where individuals are true heterozygotes. The presence of null alleles in a population will bias allele frequencies and inflate the number of homozygous genotypes, thus reducing the

observed heterozygosity (DeWoody & Avise, 2005) . Nevertheless, the presence of null alleles is unlikely in this study, as output from Microchecker found no evidence for null alleles. Significant deviations from HWE could also result from selection pressure that may be occurring at the molecular or the phenotypic level. For example, at the molecular level *LR7319B* is a di-nucleotide repeat SSR and possibly mutations in repeat number at this locus may interrupt the reading of downstream coding regions (frame-shift mutation), therefore having a deleterious effect on the organism, leading to selection pressure to maintain repeat numbers that do not interrupt the reading frame (Li et al., 2004). In this study, it may be argued that the sample size used is rather small, thus the same approach as Choi et al. (1999) was also used to study genetic variation using microsatellite markers in soybean. The significant violation from HWE at this locus, however, could simply be caused by the limited sample size of individuals per population used in this study (Wang et al., 2005), or as a result of undetected nulls.

5.5 Population genetic structure among six mungbean germplasms

Population structure analysis of molecular marker data from six primer pairs divided the populations into distinct groups (Figure 4.20). Populations 40995(2) and 40031(4) were composed one group, while 40593(5) and 40714(6) comprised a second cluster. Corresponding to Population structure 40521(3) was observed to be least similar compared to the other populations. Genetic structuring of populations can be influenced by the combination of factors; gene flow, natural selection and genetic drift (Freeland *et al.*, 2010). Results of previous studies on genetic relatedness showed that the genetic distance values within the cultivated beans were higher than in the wild beans (Kumar et al., 2003). In general, population differentiation is inversely correlated with gene flow and directly correlated with genetic drift (Frankham *et al.*, 2002; Freeland *et al.*, 2010).

Allelic frequencies were observed in low and similar frequencies at each locus among all populations. When gene flow is low or being restricted between populations, allele frequencies are expected to diverge as the forces of genetic drift and selection act independently in each population (Slatkin, 1987).

The average number of alleles obtained from six microsatellite markers for all populations were rather similar, except 40714 and 40521 that were 5.5 and 4.8 respectively. These two populations show high levels of variation compared to other four populations. High level of variation in mungbean suggests that it is a good source of genes for mungbean development. This is a good sign, especially when comparisons are made with other plant species. In the garden pea (*Pisum sativum*) the average number of alleles obtained from 31 microsatellite markers was 3.6 (Burstin *et al.*, 2001), compared to 5.5 or 4.8 that were observed in two mungbean populations in the current study. In alfalfa reported 3.2 alleles per locus with four microsatellite markers (Diwan *et al.*, 1997), and in barley, 2.1 alleles per locus were observed with 15 microsatellite markers (J. Becker & Heun, 1995). The primary source of the gene pool used in mungbean improvement program comes from the wild mungbean (*V. radiata var. sublobata*) (Lawn *et al.*, 1988).

5.6 Heterozygosity level in mungbean (*Vigna radiate L.Wilczek*)

The observed heterozygosity was higher than expected heterozygosity for six populations of mungbean (Table 4.10). All of these populations showed excess in heterozygosity as shown by the F_{IS} values (Table 4.10). Negative values indicate excess in heterozygosity while positive values indicate heterozygosity deficiency (Hedrick, 2000). Excess heterozygosity could be caused by either outbreeding or overdominance (Goossens *et al.*, 2003).

CHAPTER 6

Summary and conclusion

Research reported in this manuscript was conducted on 20 mungbean germplasms collected from different agro-ecological zone in Pakistan under field condition and molecular experiment to estimate the genetic diversity of mungbean germplasm on the basis of morphological and agronomic characteristics, considering the reasons of low yield, through analysis of yield and yield contributing characters and finally, to evaluate the difference of genetic makeup and to characterize the high yielding potential genotypes within 20 mungbean advance line.

Twenty mungbean accessions were evaluated for various agro-morphological traits under field condition at RCBD. Data were recorded on eight quantitative traits (plant height, number of fruiting branches per plant, number of pod per plant, number of pod clusters per plant, pod length, number of grains per pod, 1000 grain weight and total grain yield per plan). Six genotypes were selected on the basis of grain yield graph form Duncan`s Multiple Range Test (DMRT) to evaluate genetic differentiation using six microsatellite primers.

