"CHARACTERIZATION OF WINGED BEAN [*Psophocarpuste tragonolobus* (L.) DC.] BASED ON MOLECULAR MARKERS"

A thesis submitted to the

MAHATMA PHULE KRISHI VIDYAPEETH, RAHURI - 413722,
DIST. AHMEDNAGAR, MAHARASHTRA,
INDIA

by

Mr. TRAN QUANG DIEU

(Reg. No. 015/052)

in partial fulfillment of the requirements for the degree

of

MASTER OF SCIENCE (AGRICULTURE)

in

GENETICS AND PLANT BREEDING

DEPARTMENT OF AGRICULTURAL BOTANY
POST GRADUATE INSTITUTE
MAHATMA PHULE KRISHI VIDYAPEETH,
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Approved by

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CANDIDATE'S DECLARATION

I hereby declare that this thesis or part

thereof has not been submitted

by me or any other person to

this or any other University

or Institute for a

Degree or

Diploma.

Place : M.P.K.V., Rahuri

Date : / /2017 **(Tran Quang Dieu)**

Dr. Suresh S. Dodake
Wheat Specialist,
Agriculture Research Station,
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CERTIFICATE

This is to certify that the thesis entitled**,** **"CHARACTERIZATION OF WINGED BEAN [*Psophocarpus tetragonolobus* (L.) DC.] BASED ON MOLECULAR MARKERS**, submitted to MAHATMA PHULE KRISHI VIDYAPEETH, RAHURI, DIST. AHMEDNAGAR, M.S. for the degree of **MASTER OF SCIENCE (AGRICULTURE)** in **GENETICS AND PLANT BREEDING**, embodies the results of a bonafide research carried out by **Mr. TRAN QUANG DIEU,** under my guidance and supervision and that no part of the thesis has been submitted for any other Degree or Diploma or publication in other form.

The assistance and help received during the course of this investigation have been duly acknowledged.

Place : M.P.K.V., Rahuri
Date: / /2017

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CERTIFICATE

This is to certify that the thesis entitled, **"CHARACTERIZATION OF WINGED BEAN [*Psophocarpus tetragonolobus* (L.) DC.] BASED ON MOLECULAR MARKERS",** submitted to the MAHATMA PHULE KRISHI VIDYAPEETH, RAHURI, DIST. AHMEDNAGAR, M.S. for the degree of **MASTER OF SCIENCE (AGRICULTURE)** in **GENETICS AND PLANT BREEDING**, embodies the results of a bona-fide research carried out by **Mr. TRAN QUANG DIEU,** under the guidance and supervision of **Dr. S. S. DODAKE,** Wheat Specialist, Agriculture Research Station, Niphad, Dist. Nashik, MPKV, Rahuri and that no part of the thesis has been submitted for any other Degree or Diploma.

Place : M.P.K.V., Rahuri
Date : / /2017

(B. R. Ulmek)
Associate Dean

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Place : M.P.K.V., Rahuri
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Tran Quang Dieu

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LIST OF ABBREVIATIONS

%	:	Percent
/	:	or, per
µg	:	Microgram
µl	:	Microlitre
µM	:	Micromolar
bp	:	Base pair
CTAB	:	Cetyl Tri-methyl Ammonium Bromide
dNTPs	:	Deoxynucleotide triphosphates
EDTA	:	Ethylene diamine tetra acetic acid
et al.	:	Et alli (and other)
kg	:	Kilogram
M	:	Molar
mg	:	Milligram
ml	:	Milliliter
mM	:	Millimolar
NaCl	:	Sodium Chloride
ng	:	Nanogram
rpm	:	Revolutions per minute
ISSR	:	Inter Simple Sequence Repeat
Taq	:	*Thermusaquaticus*
TBE buffer	:	Tris base, Boric Acid, EDTA buffer
TE buffer	:	Tris base, EDTA buffer
Tris buffer	:	Tris (hydroxymethyl) aminomethane
U	:	Unit
UV	:	Ultra violet
V	:	Volt
v/v	:	Volume by volume
viz.	:	Namely
w/v	:	Weight by volume

ABSTRACT

CHARACTERIZATION OF WINGED BEAN [*Psophocarpus tetragonolobus* (L.) DC.] BASED ON MOLECULAR MARKERS

by

Mr. Tran Quang Dieu

A candidate for the degree

of

MASTER OF SCIENCE (AGRICULTURE)

in

GENETICS AND PLANT BREEDING

Research Guide	**:**	**Dr. S. S. Dodake**
Discipline	**:**	**Genetics and Plant Breeding**

The present study was undertaken with the objective to estimate genetic diversity and establish a genetic relationship among winged bean genotypes using ISSR markers.

Twenty winged bean genotypes received from All India Co-ordinated Research Network on Potential Crop, Department of Agricultural Botany, MPKV, Rahuri was sown and used for assessment of molecular polymorphism, diversity analysis.

Total 16 ISSR primers were used for this study, out of which, only 11 primers successfully amplified and all of them were polymorphic.

On PCR amplification, 124 bands position were generated from 20 winged bean genotypes with the size ranging from 150bp to 2000bp. Out of which, 76 were polymorphic with 61.3% polymorphism. However, polymorphism information content (PIC) value for ISSR ranged from 0.06 to 0.39, while their resolving power (Rp) ranged between 0.9 to 4.7.

The Dice similarity coefficient values based on ISSR primers analysis ranged from 0.72 to 0.98. Out of 190 genotype combinations, EC-142667 and EC-27885 won closet with similarity value 0.98; while EC-178310 and EC-178269 won most distinct with similarity index 0.72.

Dendrograms were constructed based on unbiased measure of genetic distance by UPGMA method from two major clusters with four main group. First cluster composed of a single distinct genotype EC14266; while second cluster had two sub-cluster IIa (4 genotypes) and IIb (15 genotypes).

The two and three-dimension principle component analysis (PCA) based on ISSR data showed similar clustering of the 20 winged bean as evident from dendrogram tree analysis. Genotype EC14266 (Group I) was placed isolated separated from 12 genotypes by 2nd component (Y axis). Two other groups, group IIb1 (EC-178268, EC-178269 and EC-178311) and group IIa (EC-178291, EC-178310, EC-121921, EC-251020) were also placed on same side of Y axis. These 8 genotypes were thus showing clear variability from rest 12 genotypes placed closely on another half showing limited

divergence. The X axis (-0.33 to +0.32) and Y axis (-0.36 to +0.18) showed limited scale of divergence in winged bean genotypes studied.

Hence, the genetic distance estimates based on the ISSR markers indicate the closed relationship and narrow genetic background between winged bean accession.

I. INTRODUCTION

Psophocarpus tetragonolobus (L.) DC. (Fam. Fabaceae), popularly known as winged bean or Goa bean is a tropical legume found growing abundantly in hot, humid equatorial countries like India, Burma, Sri Lanka, Thailand and the Philippines. It is also called a wonder legume as it has the high protein content in the seeds and therefore considered as a versatile legume (Peyachoknagul *et al*., 1989). It is a diploid ($2n = 2x = 18$), self-fertilizing leguminous crop with multifarious usage (NAS, 1975). It can be grown as a grain legume, green vegetable, tuber-crop or a forage and cover-crop (Khan, 1982). Seeds of winged bean contain some pharmacologically active anti-nutrients such as trypsin and chymotrypsin inhibitors (Kortt, 1980), haematoglutins and amylase inhibitors. There are other unfavorable compounds like tannins (proanthocyanidins), which have been reported to be present in the seeds of winged bean (Kantha *et al.*, 1986). Tannins are polyphenolic compounds and are either hydrolysable or condensed. It can interact and precipitate with protein and therefore, reduce food protein quality in monogastric organisms (Cabrera and Martin, 1986). Knowledge of genetic diversity within and among genotypes of any crop is fundamental to estimate the potential of genetic gain in breeding programs and for effective conservation and sustainable utilization of available genetic resources (Sakiyama, 2000). All food legumes are valuable sources of proteins, vitamins and minerals and occupy an important place in human nutrition.

Biotechnology and genetic engineering hold great potential for plant breeding. During the past few years, new strategies based on molecular markers have been proposed to reduce time and effort in plant breeding. Molecular markers which are associated with economically important traits, based on direct analysis of the genomic DNA have been used widely for genetic mapping, DNA fingerprinting, phylogenetic and evolutionary studies, heterosis breeding, gene tagging and marker assisted selection (MAS) by plant breeders as a selection tool (Paterson *et al.*, 1991; Darvasi and Soller, 1994).

The presence of genetic diversity is crucial for improving the quality of any plant species. An understanding of the magnitude and pattern of genetic diversity in crop/forestry plants has important implications in breeding programs and for conservation of genetic resources (Charu *et al*., 2013). It is important to find out the amount of genetic variability by the way of morphological, biochemical and molecular markers. Characterization of diversity has long been based on morphological traits mainly. However, morphological variability is often restricted, characters may not be obvious at all stages of the plant development and their appearance may be affected by environment. Nowadays, a genotype of different genetic markers has been proposed to assess genetic variability as a complementary strategy to more traditional approaches in genetic resources management. Molecular

marker tools provide valuable data on diversity through their ability to detect variation at the DNA level.

Assessment of genetic variations and relationships among these leguminous crops may therefore, play a significant role in breeding programs to improve grain yield, oil and protein content. Winged bean is of rapidly increasing interest as a high-protein multipurpose crop. The breeding of winged bean as a grain legume requires the development of an improved ideotype with the highest nutritional content and lowest anti-nutritional factors (Lazaroff, 1989).

Over the years, the methods for detecting and assessing genetic diversity have extended from analysis of discrete morphological traits to advanced biochemical and molecular traits. Several DNA based molecular markers are now currently used in diversity study of plants. Amongst them inter simple sequence repeat (ISSR) markers are commonly used to characterize the genetic diversity of crop plants and can be immensely helpful in identifying the potential elite genotypes. The utility of this marker as a potential tool for documenting the genetic variations in several legume crops like Chickpea (Sant *et al.*, 1999; Collard *et al.*, 2003). Lens (Durán *et al.*, 2004), Lentil (Fikiru *et al.*, 2007), Groundnut (Mondal *et al.*, 2009), *Cajanas cajan* (Malviya and Yadav, 2010), Cluster bean (Pathak *et al.*, 2010), Mung bean and Black gram (Tantasawat *et al.*, 2010) etc., have been clearly established over the period of time. However, no such reports on

genetic diversity assessment studies using molecular markers separately are available in winged bean. Thus, in the present study, ISSR marker was used to evaluate the extent of genetic diversity among 20 winged bean genotypes and their efficiency in analyzing the genetic variations will be compared.

Keeping in view the above points, present research work will focus on the following objectives:

- To estimate genetic diversity and establish a genetic relationship among winged bean genotypes using ISSR marker

II. REVIEW OF LITERATURE

2.1 Winged bean

The winged bean is a species that belongs to the genus *Psophocarpus* which includes 6-9 different species (Khan, 1982). Species in the *Psophocarpus* genus are perennial herbs grown as annuals. They are capable of climbing by twining their stems around a support. In addition, they have tuberous roots and pods with wings. Winged bean is nutrient-rich, and all parts of the plant are edible. Its leaves can be eaten like spinach, flowers can be used in salads, tubers can be eaten raw or cooked, and seeds can be used in similar ways as the soybean. The winged bean is an underutilized species but has the potential to become a major multi-purpose food crop in the tropics of Asia, Africa and Latin America (Khan, 1982).

The winged bean plant grows as a vine with climbing stems and leaves, 3–4 m in height. It is an herbaceous perennial, but can be grown as an annual. It is generally taller and notably larger than the common bean (*Phaseolus vulgaris)*. The bean pod is typically 15–22 cm (6–9 inches) long and has four wings with frilly edges running lengthwise. The skin is waxy and the flesh partially translucent in the young pods. When the pod is fully ripe, it turns an ash-brown color and splits open to release the seeds. The large flower is pale blue. The winged beans themselves are similar to soybeans in both use and nutritional content (being 29.8% to 39% protein) (Amoo *et al.*, 2006).