In conclusion all the twenty mungbean accessions under study were grouped into 3 main clusters and 1 minor cluster on average linkage basis. The results of cluster analysis can be applied for crossing program for mungbean improvement. Variance was further studied by principal component analysis (PCA). The first three principal components and factors with Eigen values more than one explained 78 % of total variability.

High variance was observed for grain yield, plant height and 1000 grain

weight. Results of genotypic and phenotypic correlation coefficients among different characters revealed that grain yield per plant was significantly and positively correlated with pod length and 1000 grain weight. In general, this revealed that the selection for these economic traits is effective in developing high yielding varieties of mungbean. Genetic advance expected in the next generation for number of pod per plant, number of pod cluster and grain yield indicated that these characters are supported by additive gene affects, so these characters would be best for phenotypic selection.

In genetic study, microsatellite markers were used to consider the level of genetic variability in six selected mungbean germplasms on the basis of phenotypic variability. Twenty two microsatellite primers initially were tested, of these 9 SSR loci failed to amplify the mungbean genome or gave irreproducible banding patterns and were subsequently rejected. From 13 remaining SSR loci successfully amplified, six potential polymorphic loci were observed from microsatellite banding profiles on the gel images.

Results from this study showed significant departure from HWE in locus *LR7319B* suggesting that this locus is not assorting randomly. An investigation on the level of variability using six microsatellite primers, revealed the observed heterozygosity was higher than expected heterozigosity for all population, all of these population showed excess in heterozygosity as shown by the negative Fis values.

Genetic diversity measures showed that average number of alleles obtained from six microsatellite markers for populations 40714 and 40521 were significantly high compared to other four populations. 40714 also showed the highest allelic richness between all populations. High level of variation in these two germplasms can be explained the different genetic makeup between these two and the other six populations of mungbean. The high level of microsatellite markers variation which suggests that it is

a good resource for mungbean development.

Through this study, morphological and agronomic traits and characterization of microsatellite markers provide the basis for plant breeding and crossbreeding programs. Via the study genetic diversity of mungbean germplasms, and the contribution in yield components on yield and genetic variability available in the different accessions of mungbean.

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APPENDIXES

APPENDIX A

Table 1: Genotypic scoring for all individuals based on six microsatellites loci

npops = 6											
nloci = 6											
VJ31122A		VJ31122B		LR7323A		LR7323B		LR7322B		LR7319B	
Pop = NM19-19											
217	217	209	197	201	211	281	281	189	177	249	261
217	217	209	197	201	211	281	269	189	175	249	261
217	217	209	197	199	211	281	269	187	175	249	261
217	217	209	197	203	211	281	269	185	175	261	249
217	217	209	197	199	211	281	269	189	189	249	261
217	205	209	197	211	201	281	269	187	171	249	261
217	205	209	197	199	211	281	269	187	171	249	261
217	205	209	197	199	211	281	281	187	171	249	261
217	205	209	209	199	211	281	269	187	173	261	249
217	205	209	197	199	211	281	269	187	175	261	249
217	205	209	197	199	211	281	269	189	173	249	261
217	217	209	197	199	211	281	281	189	173	261	249
217	205	209	197	199	211	269	281	189	173	261	249
217	205	209	193	199	211	281	269	187	173	249	261
217	205	209	209	199	211	281	269	189	173	249	261
217	205	209	209	211	199	281	269	187	173	249	261
217	205	209	197	211	199	281	269	187	175	249	261
217	205	209	197	199	211	281	269	187	175	249	261
Pop = 40995											
217	217	209	197	199	199	291	281	179	173	249	261
217	217	209	209	199	199	291	291	177	169	261	249
217	217	209	197	199	193	291	291	177	169	249	261
217	217	209	197	199	193	291	291	179	169	249	261

217	217	209	209	199	199	291	291	179	175	261	261
217	217	209	209	199	199	291	281	179	175	249	261
217	205	209	193	199	199	281	291	179	173	249	261
217	205	209	197	199	195	291	281	179	169	261	249
217	217	209	209	199	199	291	281	179	171	261	245
217	217	209	209	199	195	291	291	179	169	261	249
217	205	209	197	199	195	291	281	175	169	249	261
217	205	209	197	199	197	291	281	179	169	261	249
217	217	209	209	199	195	291	291	179	179	261	249
217	217	213	201	199	195	291	281	179	175	249	261
217	217	209	209	199	197	291	281	179	179	261	249
Pop = 40031											
217	217	209	209	199	199	281	281	185	185	261	261
217	205	209	189	199	193	291	281	179	173	249	261
217	217	209	209	199	199	291	291	179	179	261	261
217	217	209	209	199	199	281	291	179	179	241	261
217	217	209	209	199	199	281	281	179	189	261	249
217	205	209	197	211	199	281	281	185	171	249	261
217	205	209	197	199	193	291	291	179	169	261	249
217	217	209	209	199	211	281	281	179	171	261	261
217	197	209	197	199	211	281	269	185	171	261	249
217	205	209	209	199	211	281	269	185	171	249	261
217	205	209	209	199	199	281	291	179	189	249	261
Pop =40521											
217	217	209	197	199	211	281	281	187	175	249	261
217	217	209	209	203	211	281	269	189	179	263	263
217	217	209	209	199	211	181	281	189	183	261	249
217	217	209	209	207	197	281	281	189	177	249	261

217	205	201	213	207	197	281	269	187	177	249	261
217	205	209	209	199	199	291	281	179	169	249	261
217	205	209	209	199	211	281	281	187	173	249	261
217	217	209	209	201	211	281	281	187	175	261	261
217	217	209	197	199	211	281	269	185	175	261	249
217	217	209	209	201	211	281	281	179	187	261	249
217	205	209	197	199	211	281	269	185	171	261	249
217	205	209	209	207	199	269	281	185	171	261	249
217	205	209	197	211	199	281	269	185	171	249	261
217	205	209	209	199	211	281	269	185	171	249	261
217	217	209	209	199	211	281	269	185	171	249	261
217	205	209	209	199	211	281	269	185	171	249	261
217	205	209	209	199	211	281	269	187	173	249	261
217	205	209	197	199	211	281	269	185	171	261	261
217	217	209	209	199	211	281	281	179	189	261	249
217	217	209	209	199	211	281	281	185	185	261	261
Pop = 40593											
217	217	197	197	199	195	291	291	179	169	261	249
217	217	209	209	?	?	291	281	179	179	261	261
217	217	209	197	199	199	291	267	179	179	249	261
217	217	209	209	?	?	291	291	179	179	261	261
217	205	209	209	199	213	281	291	179	193	261	249
217	217	209	189	199	197	291	281	179	169	261	249
217	205	209	209	199	199	291	281	177	169	261	249
217	205	209	209	203	213	281	269	189	175	249	261
217	205	209	209	203	213	281	281	189	175	249	261

217	217	209	209	199	197	291	289	179	179	261	261
217	205	209	209	?	?	281	291	179	179	261	249
Pop = 40714											
217	217	209	197	211	197	281	269	191	175	249	261
217	217	209	209	199	213	281	291	179	193	249	261
217	205	209	209	199	213	273	273	179	193	261	249
217	205	209	197	199	191	291	291	179	171	249	261
217	217	209	209	213	199	281	269	181	193	249	261
217	217	209	209	203	213	281	281	179	189	261	261
217	217	213	197	199	199	291	281	179	179	249	261
217	205	185	185	213	199	281	281	191	171	249	261
217	217	209	197	199	213	281	291	191	173	261	261
217	205	209	197	203	213	281	256	191	175	249	261
217	205	209	197	211	201	281	269	191	175	249	261
217	205	209	209	201	213	281	269	189	175	249	262
217	205	209	189	211	199	281	269	189	189	261	249
217	205	209	197	213	203	281	269	189	171	249	261
217	217	209	209	199	197	291	289	179	173	261	261

APPENDIX B

Table 2: Comparisons of linkage disequilibrium values for each locus pair combinations.
Disequilibrium values were compared between all pairs of loci in this study and p-values were calculated.