There is abundant variation in the appearance of winged bean. The shape of its leaves ranges from ovate, deltoid, ovate-lanceolate, lanceolate and long lanceolate (Khan, 1982). The leaves of winged bean also vary in color appearing as different shades of green. Stem color is commonly green, but can vary from shades of green to shades of purple (Erskine, 1980).

Pod shape is most commonly rectangular, but can also appear flat. Pod color may also vary from shades of cream, green, pink or purple. The exterior surface of the pod also varies in texture. Pods can appear smooth or rough depending on genotype. Seed shape is often round, but oval and rectangular seeds are also found. Seed color changes based on environmental factors and storage conditions (Khan, 1982). Seeds may appear white, cream, brown or dark tan in appearance. The shape of winged bean tuberous roots also shows variation (NAS, 1975).

The winged bean is rich in protein and tocopherol, an antioxidant that increases vitamin A use in the body (NAS, 1975). Its ability to grow in heavy rainfall makes the species a good crop to adequately nourish the people of tropical equatorial countries in Africa. The winged bean can also be used to produce winged bean milk made from water, winged beans, and emulsifier (Yang and Tan, 2011). Winged bean milk has similar characteristics as soymilk, but without the same bean-rich flavor. Winged bean has also been reported as an effective remedy for smallpox and as a cure for vertigo in Malaysia (Duke and DuCellier, 1993).

A study by Mohanty *et al*. (2014) showed that fatty oil from fully mature seeds of winged bean contained high proportion of unsaturated fatty acids (75.5 %), while immature seeds contained higher percentage of saturated fatty acid (61.3 %). Further, unsaponification matter (0.25 %) of fatty oil was calculated as stigmasterol (66.4 %) and β-sitosterol (25.1 %). The fatty oil of fully mature seeds was enriched with mono-unsaturated fatty acids (38.6 %) and poly-unsaturated fatty acids (36.9 %) without trans-fatty acids, thus meeting the edible oil standard.

The winged bean also provides many opportunities for economic benefit. Many parts of the winged bean can be sold. Mature seeds can bring in a high price. There is evidence of smoked pods, uncooked tubers, cooked tubers, dry seeds and leaves being sold in domestic markets in South East and South Asia. Winged bean also has the potential to be used as animal feed for livestock and poultry. The winged bean also has the potential to be used as a replacement for fish meal used to raise African catfish (*Clarias gariepinus*), a highly-valued food fish in Africa. Feeding fish represents a large portion of operating cost for fish farmers and fishmeal is scarce and high-priced. Winged bean can be used as the primary protein source for fish feed to reduce farmer dependence on fish meal availability (Fagbenro, 1999)

Winged bean also shows potential as a cover crop and a restorative crop. Planting winged bean uniform with the ground can reduce weeds and functions well as a cover crop. The winged bean can also function

effectively as a restorative crop that can improve nutrient poor soil with nitrogen when it is turned over into the soil (Lynd *et al.*, 1983).

2.2 Assessment of plant genetic diversity

The importance of plant genetic diversity is now being recognized as a specific area since exploding population with urbanization and decreasing cultivable lands are the critical factors contributing to food insecurity in developing world. Scientists realized that plant genetic diversity can be captured and stored in the form of plant genetic resources such as gene bank, DNA library, and so forth, in the biorepository which preserve genetic material for long period (Govindaraj *et al*., 2015).

Knowledge about germplasm diversity and genetic relationships among breeding materials is an invaluable component in crop improvement strategies. Various methods are currently available for analysis of genetic diversity in germplasm accessions, breeding lines and populations. These methods have relied upon pedigree data, morphological data, agronomic performance data, biochemical data, and more recently molecular data (Mohammadi and Prasanna, 2003).

The assessment of genetic diversity within and between populations is being performed at the molecular level using various techniques such as DNA analysis, which measure levels of variation directly. Genetic diversity may also be estimated using morphological, and biochemical characterization and evaluation:

i) Morphological characterization does not demand expensive technology but depend upon the available of land and time, making it possibly more expensive than molecular assessment. Morphological traits are often susceptible to phenotypic plasticity; conversely, this allows assessment of diversity in the presence of environmental variation.

ii) Biochemical analysis is based on the separation of proteins into specific banding patterns. It is a fast method which requires only small amounts of biological material. However, the number of enzymes are limited caused in limitation of the resolution of diversity

iii) Molecular analyses comprise a large variety of DNA molecular markers, which can be employed for analysis of variation. Different markers have different genetic qualities (they can be dominant or co-dominant, can amplify anonymous or characterized loci, can contain expressed or non-expressed sequences, etc.).

Genetic markers is not a new concept; in the nineteenth century, Gregor Mendel employed phenotype-based genetic markers in his experiments (Mendel, 1865). Later, phenotype-based genetic markers for *Drosophila melanogaster* led to the findings of the theory of genetic linkage, occurring when particular genetic loci or alleles for genes are inherited jointly (Morgan, 1910). Thus, the limitations of phenotype-based genetic markers led to the development of DNA-based markers, i.e., molecular markers (Mondini *et al.*, 2009).

2.3 Molecular Markers

A molecular marker can be defined as a genomic locus, detected through probes or specific primers which, in virtue of its presence, distinguishes unequivocally the chromosomic trait which it represents as well as the flanking regions at the 3' and 5' extremity (Barcaccia *et al.*, 2000).

A DNA marker is a DNA sequence observed in minimum two easy to distinguish version, which reveals individual polymorphism. The preferred marker should demonstrate the widest possible range of variation in the analyzed trait, and it should not be affected by environmental factors. Molecular markers facilitate analyses of variations between individuals, regardless of their development stage which is particularly useful in sex determination studies of plants. A number of reviews have been published on different aspects of molecular markers and their application in crop improvement.

Molecular markers are used to trace a desired gene in examined phenotypes, based on naturally occurring polymorphism, which can be identified. The technique exploits the fact that marker locus identifies a chromosomal segment and enable that segment to be monitored in subsequent generation. They have advantages over traditional phenotypic markers because they are phenotypically neutral as alternates alleles at molecular loci and cause no obvious changes in the phenotypic expression

(Tanksley, 1983). Molecular markers have emerged as a fascinating technology for evaluating genetic diversity (Naik *et al.*, 2017, Wong *et al.*, 2017, Zebarjadi *et al.*, 2016, Zong *et al.*, 2015, Charu *et al.*, 2013; Wang *et al.*, 2011; Goswami and Tripathi, 2010), genetic structure variation and fingerprinting (Hardesty *et al.*, 2005; Bhagyawant and Srivastava, 2008; Shanjani *et al.*, 2009). In addition, molecular marker techniques can also offer reliable tools for the early determination of sex in plants before they enter the reproductive stage (Milewicz and Sawicki, 2013).

The commonly used polymerase chain reaction (PCR)-based DNA marker systems are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and more recently simple sequence repeats (SSRs) or microsatellites (Staub *et al.*, 1996; Gupta and Varshney, 2000). The major limitations of these methods are low reproducibility of RAPD, high cost of AFLP and the need to know the flanking sequences to develop species specific primers for SSR polymorphism. ISSR-PCR is a technique that overcomes most of these limitations.

2.4 Inter Simple Sequence Repeats (ISSR)

In inter simple sequence repeat (Zietkiezicz *et al.*, 1994), primers based on microsatellites are utilized to amplify inter simsple sequence repeat DNA sequences. ISSR technique is a PCR based method, which involves amplification of DNA segment present at an amplifiable distance in between

two identical microsatellites repeat regions oriented in opposite direction. The technique uses microsatellites, usually 16–25 bp long, as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter-SSR sequences of different sizes (Reddy *et al.*, 2002).

The microsatellite repeats used as primers can be di-nucleotide, tri-nucleotide, tetranucleotide or penta-nucleotide. The main advantage of ISSRs is that there is no sequence data prerequisite for primer construction are needed. Furthermore, ISSRs are randomly distributed throughout the genome. This is mostly dominant marker, though occasionally exhibits as codominance. Because of the multi locus fingerprinting profiles obtained, ISSR analysis can be applied in studies involving genetic identity, clone and strain identification, and taxonomic studies of closely related species

2.5 Genetic diversity studies using ISSR markers

Durán *et al*. (2004) concluded that ISSR markers are valuable tools for Lens genetic mapping and they have a high potential in the generation of saturated *Lens* maps. A Lens map was developed based on the segregational analysis of five kinds of molecular and morphological genetic markers in 113 F2 plants obtained from a single hybrid of *Lens culinaris ssp. Culinaris* x *L. c. ssp. orientalis*.

Awasthi *et al.* (2004) used PCR based marker assays including RAPD and ISSR to study the genetic diversity and interrelationships among twelve domesticated and three wild mulberry species (genus Morus). RAPD

analysis using 19 random primers generated 128 discrete bands, ranging from 500 – 3000bp in size, 119 of these were a few putative specific amplification products which could be useful for germplasm classification and introgression studies. Cluster analysis of RAPD and ISSR data resulted into two clusters, one comprising polyploid wild species and the other with domesticated species.

Fikiru *et al*. (2007) used 4 ISSR primers (UBC primer set #9, Vancouver BC, Canada) assess the level of genetic diversity, genetic structure and genetic distance, and to indirectly estimate the level of gene flow among 70 Ethiopian lentil landraces. A total of 47 bands were amplified, out of which, 28 were polymorphic and no unique bands were observed. There was high genetic differentiation (GST = 0.455) but intermediate gene flow level (Nm = 0.60) among populations. The Inter-population genetic distance (D) ranged from 0.012 to 0.228. The results provide important baseline for future germplasm conservation and improvement programs.

Chen *et al.* (2008) assessed genetic diversity and genetic relationships of 92 cultivars of sacred lotus (*Nelumbo nucifera* Gaertn.). Their results showed that lotus exhibited a low level of genetic diversity which may result from its asexual mode of reproduction and long-term artificial selection. Clustering analyses indicated that these cultivars divided into two clades. Most cultivars of Chinese lotus species origin were included in one clade,

and other American lotus species origin was in the other clade. Seven cultivars native to Thailand formed a distinct subclade among the cultivars of Chinese lotus species origin. Genetic differentiation between two subspecies, and between cultivars from Thailand and other cultivars could be attributed to geographic isolation.

Terzopoulos and Bebeli (2008) described the genetic diversity of 20 faba bean (*Vicia faba* L.) local populations, five minor-type and 15 Mediterranean-type populations using 5 ISSR markers. Based on Nei's standard genetic distances, both cluster analysis using UPGMA and principal coordinate analysis (PCoA) separated four minor-type populations from the Mediterranean-type populations. The groups studied are promising for the production of synthetic varieties.

Bhagyawant and Srivastava (2008) used 10 ISSR primers to analyze genetic diversity of chickpea germplasm. Amplification of genomic DNA of the 12 genotypes using ISSR analysis yielded 492 visible bands. The primers based on poly (ATG) and (GAA) motifs produced least number of fragments (36) whereas, primers (AC) T and (AC) TT, produced maximum number of fragments (96). The results obtained in the investigation show that the ISSR primers are informative markers which can be examined to correlate banding patterns and agronomic characteristics.

Geleta and Bryngelsson (2009) used ISSR technique to study genetic variation of the Campanulaceae (*Lobelia rhynchopetalum*), based on ten

populations sampled from Bale and Simen mountains in Ethiopia. The percentage of polymorphic loci across all samples and within population was 78% and 27%, respectively. Regardless of a high total genetic variation, the species has quite low variation within populations. All genetic variation analyses revealed higher variation among populations than within populations.

Lisek and Rozpara (2009) used ISSR technique to determine the genetic similarity between 18 cultivars of sour cherry (*Prunus cerasus* L.), 24 cultivars of sweet cherry (*Prunus avium* L.) and 9 types of rootstocks for plants of these species. The highest degree of DNA polymorphism was observed in the case of the rootstocks (71.2%); 50.7% for sour cherry and 39.5% for sweet cherry, respectively. It was possible to distinguish between types of rootstocks using only two primers (827, 841), cultivars of sour cherry using also two primers (825, 841), whereas in order to distinguish the sweet cherry cultivars, three primers had to be used: 830, 841 and 843. Obtained ISSR markers allow the identification of tested genotypes as well as their more accurate characterization. Results of the research may find application in gene banks of Prunus genotypes, and in orchard and nursery practice.