Pop	Locus 1	Locus 2	P Value	Order		P Value	Orig	Rank	FDR	Significant
NM19-19	VJ31122A	VJ31122B	0.356482	A1		0.003024	A35	1	0.00058824	FALSE
NM19-19	VJ31122A	LR7323A	0.189234	A2		0.00736	A49	2	0.00117647	FALSE
NM19-19	VJ31122B	LR7323A	0.730458	A3		0.011032	A32	3	0.00176471	FALSE
NM19-19	VJ31122A	LR7323B	0.244142	A4		0.01734	A78	4	0.00235294	FALSE
NM19-19	VJ31122B	LR7323B	0.66534	A5		0.01895	A9	5	0.00294118	FALSE
NM19-19	LR7323A	LR7323B	1	A6		0.02801	A43	6	0.00352941	FALSE
NM19-19	VJ31122A	LR7322B	0.105868	A7		0.03116	A29	7	0.00411765	FALSE
NM19-19	VJ31122B	LR7322B	0.227386	A8		0.04611	A51	8	0.00470588	FALSE
NM19-19	LR7323A	LR7322B	0.01895	A9		0.051838	A11	9	0.00529412	FALSE
NM19-19	LR7323B	LR7322B	0.573424	A10		0.06274	A81	10	0.00588235	FALSE
NM19-19	VJ31122A	VJ31122B	0.051838	A11		0.06957	A62	11	0.00647059	FALSE
NM19-19	VJ31122A	LR7323A	0.69455	A12		0.08477	A79	12	0.00705882	FALSE
NM19-19	VJ31122B	LR7323A	0.597852	A13		0.087702	A14	13	0.00764706	FALSE
40995	VJ31122A	LR7323B	0.087702	A14		0.105868	A7	14	0.00823529	FALSE
40995	VJ31122A	LR7323B	0.8609	A15		0.12122	A55	15	0.00882353	FALSE
40995	LR7323A	LR7323B	0.26602	A16		0.15616	A34	16	0.00941176	FALSE
40995	VJ31122A	LR7322B	0.37448	A17		0.17856	A42	17	0.01	FALSE
40995	VJ31122B	LR7322B	0.348148	A18		0.18001	A66	18	0.01058824	FALSE
40995	LR7323A	LR7322B	0.593566	A19		0.189234	A2	19	0.01117647	FALSE
40995	LR7323B	LR7322B	0.60473	A20		0.1973	A85	20	0.01176471	FALSE
40995	VJ31122A	LR7319B	1	A21		0.217158	A25	21	0.01235294	FALSE

40995	VJ31122B	LR7319B	1	A22		0.227386	A8	22	0.01294118	FALSE
40995	LR7323A	LR7319B	1	A23		0.23094	A48	23	0.01352941	FALSE
40995	LR7323B	LR7319B	0.656444	A24		0.244142	A4	24	0.01411765	FALSE
40995	LR7322B	LR7319B	0.217158	A25		0.255988	A31	25	0.01470588	FALSE
40995	VJ31122A	VJ31122B	0.628464	A26		0.26602	A16	26	0.01529412	FALSE
40995	VJ31122A	LR7323A	0.720542	A27		0.27491	A71	27	0.01588235	FALSE
40995	VJ31122B	LR7323A	0.366454	A28		0.277938	A38	28	0.01647059	FALSE
40521	VJ31122A	LR7323B	0.03116	A29		0.27865	A64	29	0.01705882	FALSE
40521	VJ31122B	LR7323B	0.735394	A30		0.28489	A46	30	0.01764706	FALSE
40521	LR7323A	LR7323B	0.255988	A31		0.348148	A18	31	0.01823529	FALSE
40521	VJ31122A	LR7322B	0.011032	A32		0.356482	A1	32	0.01882353	FALSE
40521	VJ31122B	LR7322B	0.483578	A33		0.366454	A28	33	0.01941176	FALSE
40521	LR7323A	LR7322B	0.15616	A34		0.37448	A17	34	0.02	FALSE
40521	LR7323B	LR7322B	0.003024	A35		0.39356	A70	35	0.02058824	FALSE
40521	VJ31122A	LR7319B	0.581288	A36		0.41037	A74	36	0.02117647	FALSE
40521	VJ31122B	LR7319B	1	A37		0.42812	A57	37	0.02176471	FALSE
40521	LR7323A	LR7319B	0.277938	A38		0.45809	A59	38	0.02235294	FALSE
40521	LR7323B	LR7319B	0.823302	A39		0.46685	A63	39	0.02294118	FALSE
40521	LR7322B	LR7319B	0.759616	A40		0.47873	A52	40	0.02352941	FALSE
40521	VJ31122A	VJ31122B	0.90586	A41		0.483578	A33	41	0.02411765	FALSE
40521	VJ31122A	LR7323A	0.178556	A42		0.5295	A80	42	0.02470588	FALSE
40521	VJ31122B	LR7323A	0.028012	A43		0.53755	A73	43	0.02529412	FALSE

40521	VJ31122A	LR7323B	0.637428	A44		0.54492	A56	44	0.02588235	FALSE
40521	VJ31122B	LR7323B	0.889334	A45		0.56478	A47	45	0.02647059	FALSE
40521	LR7323A	LR7323B	0.284886	A46		0.57105	A60	46	0.02705882	FALSE
40521	VJ31122A	LR7322B	0.564778	A47		0.57326	A72	47	0.02764706	FALSE
40521	VJ31122B	LR7322B	0.23094	A48		0.573424	A10	48	0.02823529	FALSE
40031	LR7323A	LR7322B	0.007356	A49		0.581288	A36	49	0.02882353	FALSE
40031	LR7323B	LR7322B	0.716436	A50		0.593566	A19	50	0.02941176	FALSE
40031	VJ31122A	LR7319B	0.046114	A51		0.597852	A13	51	0.03	FALSE
40031	VJ31122B	LR7319B	0.478732	A52		0.60473	A20	52	0.03058824	FALSE
40031	LR7323A	LR7319B	0.789158	A53		0.628464	A26	53	0.03117647	FALSE
40031	LR7323B	LR7319B	0.63475	A54		0.63475	A54	54	0.03176471	FALSE
40031	LR7322B	LR7319B	0.121222	A55		0.63743	A44	55	0.03235294	FALSE
40031	VJ31122A	VJ31122B	0.54492	A56		0.64788	A83	56	0.03294118	FALSE
40031	VJ31122A	LR7323A	0.428124	A57		0.656444	A24	57	0.03352941	FALSE
40031	VJ31122B	LR7323A	0.854482	A58		0.66138	A84	58	0.03411765	FALSE
40031	VJ31122A	LR7323B	0.458094	A59		0.66534	A5	59	0.03470588	FALSE
40593	VJ31122B	LR7323B	0.571046	A60		0.69455	A12	60	0.03529412	FALSE
40593	LR7323A	LR7323B	1	A61		0.71644	A50	61	0.03588235	FALSE
40593	VJ31122A	LR7322B	0.069566	A62		0.720542	A27	62	0.03647059	FALSE
40593	VJ31122B	LR7322B	0.466854	A63		0.730458	A3	63	0.03705882	FALSE
40593	LR7323A	LR7322B	0.27865	A64		0.735394	A30	64	0.03764706	FALSE
40521	VJ31122A	LR7323B	0.637428	A65		0.759616	A40	65	0.03823529	FALSE

40593	VJ31122A	LR7319B	0.180012	A66		0.77937	A82	66	0.03882353	FALSE
40593	VJ31122B	LR7319B	1	A67		0.78916	A53	67	0.03941176	FALSE
40593	LR7323A	LR7319B	1	A68		0.823302	A39	68	0.04	FALSE
40593	LR7323B	LR7319B	0.878182	A69		0.85448	A58	69	0.04058824	FALSE
40593	LR7322B	LR7319B	0.393558	A70		0.8609	A15	70	0.04117647	FALSE
40714	VJ31122A	VJ31122B	0.274914	A71		0.86384	A75	71	0.04176471	FALSE
40714	VJ31122A	LR7323A	0.573264	A72		0.87818	A69	72	0.04235294	FALSE
40714	VJ31122B	LR7323A	0.537554	A73		0.88933	A45	73	0.04294118	FALSE
40714	VJ31122A	LR7323B	0.410368	A74		0.90586	A41	74	0.04352941	FALSE
40714	VJ31122B	LR7323B	0.86384	A75		0.95097	A76	75	0.04411765	FALSE
40714	LR7323A	LR7323B	0.950968	A76		0.97488	A65	76	0.04470588	FALSE
40714	VJ31122A	LR7322B	1	A77		1	A6	77	0.04529412	FALSE
40714	VJ31122B	LR7322B	0.01734	A78		1	A21	78	0.04588235	FALSE
40714	LR7323A	LR7322B	0.084768	A79		1	A22	79	0.04647059	FALSE
40714	LR7323B	LR7322B	0.529498	A80		1	A23	80	0.04705882	FALSE
40714	VJ31122A	LR7319B	0.062742	A81		1	A37	81	0.04764706	FALSE
40714	VJ31122B	LR7319B	0.779366	A82		1	A61	82	0.04823529	FALSE
40714	LR7323A	LR7319B	0.647882	A83		1	A67	83	0.04882353	FALSE
40714	LR7323B	LR7319B	0.661378	A84		1	A68	84	0.04941176	FALSE
40714	LR7322B	LR7319B	0.197298	A85		1	A77	85	0.05	FALSE

APPENDIX C

Table 3: Genotypic frequencies of six microsatellites loci for all six population of mungbean