Mondal *et al.* (2009) used 21 ISSR primers to analyze genetic diversity among twenty cultivated groundnuts (*Arachis hypogaea* L.) genotypes. Out of 154 amplicons produced by 21 ISSR primers, 115 were

found polymorphic (74.67%). The 3'-anchored primers based on poly 'GA' and poly 'AG' motifs produced higher proportion of polymorphism of 74.85% and 77.27%, respectively. Based on Kruskal Wallis one way ANOVA, UBC 810540 was found associated with both rust and late leaf spot (LLS) resistance and UBC810500 with LLS resistance.

Tantasawat *et al.* (2010) evaluated genetic relationship of 17 mung bean (*Vigna radiata* (L.) Wilczek) and 5 black gram (*Vigna mungo* (L.) Hepper) genotypes by means of 18 ISSR analysis and morphological characters. Pair wise coefficients of ISSR-based genetic similarity between all genotypes ranged from 0.70 to 0.99 with an average of 0.86, suggesting quite narrow genetic base of mung bean and black gram that might limit continued breeding success. UPGMA analysis based on ISSR exhibited 2 major clusters. It appeared that ISSR was more effective for classification at the species level although no clear separation at the subspecies level was found. All 22 mung bean and black gram genotypes can be effectively distinguished by only 6 ISSR primers with the highest PIC values, suggesting the applicability of ISSR analysis for variety identification.

Liu and Lin (2011) used ISSR markers for preliminary assessment of Genetic Diversity in Cultivated *Glycyrrhiza* uralensis, *G*. inflate and *G*. glabra. Five efficient ISSR primers were screened and optimized for detecting the genetic diversity. From the UPGMA clustering results, genetic diversity was indicated by Nei's similarity coefficients ranged from 0.45 to

0.51. The higher genetic diversity in G. inflate was useful to more broad breeding. The result suggest that provides an optimized method for assessment genetic diversity of cultivated *Glycyrrhiza* uralensis, *G.* inflate and *G.* glabra using ISSR markers which is useful for further investigation in breeding.

Dos Santos *et al.* (2011) assessed genetic variation among 45 accessions of sweet, purple, and yellow passion fruit were assessed using 18 ISSR primers. Nei's genetic distance between accessions ranged from 0.04 to 0.35. Clustering using the neighbor-joining method resulted in the formation of 11 major clusters. It was not possible to classify the accessions according to their geographic origin, showing that there is no structure in the gene bank. The overall mean Shannon–Weaver diversity index was 0.32, indicating good resolution of genetic diversity in passion fruit germplasm using ISSR markers. These results indicate that ISSR can be useful for genetic diversity studies, to provide practical information for parental selection and to assist breeding and conservation strategies.

Aruna *et al.* (2012) analyzed diversity of sorghum for resistance to shoot pests using 26 ISSR primers. Cluster analysis based on ISSR markers resulted in two major groups, resistance sources, and the susceptible control and hybrid parental lines. It was noted that the susceptible elite hybrid parents and susceptible control were genetically similar, and resistance

sources were genetically diverse as a group, offering the possibility that they may have diverse resistance mechanisms.

Kebour *et al.* (2012) used ISSR markers for the study of genetic polymorphism of the Pistachio fruit in Algeria. Fifty four percent of polymorphism bands was recorded. The matrix has a genetic distance of 0,61 to 0,82 with a mean of 0,56, assumed that the varieties are characterized by a high degree of genetic diversity at the DNA level. Principle coordinate analysis (PCA) based on genetic similarity matrices were used to visualize the genetic relationships among genotypes, confirmed the results of cluster analysis.

Gaafar *et al.* (2014) assessed genetic diversity in the endangered populations of *Breonadia salicina* (Rubiaceae) growing in The Kingdom of Saudi Arabia using 14 ISSR primers selected from 43 primers. The amplification gave rise to 211 amplified loci, of which 68 were polymorphic. Nei's gene diversity and Shannon's information index were 0.086 and 0.125, respectively. A hierarchical analysis of molecular variance revealed a high level of genetic differentiation among populations (17% of total variance, P = 0.001), consistent with the gene differentiation coefficient (GST = 0.256). Nevertheless, the evaluated genetic diversity was very low within populations; while relatively high among populations, levels were insufficient for long term survival.

Alam *et al.* (2015) evaluated genetic diversity and relationships among 45 collected purslane accessions using 28 ISSR primers. The genetic diversity as estimated by Shannon's information index was 0.513, revealing a quite high level of genetic diversity in the germplasm. The UPGMA dendrogram based on Nei's genetic distance grouped the whole germplasm into 7 distinct clusters. Based on the constructed dendrogram using ISSR markers those accessions that are far from each other by virtue of genetic origin and diversity index are strongly recommended to select as parent for future breeding program to develop high yielding and stress tolerant purslane variety in contribution to global food security.

Al-Turki and Basahi (2015) assessed 27 landraces of Hassawi rice growing in Al-Ahsa region of Saudi Arabia and deposited at King Abdulaziz City by using 14 primers to estimate genetic diversity. The analysis of ISSR polymorphism divided the examined rice landraces into two groups. The results indicated that ISSR fingerprints are efficient in the identification and resolution of genetic diversity between the landraces of the Hassawi rice and will be an efficient method in the authentication of the rice germplasm in the gene bank of Saudi Arabia.

Zebarjadi *et al.* (2016) used 14 specific ISSR primers to assess genetic diversity of 21 Harmal (*Peganum harmala* L.) accessions collected from different regions of Iran. The results based on cluster analysis categorized all accessions into three groups that did not completely match to

their geographic pattern place of collection. Result of principle coordinate analysis of samples also showed distribution pattern similar to cluster analysis.

Anjali *et al.* (2016) analyzed intraspecific variation in *Elettaria cardamomum Maton* (Cardamom) by flow cytometry, cytological studies and ISSR analysis. The nuclear DNA content was estimated in various sections of the species representing individuals from wild and cultivar genotypes following *Zea mays* L. Twenty-six ISSR primers were used for genetic diversity analysis of the thirty accessions of cardamom. Among the cardamom genotypes, C53 (feral from Bonacaud) showed a very prominent level of genetic diversity and was lowest for C96 (Avinash-I, a released variety from Indian Institute of Spices Research, Kozhikode).

Haritha *et al.* (2016) used 17 ISSR primers to determine genetic diversity and phylogenetic relationships in 90 genotypes of wild and cultivated species of *Oryza* from different geographical regions of the world. Un-weighted pair group method with arithmetic mean dendrogram and population structure based on the 17 primers separated all genotypes into 4 major clusters with a genetic similarity of 53%–100%. The study would help to identify closely related or distantly related accessions for use in introgression of new variability into rice cultivars. It would also help to identify duplicates in wild accessions in germplasm banks, to study genetic

differentiation in wild rice populations from different locations and decide on populations or accessions for conservation.

Gautam *et al.* (2016) estimated genetic diversity in 13 accessions of chickpea including cultivated and wild by 10 ISSR primers. A total of 150 bands were amplified in a molecular weight range of 100-2000 bps revealing an average of 21.4 bands per primers and 1.64 bands per primer per genotype. The cophenetic correlation between ultrametric similarities of the tree and similarity matrix was high, indicated the cluster analysis represents the similarity data. Based on genetic origin and diversity index viz. ICC-14051, ICC-13441, ICC-15518, ICC-12537, and ICC-17121 recommended to be selected as a parent in future breeding programmes for chickpea.

Tabin *et al.* (2016) used 17 ISSR primers to evaluate genetic diversity of three *Rheum* species, namely, *Rheum emodi*, R. *spiciforme* and R. *webbianum* from Kashmir Himalaya. Total 17 primers produced 94 loci in a size range of 100 bp to 2500 bp. Mean Polymorphism Information Content (PIC) and Marker Index (MI) were 0.449 and 2.58, respectively. Dendrograms of each species showed at least two groups while combined dendrogram showed three groups with intermixing, indicating possible high levels of cross breeding between species. Analysis of Molecular Variance (AMOVA) partitioned 96% genetic variance within species and 4% among species. Bayesian model based STRUCTURE analysis detected three gene

pools for Rheum germplasm prevailing in this region and showed high admixture within individuals. Although different geographical populations of these three species from Kashmir Himalaya showed a high level of genetic diversity.

Nath et al. (2017) assessed genetic diversity in green gram [*Vigna radiata* (L.) Wilczek] using 25 ISSR primer. Total 109 bands were scored, out of which, 88 bands were polymorphic and the level of polymorphism was 81 per cent. The average number of bands per primer was found to be 6.22 and average numbers of polymorphic bands per primer were 4.89. The similarity values ranged from 0.43 to 0.80 indicated the high level dissimilarity between green gram genotypes.

Muhammad *et al.* (2017) evaluated twenty one maize genotypes using 20 ISSR primers to estimate the genetic diversity. Total of 190 polymorphic bands with an average of 9.5 alleles per primer were identified. The coefficient of genetic similarity ranged from 0.888 to 0.118%. The highest similarity was found between accessions 12 (015224) and 9 (015114), whereas the lowest similarity was found between genotypes 20 (EV-5098) and 14 (015030). The PIC value ranged from 0.17 to 0.47. A dendrogram was generated based on Jaccard's distance matrix and the identified genotypes could be used as parents for future development of diverse populations.

Handayani and Rahayu (2017) studied genetic diversity of 8 Lai (*Durio kutejensis*) local cultivars of Batuah (Indonesia) using 10 ISSR primers. The result shows that similarity coefficient of cultivars ranged between 0,34 - 0,58. Seven cultivars were grouped in the same cluster with 0,44 of similarity coefficient, while Lai Durian was separated in 0,34 of similarity coefficient. Genetically, Lai Kuning and Lai Belimbing were found to be the most similar existing cultivar with the similarity coefficient of 0,58.

The clonal fidelity among the in vitro-regenerated plantlets was also assessed using ISSR marker in *Tylophora indica* (Burm. f.) Merrill (Sharma *et al.*, 2014), *Dendrocalamus strictus* (Goyal *et al.*, 2015), *Nothapodytes nimmoniana* (Prakash *et al.*, 2016) and *Saccharum officinarum* L. (Thorat *et al.*, 2017). The true-to-type nature of the micropropagated plants was confirmed based on their monomorphic banding profiles with that of the mother plants.

Not only in plants, ISSR markers also used for estimating the genetic diversity of pathogens cause the disease of plant *viz.* invasive aspergillosis caused by *Aspergillus terreus* (Neal *et al.*, 2011), Downy mildew (Innark *et al.*, 2014) or parasitic fungus (Qi *et al.*, 2015). These studies revealed that the relationship between sib-ship and geographical distribution was intensive and provides evidence of a population structure linked to geographical origin of pathogens.

2.6 Diversity of winged bean based on molecular markers

Although winged bean is an amazing potential vegetable crop (Singh *et al.*, 2013), there was only few reports on molecular characterization and evaluation of genetic diversity at present.

Mohanty *et al*. (2013) used 13 and 7 primers of RAPD and ISSR respectively to estimate the genetic diversity of 24 winged bean genotypes. The significant correlation (r = 0.839) between similarity matrices was obtained. ISSR technique was found more efficient than RAPD to unravel polymorphism in Winged Bean genotypes. There were little significant correlations between the physiological clustering patterns and those obtained by cumulative analysis of RAPD and ISSR data except for few genotypes in which the inter cluster distance was least. The results obtained in the study suggested that physiological diversity in the genotypes of winged bean need not be necessarily related to genetic diversity.

Chen *et al.*, (2015) used ISSR marker with 5 primers to evaluate genetic diversity of 45 germplasm of Winged Bean. The result showed that the genetic dissimilarity coefficients ranging from 0.73 to 0.97, had a little genetic variation. The dendrogram of 45 winged bean genotypes did not show any clear pattern of clustering according to the land cultivation. The genetic distance and genetic identity indicated that it had a closed relationship and narrow genetic background. Only two genotype name Tu3

and M26 is obvious distantly, and the two accessions facilitate gene exchange and genetic improvement of Winged Bean.

Wong *et al*. (2016) developed an expressed sequence dataset based on Illumina Mi-Seq sequencing of pooled cDNA libraries prepared from leaf, root, pod and reproductive tissues from winged bean genotypes. A complete assembly of all four types of tissues produced 198,554 contigs. Out of the total 24,598 SSR motifs identified, 84 SSR markers were tested and verified through PCR amplification, giving rise to a set of 26 polymorphic SSR markers. Twenty-six polymorphic markers were used to assess the genetic diversity of 26 winged bean accessions and to generate a dendrogram that indicated the genetic relationships between all the accessions. Information from this study could facilitate the development of improved cultivars of winged bean and help to elevate its importance for nutritional security and sustained livelihoods through a directed breeding programmes.

The same study was also conducted by Wong *et al.* (2017) to develop gene-based SSR markers in winged bean for diversity assessment. In conclusion, total of 18 SSR markers targeting di- and tri-nucleotide repeats have been validated as polymorphic markers based on an initial assessment of nine genotypes originated from five countries. A cluster analysis revealed provisional clusters among this limited, yet diverse selection of germplasm. The developed assembly and validated genic SSRs in this study provide a

foundation for a better understanding of the plant breeding system for the genetic improvement of winged bean.

III. MATERIALS AND METHODS

The present investigation entitled "Characterization of Winged Bean [*Psophocarpus tetragonolobus* (L.) DC.] genotypes based on molecular markers" was carried out at State Level Biotechnology Centre, Mahama Phule Krishi Vidyapeeth, Rahuri, Dist. Ahmednagar during the year 2016-2017.

The details of materials used and method adopted in the present study are mentioned under following subheadings.

3.1 Materials

3.1.1 Winged bean genotypes

A set of 20 winged bean genotypes (Table No. 3.1) received from Plant Breeder, All India Co-ordinated Research Network on Potential Crop, Department of Agricultural Botany, MPKV, Rahuri was sown and used for assessment of molecular polymorphism, diversity analysis.

3.1.2 Chemicals

All the chemicals used in the present study were either analytical grade or molecular biology grade and procured from different firms *viz:*

1. Bangalore Genei, Bangalore
2. Genetics biotech Asis Pvt. Ltd., New Delhi
3. Himedia Laboratory Pvt. Ltd., Mumbai
4. Chromous Biotech Pvt.Ltd

Table No. 3.1 Winged bean germplasm used in the present study

Sr. No.	Genotype	Sr. No.	Genotype
1	IC-95221	11	EC-178291
2	EC-27885	12	EC-178310
3	EC-38821	13	EC-178311
4	EC-121921	14	EC-178317
5	EC-142653	15	EC-178318
6	EC-142661	16	EC-178319
7	EC-142666	17	EC-178332
8	EC-142667	18	EC-198327
9	EC-178268	19	EC-251020
10	EC-178269	20	EC-178331

3.1.3 Glasswares and equipment

Glasswares used in various experiments were of Borosil brand. Different equipment used for this study includes ultra-centrifuge, water bath, dry bath, PCR thermos-cycler, horizontal gel electrophoresis, gel documentation unit, shaker, etc.

3.2 Methodology

3.2.1 Isolation of DNA

The genomic DNA was extracted from leaves of seedlings from 20 genotypes of Winged Bean following CTAB method described by Aljanabi *et al*. (1999) with some modifications.

Reagents

1. CTAB Extraction buffer (2% CTAB, 2.2M NaCl, 0.1 M Tris HCl pH 8.0, 50 mM EDTA pH 8.0, 0.2% Beta-MercaptoEthanol – added at the time of use)
2. 20% Cetyl Trimethyl Ammonium Bromide (CTAB)
3. 10% Polyvinylpyrrolidone(PVP)
4. 5% N-Lauroyl-sarcosine
5. Chloroform : Isoamyl Alcohol (24:1)
6. RNase 10mg/ml, stored at -20^{o}C
7. Chilled Isopropanol
8. Phenol : Chloroform : Isoamyl Alcohol (25:24:1) (P:C:I)
9. 70% Ehanol
10. TE buffer (10 mM Tris and 1mM EDTA, autoclave before use)
11. Sodium Acetate pH 5.2
12. Liquid Nitrogen

Glasswares and equipments

1. Sterile Eppendorf tubes
2. Micropipettes
3. Conical flask
4. Beakers
5. Centrifugation
6. Water bath

Procedures

1. CTAB extraction buffer was prepared and warmed at 65°C in water bath.
2. Leaf samples (200 mg) were grinded in liquid nitrogen using mortar and pestle.
3. The powder was transferred into 2ml tube contained 1ml warm CTAB extraction buffer (65°C)
4. Then, the following reagents were added:
    - 200 µl 5% N-Lauryl-Sarcosine
    - 200 µl 10% PVP
    - 200 µl 20% CTAB
5. Tubes were mixed by inverting and incubated for 1 hours in water bath at 65°C. During incubation, tubes were inverted every 8 – 10 minutes to prevent the tissue separation from extraction buffer
6. After incubation, tubes were cooled down on ice for 5 minutes, and centrifugation was carried out at 12,000 rpm for 10 minutes at 4°C.
7. After centrifugation, equal volume of P:C:I (25:24:1) was added and mixed by inversion; then centrifuged againat 12,000 rpm for 10 minutes at 4°C.
8. After centrifugation, the top aqueous phase was collected into new tubes and added 5 µl RNAse then incubated for 30 minutes at 37°C.

9. After incubation, equal volume of chilled Isopropanol, followed by 200 µl 6M NaCl were added and mixed by inversion.
10. Tubes were incubated in depthrefrigerator at -20°C for 1 hours.
11. After incubation, tubes were centrifuged at 12,000 rpm for 10 minutes at 4°C.
12. After centrifugation, the supernatant was discarded, pellet was collected and washed with 70% ethanol.
13. Supernatant was discarded and pellet was air dried.
14. Finally, pellet was dissolved in 60 µl TE buffer and store at 4°C.

3.2.2 DNA quantification and purity analysis

Concentration of DNA was measured using Spectrophotometer (Nanodrop, ND-1000, USA) at 260 and 280 nm. The absorbance of various DNA samples was measured at a wave-length of 260nm against TE buffer blank. Good DNA preparation will show the ratio of A_{260}/A_{280} at 1.8. The following inferences can be drawn from this ratio about the quality of DNA

- A_{260}/A_{280} = 1.8 indicates pure DNA
- A_{260}/A_{280}> 1.8 indicates RNA contamination
- A_{260}/A_{280}< 1.8 indicates protein contamination

The integrity of genomic DNA was determined by running the DNA on 0.8% agarose gel, and visualized on gel documentation unit (Gel Logic 1500 imaging system). Based on this concentration, the stock genomic DNA was diluted to working concentration of 50 ng/µl for PCR assays.

Agarose gel electrophoresis

Reagents

1. 1X Tris Borate EDTA (TBE) buffer
2. 6x gel loading dye
3. Lambda DNA
4. Agarose Low EEO
5. Ethidium Bromide

Equipment

1. Horizontal gel electrophoresis unit (Biorad sub cell model 96 USA)
2. UV visualize and gel documentation unit (Gel logic 1500 imaging system)
3. Microwave Oven

Protocol

1. The Pyres gel casting plate open ends were sealed with cello tape and the comb was placed properly in casting plate kept on a perfectly horizontal platform.
2. 0.8% agarose was prepared add added to 1X TBE buffer (0.8 gr/ 100ml)
3. The mixture was boiled using microwave oven until the agarose dissolved completely and then allowed to cool. 5 µl of Ethidium bromide (DNA intercalating agent) was added when the

temperature reached 55 – 60°C just prior to solid as a staining agent.

4. After that, it was poured into the gel mound and allowed to solidify.
5. The comb and the cello tape were removed carefully after solidification of agarose.
6. The casted gel was placed in the electrophoresis unit wells towards the cathode and submerged with 1X TBE buffer to a depth of about 1cm.
7. 1 µl of DNA sample was mixed well with 3 µl of loading dye on parafilm by pipetting several times and loaded into the well.
8. Electrophoresis was run at 80V for 60 minutes.

3.2.3 DNA amplification by ISSR primers

Amplification reaction mixture was prepared in 0.2 ml thin wallet flat capped PCR tubes, contained the following components (Table No. 3.2). The total volume of each reaction mixture was 20 µl. Total of 16 primers were used for this study, but only 11 primers got amplified (Table No. 3.4).

Procedure

The 20 µl reaction mixture was gently vortexed and spinned down. The DNA amplification was carried out in the Corbett Research PCR Palm Cycler. The PCR conditions set for amplification were tabulated as

following (Table No. 3.3). The samples were placed in thermal cycler until it reached 50°C.

Table No. 3.2 Composition of PCR reaction mixture

PCR reaction component	Initial concentration	Volume collected
Genei Tag DNA buffer B	10X	2 µl
$MgCl_2$	25 mM	2 µl
dNTP mix	2.5 mM	2 µl
Primer	25 picomoles	1 µl
GeneiTaq DNA polymerase	3 Units/µl	0.33 µl
Template DNA	50 ng	1 µl
Sterilized distilled water	----	11.67 µl
Total volume		**20 µl**

Table No. 3.3 PCR programme set in thermal cycler

Name of the step followed	Temperature	Time	Cycle
Initial denaturation	94°C	5 min	1 cycle
Denaturation	94°C	1 min	35 cycles
Annealing	Depends on primer	1 min	
Extension/ Elongation	72°C	1 min	
Final extension	72°C	10 min	1 cycle
Final hold	4°C	Till retrieval	-

Table No. 3.4 List of ISSR primers used in this study

Sr. No.	Primers name	Sequences of primers (5' - 3')	Annealing temperature (°C)
1	ISSR-8932801	AGCAGCAGCAGCAT	48.5
2	ISSR-8932802	CACACACACACAAT	42.0
3	ISSR-8932803	CACACACACACAAC	44.0
4	ISSR-8932804	CACACACACACAGT	44.0
5	ISSR-8932806	CACACACACACAGA	44.0
6	ISSR-8932807	CACACACACACAAA	42.0
7	ISSR-8932808	GTGTGTGTGTGTTA	44.0
8	ISSR-8932809	GTGTGTGTGTGTTG	46.5
9	ISSR-8932811	GTGTGTGTGTGTCT	44.0
10	ISSR-8932812	CAGGAGAGAGAGAGA	39.0
11	ISSR-8932815	GCTGAGAGAGAGAGA	44.0

Agarose gel electrophoresis of amplified PCR products

Requirements

Electrophoresis unit (gel casting trough, gel combs, power pack)

UV transilluminator

Solutions required

- Ethidium bromide 10mg/ml
- 6X Loading dye (Bromophenol blue)
- Agarose Low EEO
- 1X TBE buffer pH 8.0
- 100bp DNA ladder marker

Procedure

1. The Pyres gel casting plate open ends were sealed with cello tape and the comb was placed properly in casting plate kept on a perfectly horizontal platform.
2. 1.5% Agarose was prepared add added to 1X TBE buffer (1.5 gr/ 100ml)
3. The mixture was boiled using micro oven until the agarose dissolved completely and then allowed to cool. 5 µl of Ethidium bromide (DNA intercalating agent) was added when the temperature reached 55 – 60^{o}C just prior to solid as a staining agent.

4. After that, it was poured into the gel mound and allowed to solidify.
5. The comb and the cello tape were removed carefully after solidification of agarose.
6. The casted gel was placed in the electrophoresis unit wells towards the cathode and submerged with 1X TBE buffer to a depth of about 1cm.
7. 20 µl of DNA sample was mixed well with 3 µl of loading dye and loaded into the well.
8. A 100bp DNA ladder marker (step up) was used as a molecular size reference for band yield from PCR.
9. Electrophoresis was run at 80V and stopped when the dye front run about 2 cm away from the well.
10. The amplified PCR products were observed under UV transilluminator in gel documentation system (Gel Logic 1500 imaging system) and image was captured.

3.2.4 Data Analysis

The clearly resolved PCR amplified ISSR bands of 20 winged bean genotypes with 11 different primers were scored manually for their presence (1) and absence (0) in the datasheet. Data was analyzed and similarity matrix was constructed from binary data with Dice similarity coefficients which were calculated as per model suggested by Nei and Li, 1979.