Population / Locus	Genotype					
	NM19-19	**40995**	**40521**	**40031**	**40593**	**40714**
VJ31122A	217/205 (12) 217/217 (7)	217/205 (4) 217/217 (12)	217/205 (10) 217/217 (10)	217/197 (1) 217/205 (5) 217/217 (5)	217/205 (5) 217/217 (6)	217/205 (9) 217/217 (8)
VJ31122B	209/197 (14) 209/209 (3)	209/193 (1) 209/197 (6) 209/209 (7) 213/201 (1)	209/197 (5) 209/209 (14) 213/201 (1)	209/189 (1) 209/197 (3) 209/209 (7)	197/197 (1) 209/189 (1) 209/197 (1) 209/209 (8)	185/185 (1) 209/189 (2) 209/197 (6) 209/209 (6) 213/197 (1)
LR7323A	211/199 (14) 211/201 (3) 211/203 (1)	199/193 (2) 199/195 (5) 199/197 (2) 199/199 (6)	199/199 (1) 207/197 (2) 207/199 (1) 211/199 (13) 211/201 (2) 211/203 (1)	199/193 (2) 199/199 (6) 211/199 **(**4)	199/195 (1) 199/197 (2) 199/199 (3) 213/199 (1) 213/203 (2)	199/191 (1) 199/197 (1) 199/199 (3) 211/197 (1) 211/199 (1) 211/201 (1) 213/199 (5) 213/201 (1) 213/203 (3)

Population / Locus	Genotype					
	NM19-19	**40995**	**40521**	**40031**	**40593**	**40714**
LR7322B	185/175 (1) 187/171 (3) 187/173 (3) 187/175 (4) 189/173 (4) 189/175 (1) 189/177 (1) 189/189 (1)	175/169 (1) 177/169 (2) 179/169 (4) 179/171 (1) 179/173 (2) 179/175 (4) 179/179 (2)	179/169 (1) 185/171 (7) 185/175 (1) 185/185 (1) 187/173 (2) 187/175 (2) 187/177 (1) 187/179 (1) 189/177 (1) 189/179 (2) 189/183 (1)	179/ 169 (1) 179/171 (1) 179/173 (1) 179/179 (3) 185/171 (3) 185/185 (1) 189/179 (2)	177/169 (1) 179/169 (2) 179/179 (6) 189/175 (2) 193/179 (1)	179/169 (1) 179/171 (1) 179/173 (1) 179/179 (2) 185/175 (1) 189/171 (1) 189/175 (1) 189/179 (1) 189/189 (1) 191/171 (1) 191/173 (1) 191/175 (3) 193/179 (2) 193/181 (1)
LR7319B	261/249 (18)	261/245 (1) 261/249 (13) 261/261 (1)	261/249 (16) 261/261 (3) 263/263 (1)	261/241 (1) 261/249 (7) 261/261 (4)	261/249 (8) 261/261 (3)	261/249 (12) 261/261 (3) 262/249 (1)
LR7323B	281/269 (15) 281/281 (5)	291/281 (9) 291/291 (7)	281/181 (1) 281/269 (11) 281/281 (7) 291/281 (1)	281/269 (2) 281/281 (4) 291/281 (3) 291/291 (2)	281/269 (1) 281/281 (1) 291/267(1) 291/281 (5) 291/289 (1) 291/291 (2)	273/273 (1) 281/256 (1) 281/269 (6) 281/281 (2) 291/281 (3) 291/289 (1) 291/291 (2)

Appendix D

Table 4: Ln P(D) value and K value

Summary of Project a1

File

Summary of Simulations

Parameter ...	Run Name	K	Ln P(D)	Var[LnP(D)]	α1	Fst_1	Fst_2	Fst_3	Fst_4	Fst_5	Fst_6
struc	struc_run_1	1	-1374.6	20.7	-	0.0076	-	-	-	-	-
struc	struc_run_2	2	-1210.5	57.7	0.0543	0.1881	0.1394	-	-	-	-
struc	struc_run_3	3	-1206.3	132.9	0.0557	0.2514	0.0176	0.2584	-	-	-
struc	struc_run_4	4	-1252.9	266.9	0.0511	0.0489	0.1889	0.2436	0.2958	-	-
struc	struc_run_5	5	-1240.4	295.3	0.0482	0.1508	0.2858	0.2820	0.2862	0.1561	-
struc	struc_run_6	6	-1241.3	314.2	0.0485	0.3437	0.0642	0.0897	0.1775	0.3097	0.4198