The binary data scored was used to calculate the similarity genetic distance using Simqual sub programme of NTSYS-pc 2.1 software (Numerical Taxonomy and Multivariate Analysis System Programme, Rohlf, 2004). Dendrogram was constructed by using distance matrix by the unweighed pair group method with arithmetic average (UPGMA) sub programme of NTSYS-pc.

The Polymorphism Information content (PIC) value was calculated according to De Riek *et al*. (2001):

$$\mathbf{PIC=1-p_i^2- q_i^2}$$

Where, p_i is the presence frequency of the i^{th} allele.

q_i is the absence frequency of the i^{th} allele

The frequency of an allele was calculated by dividing the number of genotypes where the band was found by the total number of genotypes.

The ability of the primers to distinguish between accessions was assessed by calculating their Resolving power (Rp) according to Prevost and Wilkinson (1999) as following:

$$\mathbf{Rp= \sum Ib}$$

Where Ib is band infomativeness.

Ib = 1-[2 x (|0.5 – p|)] where "p" is the proportion of accession containing the band.

Principal component analysis (PCA) was done using Eigen module of NTSYS-pc. The results were graphically express in the form of

two dimension (2D) and three dimension (3D) plots generated by graphic module of NTSYS-pc.

IV. RESULTS

The aim of this investigation was to study the genetic diversity in the available winged bean genotypes. For this purpose, ISSR marker was used to conduct the experiment. Results obtained are presented and discussed in the light of the available literature.

4.1 Genomic DNA quantification and quality estimation

The amount and purity of DNA (Table No. 4.1) was quantified by measuring the optical density (O.D) at both 260 and 280 nm wavelength Nanodrop® ND-1000 spectrophotometer (Saveen Werner, Sweden). Clear bands without any smear indicated the integrity of DNA being free from nuclease contamination. After comparing the DNA intensity with known amount unit lambda phage DNA and O.D at 260 nm wavelength by, the DNA samples were diluted to individual working concentration of 50ng/µl.

Table No. 4.1 Quality and Quantity of the total genomic DNA isolated from 20 winged bean genotypes

Sr. No.	Genotype	Quality of DNA isolated (A260/A280)	Yield of DNA (ng per µl)
1	IC-95221	1.76	745.87
2	EC-27885	1.74	400.46
3	EC-38821	1.78	844.93
4	EC-121921	1.77	840.89
5	EC-142653	1.75	967.89
6	EC-142661	1.74	1386.2
7	EC-142666	1.78	795.71
8	EC-142667	1.81	924.36
9	EC-178268	1.79	631.77
10	EC-178269	1.76	572.83
11	EC-178291	1.8	800.19
12	EC-178310	1.73	956.2
13	EC-178311	1.77	959.99
14	EC-178317	1.79	1030.57
15	EC-178318	1.73	936.32
16	EC-178319	1.76	843.91
17	EC-178332	1.79	398.87
18	EC-198327	1.74	684.73
19	EC-251020	1.8	575.81
20	EC-178331	1.79	429.85

4.2 ISSR analysis

The genomic DNA was isolated from 20 winged bean genotypes and was subjected to PCR amplification using 16 ISSR primers. Five primers failed to amplify in any sample studied. Annealing temperature for each of the primer was fixed as shown on Table No. 3.4.

Table No. 4.2 Details of the ISSR amplification of winged bean genotypes

Sr. No.	ISSR Analysis	Observation
1	Total number of primer used	11
2	Number of primer amplified the genomic DNA	11
3	Total number of polymorphic markers	11
4	Total number of bands position	124
5	Total number of polymorphic bands	76
6	Total number of monomorphic bands	48
7	Total number of unique bands	3
8	Per cent polymorphism	61.29
9	Average band position produced per primer	11.27
10	Average polymorphic band produced per primer	6.91
11	Size of amplified products	150-2000bp

Plate 1

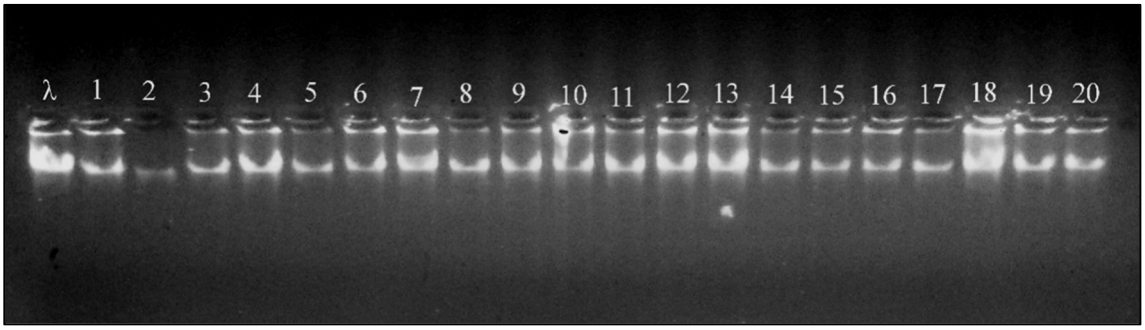


Figure 1. Agarose gel (0.8 %) electrophoretic pattern of total genomic DNA isolated from 20 winged bean genotypes

Plate 2

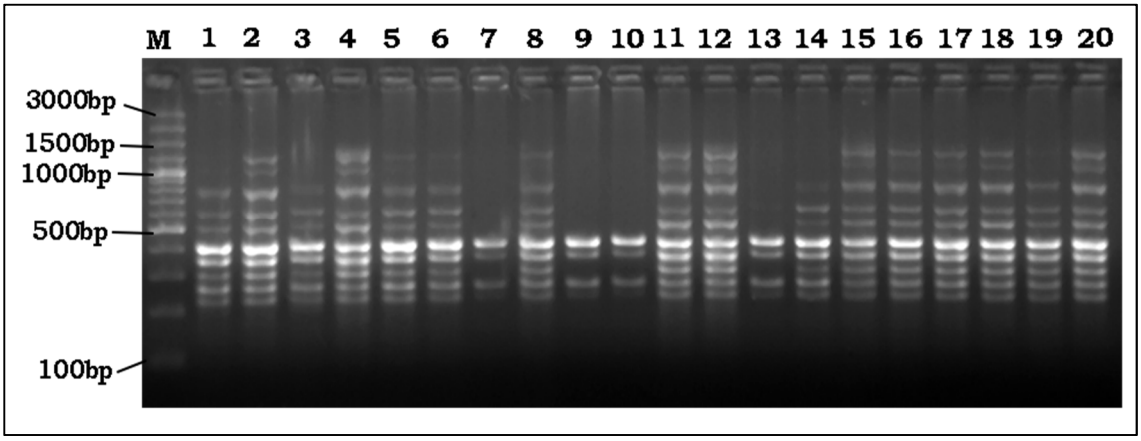


Figure 2. ISSR profile of 20 genotypes using primer ISSR-8932801

Lane λ: Lambda DNA **Lane M: 100 bp DNA ladder**

Lane	Genotypes	Lane	Genotypes	Lane	Genotypes	Lane	Genotypes
1	IC-95221	6	EC-142661	11	EC-178291	16	EC-178319
2	EC-27885	7	EC-142666	12	EC-178310	17	EC-178332
3	EC-38821	8	EC-142667	13	EC-178311	18	EC-198327
4	EC-121921	9	EC-178268	14	EC-178317	19	EC-251020
5	EC-142653	10	EC-178269	15	EC-178318	20	EC-178331

Plate 3

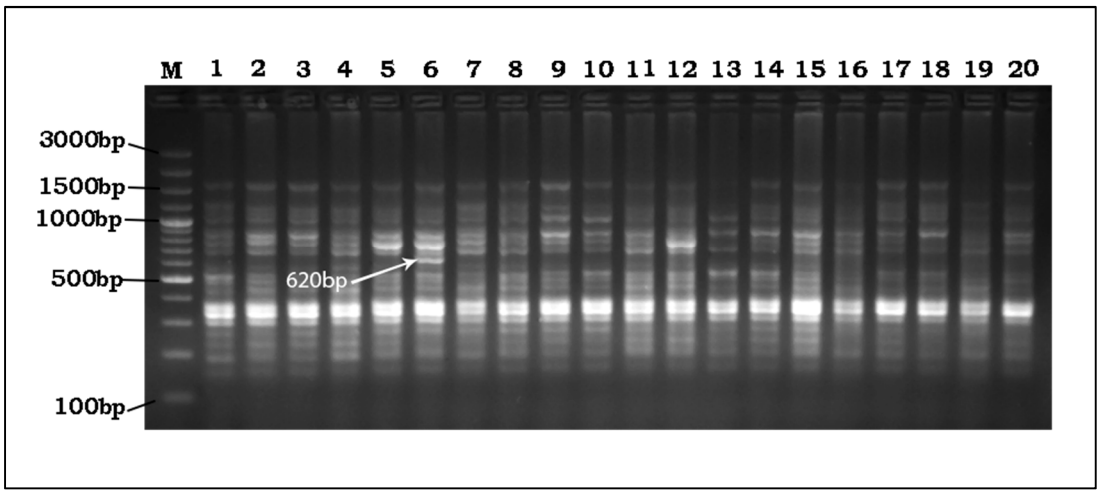


Figure 3. ISSR profile of 20 genotypes using primer ISSR-8932802

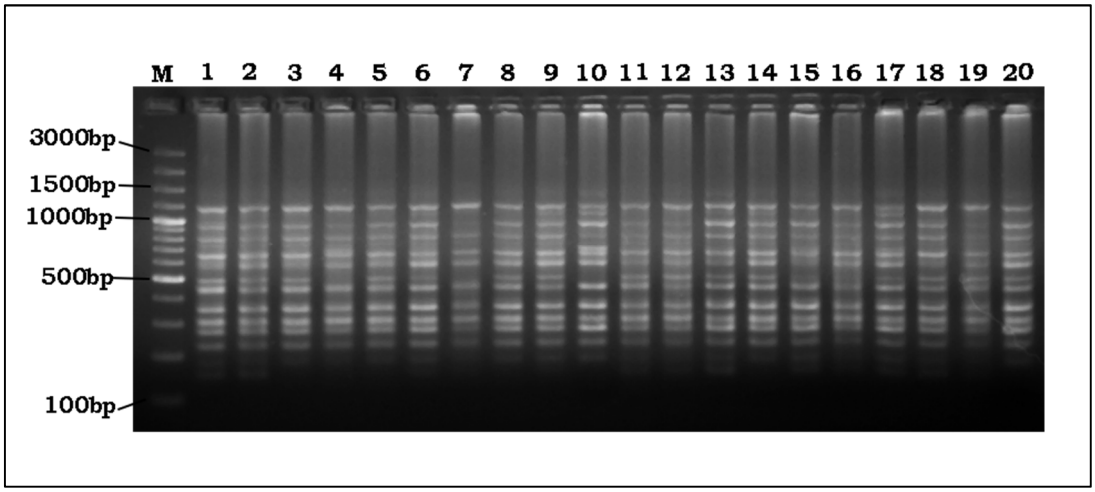


Figure 4. ISSR profile of 20 genotypes using primer ISSR-8932803

Lane M: 100bp DNA ladder

Lane	Genotypes	Lane	Genotypes	Lane	Genotypes	Lane	Genotypes
1	IC-95221	6	EC-142661	11	EC-178291	16	EC-178319
2	EC-27885	7	EC-142666	12	EC-178310	17	EC-178332
3	EC-38821	8	EC-142667	13	EC-178311	18	EC-198327
4	EC-121921	9	EC-178268	14	EC-178317	19	EC-251020
5	EC-142653	10	EC-178269	15	EC-178318	20	EC-178331

Plate 4

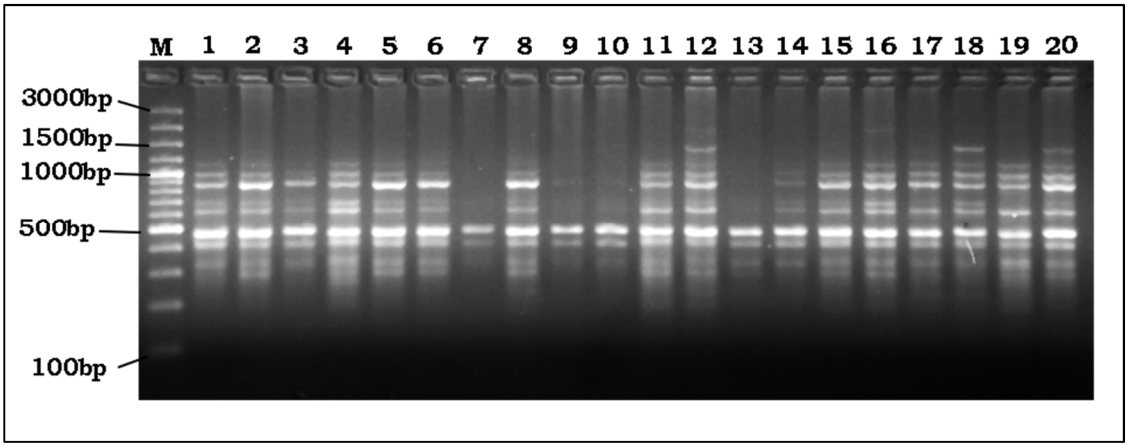


Figure 5. ISSR profile of 20 genotypes using primer ISSR-8932804

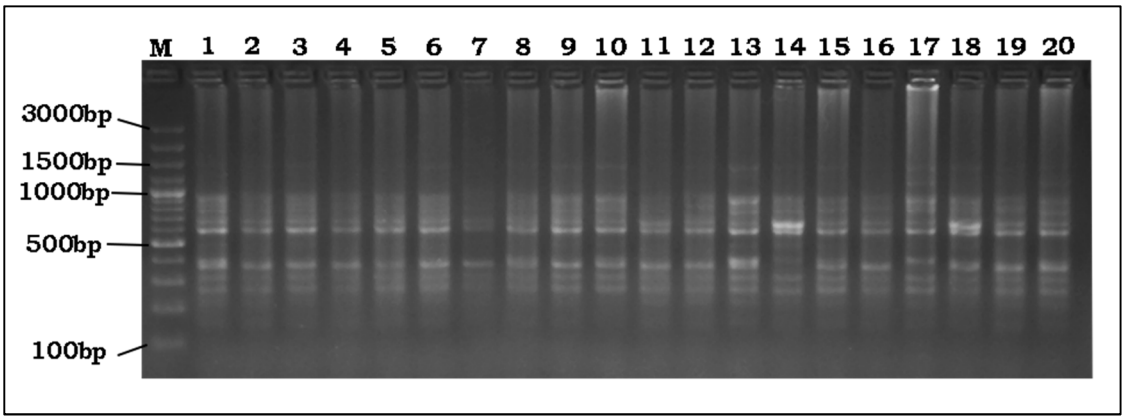


Figure 6. ISSR profile of 20 genotypes using primer ISSR-8932806

Lane M: 100bp DNA ladder

Lane	Genotypes	Lane	Genotypes	Lane	Genotypes	Lane	Genotypes
1	IC-95221	6	EC-142661	11	EC-178291	16	EC-178319
2	EC-27885	7	EC-142666	12	EC-178310	17	EC-178332
3	EC-38821	8	EC-142667	13	EC-178311	18	EC-198327
4	EC-121921	9	EC-178268	14	EC-178317	19	EC-251020
5	EC-142653	10	EC-178269	15	EC-178318	20	EC-178331

Plate 5

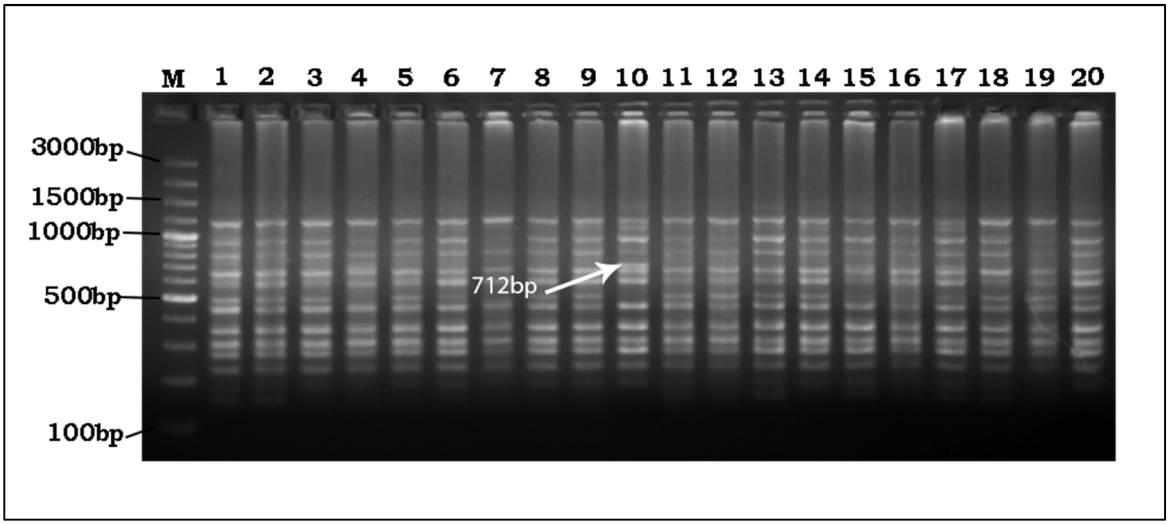


Figure 7. ISSR profile of 20 genotypes using primer ISSR-8932807

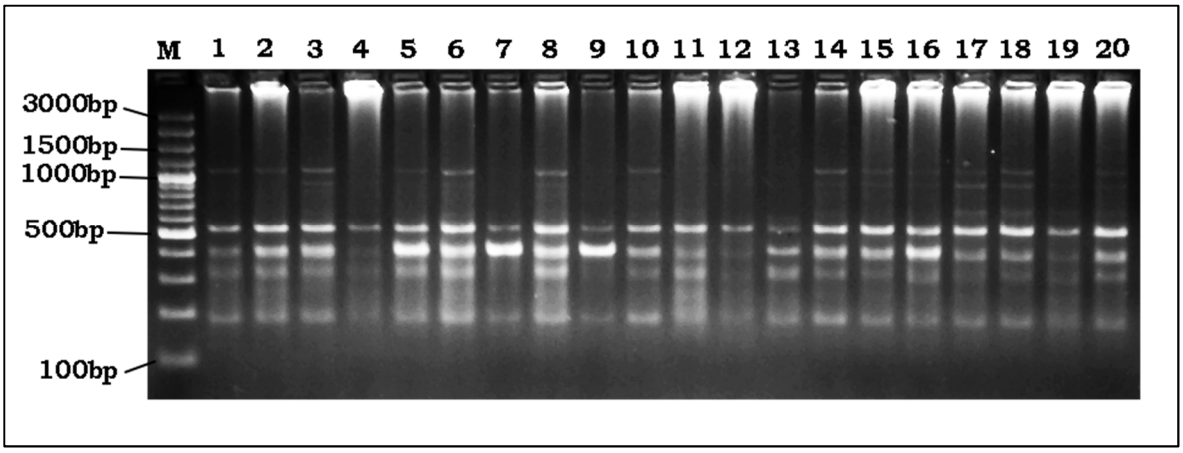


Figure 8. ISSR profile of 20 genotypes using primer ISSR-8932808

Lane M: 100bp DNA ladder

Lane	Genotypes	Lane	Genotypes	Lane	Genotypes	Lane	Genotypes
1	IC-95221	6	EC-142661	11	EC-178291	16	EC-178319
2	EC-27885	7	EC-142666	12	EC-178310	17	EC-178332
3	EC-38821	8	EC-142667	13	EC-178311	18	EC-198327
4	EC-121921	9	EC-178268	14	EC-178317	19	EC-251020
5	EC-142653	10	EC-178269	15	EC-178318	20	EC-178331

Plate 6

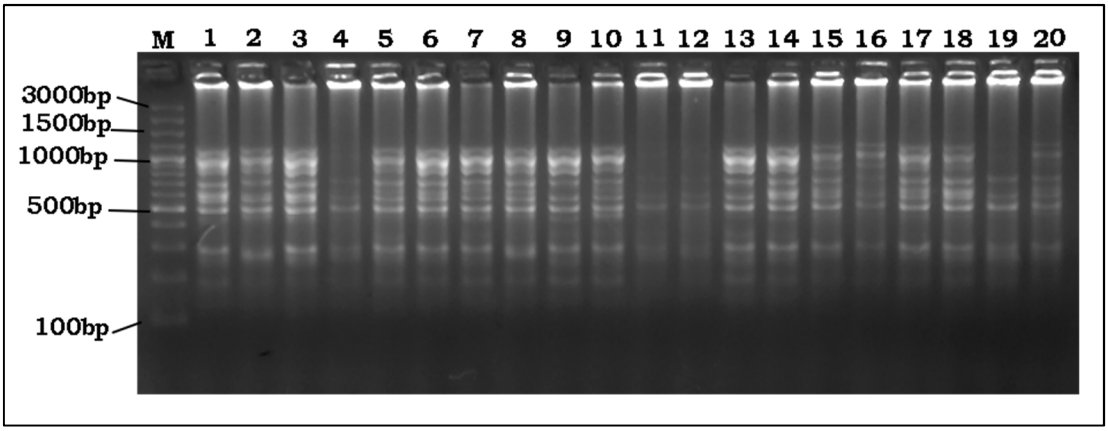


Figure 9. ISSR profile of 20 genotypes using primer ISSR-8932809

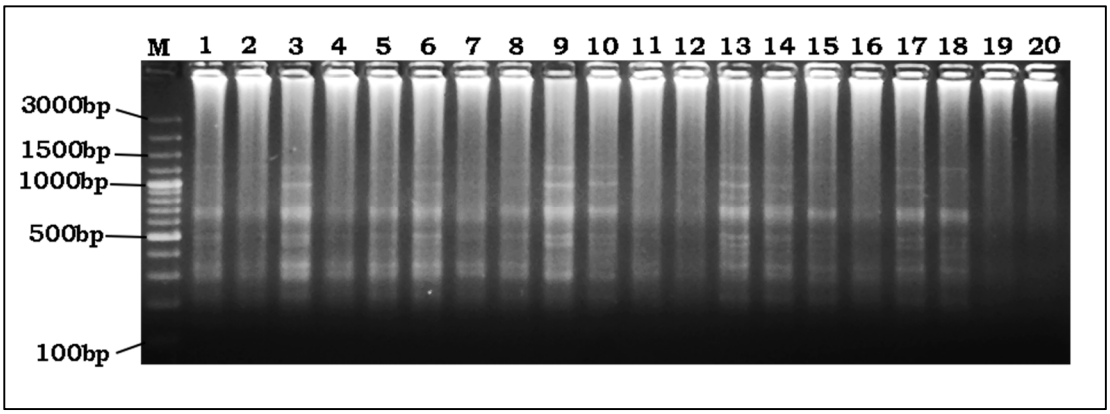


Figure 10. ISSR profile of 20 genotypes using primer ISSR-89328011

Lane M: 100bp DNA ladder

Lane	Genotypes	Lane	Genotypes	Lane	Genotypes	Lane	Genotypes
1	IC-95221	6	EC-142661	11	EC-178291	16	EC-178319
2	EC-27885	7	EC-142666	12	EC-178310	17	EC-178332
3	EC-38821	8	EC-142667	13	EC-178311	18	EC-198327
4	EC-121921	9	EC-178268	14	EC-178317	19	EC-251020
5	EC-142653	10	EC-178269	15	EC-178318	20	EC-178331

Plate 7

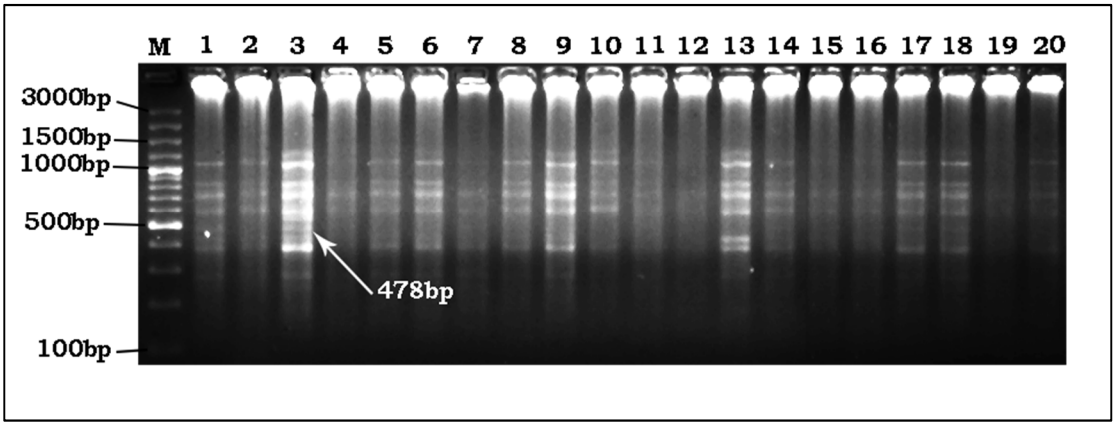


Figure 11. ISSR profile of 20 genotypes using primer ISSR-8932812

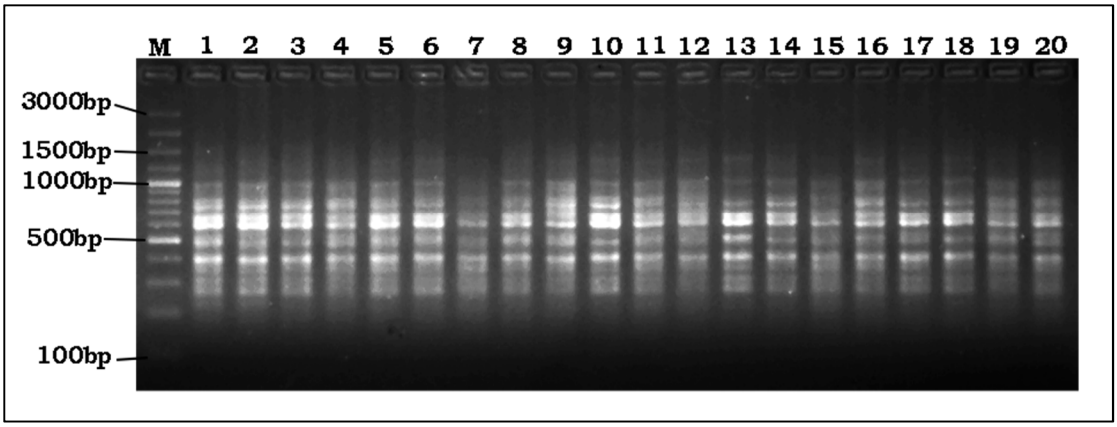


Figure 12. ISSR profile of 20 genotypes using primer ISSR-8932815

Lane M: 100bp DNA ladder

Lane	Genotypes	Lane	Genotypes	Lane	Genotypes	Lane	Genotypes
1	IC-95221	6	EC-142661	11	EC-178291	16	EC-178319
2	EC-27885	7	EC-142666	12	EC-178310	17	EC-178332
3	EC-38821	8	EC-142667	13	EC-178311	18	EC-198327
4	EC-121921	9	EC-178268	14	EC-178317	19	EC-251020
5	EC-142653	10	EC-178269	15	EC-178318	20	EC-178331

From the ISSR analysis, it was observed that a total of 124 bands were generated by amplification with 11 polymorphic primers, from which 76 bands were polymorphic, 48 bands were monomorphic and 3 unique bands. Each ISSR primer thus produced on an average 6.91 Polymorphic bands. The size of amplification ranged from 150 to 2000 bp.

Among 11 ISSR primers, ISSR-8932802 produced maximum number of 16 bands, followed by ISSR-8932807 (15 bands) and ISSR-8932803 (13 bands) and ISSR-8932809, ISSR-8932815 (12 bands). The least number of bands was amplified by ISSR-8932808 (7 bands) (Table No. 4.3).

The percentage of polymorphic bands ranged from 33.33% in primer ISSR-8932807 to 100% in primer ISSR-8932811, with an average polymorphism of 61.29%.

The polymorphism information content (PIC) values and resolving power values (Rp) werecalculated to find out the efficiency of primers in distinguishing individual genotypes. The PIC values of ISSR primers ranged from 0.06 in primer ISSR-8932803 to 0.39 in primer ISSR-8932811 with an average of 0.2 across 11 ISSR primers.

Resolving power (Rp) values of ISSR primers ranged from 0.9 in primer ISSR-8932803 to 4.7 in primer ISSR-8932811. The mean number

of bands per locus was ranged from 9.6 in primer ISSR-8932811 to 18.2 in primer ISSR-8932803. The maximum mean number of bands per accession was observed in ISSR-8932811 while the minimum was recorded in primer ISSR-8932802.

Primer ISSR-8932801 amplified 10 loci, out of which 6 were polymorphic, 4 were monomorphic. The percent of polymorphic band was 60%. The size of bands ranged from 200-1500bp.

Total 16 loci were amplified by primer ISSR-8932802 with 8 polymorphic (50%), 8 monomorphic and 1 unique. The unique locus having size 620 bp was present in genotype EC-142661.

The ISSR primer, ISSR-8932803 amplified 13 loci, out of which only 5 were polymorphic, 8 were monomorphic and no unique locus. The percent of polymorphic band was 38.4%.

ISSR-8932804 amplified 11 loci in which 9 loci were polymorphic and 2 of them were monomorphic. The same number of loci amplified (11) were observed in primer ISSR-8932806 with 7 polymorphic and 4 monomorphic. The result showed 81.8 percent of polymorphic band.

Five polymorphic loci (33.3% of polymorphic) are the result given by primer ISSR-8932807. This primer amplified total of 15 loci with 10 monomorphic and 1 unique band. Size of amplified bands ranged from 150-1500bp. The unique band was recorded in genotype EC-178269 and the locus was 712 bp in length.

Table No. 4.3 Information content of ISSR primers used for winged bean divergence analysis

Sr. No.	Primers	Total number of bands	Number of bands position	Mean number of bands/ loci	Mean number of bands/ accession	Number of Polymorphic bands	Unique bands	Percentage polymorphic bands (%)	PIC value	Rp value	Approx. band size range (bp)
1	ISSR-8932801	165	10	16.5	8.3	6	0	60.00	0.23	3.5	200-1500
2	ISSR-8932802	274	16	17.1	13.7	8	1	50.00	0.13	2.8	150-1800
3	ISSR-8932803	237	13	18.2	11.9	5	0	38.46	0.06	0.9	150-1400
4	ISSR-8932804	155	11	14.1	7.8	9	0	81.82	0.25	3.7	200-2000
5	ISSR-8932806	162	11	14.7	8.1	7	0	63.64	0.16	2.2	150-1500
6	ISSR-8932807	247	15	16.5	12.4	5	1	33.33	0.12	2.7	150-1500
7	ISSR-8932808	102	7	14.6	5.1	6	0	85.71	0.28	2.8	200-1000
8	ISSR-8932809	192	12	16.0	9.6	8	0	66.67	0.25	4.2	150-1200
9	ISSR-8932811	77	8	9.6	3.9	8	0	100.00	0.39	4.7	200-1200
10	ISSR-8932812	103	9	11.4	5.2	7	1	77.78	0.22	2.7	250-1200
11	ISSR-8932815	196	12	16.3	9.8	7	0	58.33	0.17	2.6	200-1500

Primer ISSR-8932808 showed the least (7 loci total) after amplification, out of which 6 were polymorphic and 1 monomorphic. The percent of polymorphic band was 85.7%. No unique band was recorded by this amplification.

Total 12 loci were amplified by primer ISSR-8932809. This primer showed 8 polymorphic and 4 monomorphic loci. 66.7% is the percent of polymorphic band which was amplified by this primer.

In case of primer ISSR-8932811, 100% polymorphic loci were recorded. Total 8 loci were amplified. This was the only primer which showed 100% polymorphism.

Primer ISSR-8932812 amplified total 9 loci, out of which 7 were polymorphic (77.8% polymorphic), 2 monomorphic and 1 unique. The size of bands ranged from 250-1200bp. The unique locus having size 478bp, and were presented in genotype EC-38821.

The last primer, ISSR-8932815 amplified 12 loci with 7 polymorphic and 5 monomorphic loci. This primer did not show any unique locus. The percent of polymorphic band was recorded as 55.85% by the amplification of this primer.

4.3 Genetic diversity analysis by ISSR markers

The diversity observed in the twenty winged bean genotypes mainly attributed to the genetic dissimilarities. The genetic similarity matrix was calculated on the basis of Dice similarity coefficient for ISSR

data. The pair wise similarity value ranged from 0.72 to 0.98. Maximum similarity value of 0.98 was recorded between the two genotypes EC-142667 and EC-27885. Minimum similarity value of 0.72 was observed between winged bean genotype EC-178310 and EC-178269. From this study, it is revealed that these winged bean genotypes are less divergent and it indicates that large part of the genome may be similar among themselves.

The UPGMA based dendrogram of twenty winged bean genotypes generated by NTSYSpc 2.1 software was presented in Figure 12. It was observed that two major clusters were generated with dendrogram at 81% similarity.

First major cluster **I** consists of single genotypes EC-142666 only.

Second major cluster **II** consists of 2 sub cluster **IIa and IIb**. First sub cluster **IIa** includes 2 groups **IIa1** and **IIa2**. Group **IIa1** comprises genotype EC-251020 while group **IIa2** consists 2 sub group. First sub group consists of genotype EC-178310 and second subgroup consists of 2 genotypes EC-178291 and EC-121921.

Group **IIb1** is composed of 2 sub-groups. First sub-group consists of single genotype EC-178311. Two genotypes EC-178269 and EC-178268 together make up the second sub group.

Table No. 4.4 The Dice similarity coefficient value based on ISSR markers data of 20 winged bean genotypes

	IC-95221	EC-27885	EC-38821	EC-121921	EC-142653	EC-142661	EC-142666	EC-142667	EC-178268	EC-178269	EC-178291	EC-178310	EC-178311	EC-178317	EC-178318	EC-178319	EC-178332	EC-198327	EC-251020	EC-178331
IC-95221	1.00																			
EC-27885	0.96	1.00																		
EC-38821	0.95	0.93	1.00																	
EC-121921	0.84	0.88	0.83	1.00																
EC-142653	0.94	0.96	0.93	0.87	1.00															
EC-142661	0.91	0.92	0.91	0.83	0.92	1.00														
EC-142666	0.82	0.81	0.83	0.79	0.82	0.82	1.00													
EC-142667	0.96	0.98	0.93	0.88	0.96	0.93	0.83	1.00												
EC-178268	0.86	0.86	0.91	0.77	0.86	0.85	0.87	0.86	1.00											
EC-178269	0.87	0.86	0.89	0.77	0.88	0.86	0.86	0.87	0.95	1.00										
EC-178291	0.88	0.91	0.85	0.93	0.88	0.85	0.78	0.91	0.79	0.78	1.00									
EC-178310	0.85	0.87	0.81	0.91	0.83	0.82	0.76	0.87	0.75	0.72	0.89	1.00								
EC-178311	0.85	0.85	0.86	0.74	0.85	0.85	0.81	0.84	0.92	0.90	0.78	0.73	1.00							
EC-178317	0.94	0.93	0.95	0.83	0.93	0.90	0.82	0.93	0.90	0.91	0.84	0.79	0.87	1.00						
EC-178318	0.92	0.94	0.91	0.87	0.93	0.90	0.80	0.93	0.85	0.86	0.87	0.84	0.84	0.93	1.00					
EC-178319	0.88	0.91	0.85	0.86	0.89	0.88	0.79	0.90	0.79	0.80	0.87	0.85	0.81	0.87	0.91	1.00				
EC-178332	0.91	0.92	0.93	0.81	0.93	0.91	0.79	0.91	0.86	0.87	0.85	0.81	0.86	0.93	0.91	0.87	1.00			
EC-198327	0.93	0.93	0.93	0.82	0.92	0.92	0.79	0.92	0.85	0.86	0.85	0.81	0.84	0.92	0.92	0.87	0.96	1.00		
EC-251020	0.85	0.87	0.84	0.87	0.86	0.86	0.81	0.88	0.80	0.77	0.89	0.86	0.78	0.85	0.86	0.88	0.84	0.85	1.00	
EC-178331	0.90	0.92	0.90	0.86	0.90	0.89	0.80	0.92	0.83	0.82	0.88	0.87	0.80	0.90	0.90	0.88	0.92	0.91	0.89	1.00

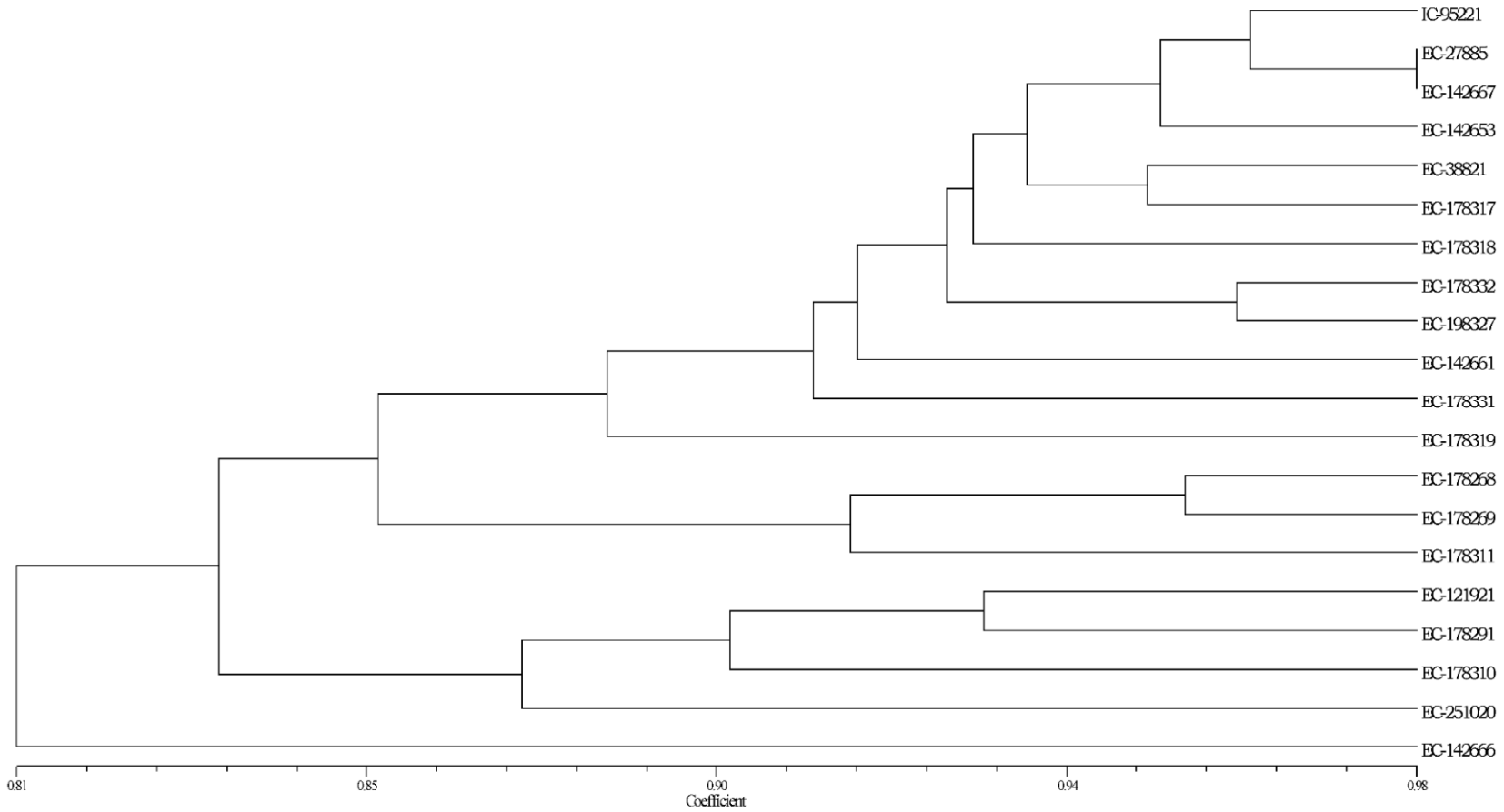


Figure 4.1 The dendrogram of 20 winged bean genotypes based on UPGMA and similarity coefficient using ISSR marker

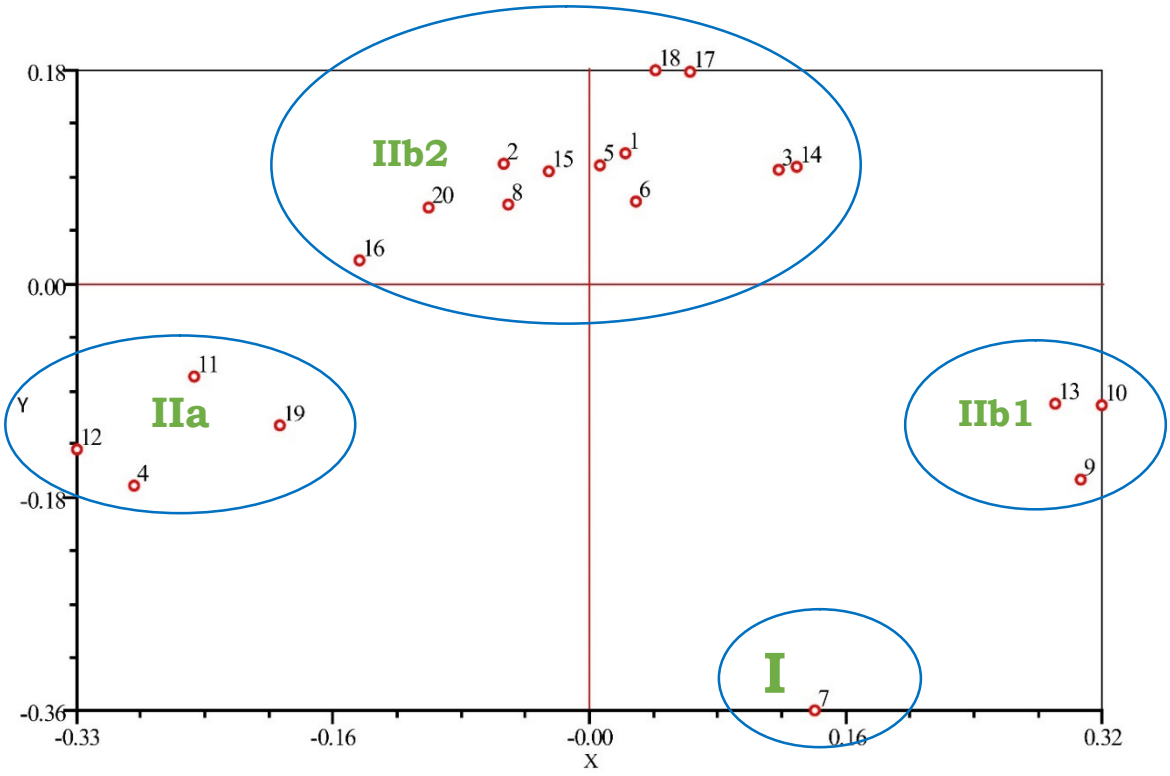
Groups **IIb2** comprises 2 sub-groups. First sub-group consists of one genotype EC-178319. Eleven genotypes including IC-9521, EC-27885, EC-142667, EC-142653, EC-38821, EC-178317, EC-178318, EC-178332, EC-178327, EC-142661 and EC-178331 belong to the second sub-group.

Among 190 combinations, 64 combinations had similarity value more than 0.9; 105 combinations had similarity index ranging from 0.8 to 0.9 and 21 combinations had similarity value less than 0.8. Combination between two genotypes EC-142667 and EC-27885 won closet with similarity value 0.98; while EC-178310 and EC-178269 won most distinct with similarity index 0.72.

The principal component analysis (PCA) almost coincided with the results of UPGMA analysis. The clustering pattern of two dimesion (2D) and three dimension (3D) analysis were in accordance with the dendrogram clustering pattern. Four main groups were constructed.

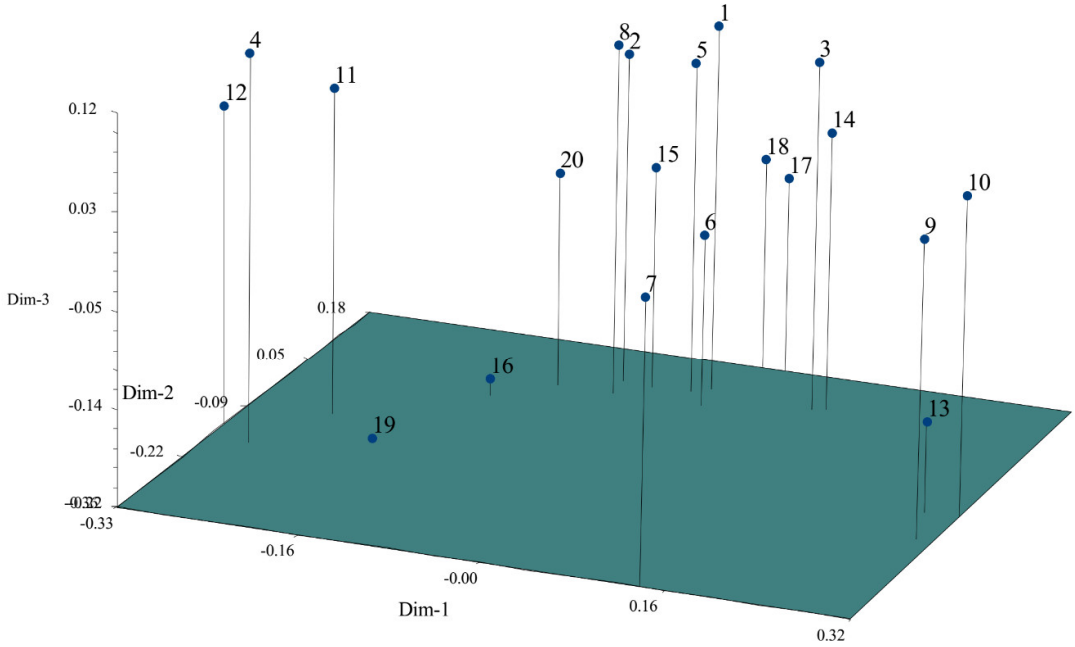
Group I consist of a single genotype EC-142666. Group II composed of 4 genotypes EC-178291, EC-178310, EC-121921 and EC-251020. Group III comprises 3 genotypes EC-178268, EC-178269 and EC-178311. Last group consists of the remains genotypes (12 genotypes). Genotype EC14266 (Group I) was placed isolated separated from 12 genotypes by 2nd component (Y axis). Two other groups, group IIb1 (EC-178268, EC-178269 and EC-178311) and group IIa (EC-178291, EC-

178310, EC-121921, EC-251020) were also placed on same side of Y axis. These 8 genotypes were thus showing clear variability from rest 12 genotypes placed closely on another half showing limited divergence. The X axis (-0.33 to +0.32) and Y axis (-0.36 to +0.18) showed limited scale of divergence in winged bean genotypes studied.



No.	Genotype	No.	Genotype	No.	Genotype	No.	Genotype
1	IC-95221	6	EC-142661	11	EC-178291	16	EC-178319
2	EC-27885	7	EC-142666	12	EC-178310	17	EC-178332
3	EC-38821	8	EC-142667	13	EC-178311	18	EC-198327
4	EC-121921	9	EC-178268	14	EC-178317	19	EC-251020
5	EC-142653	10	EC-178269	15	EC-178318	20	EC-178331

Figure 4.2 Two dimensional PCA scaling of 20 winged bean genotypes using ISSR markers



No.	Genotype	No.	Genotype	No.	Genotype	No.	Genotype
1	IC-95221	6	EC-142661	11	EC-178291	16	EC-178319
2	EC-27885	7	EC-142666	12	EC-178310	17	EC-178332
3	EC-38821	8	EC-142667	13	EC-178311	18	EC-198327
4	EC-121921	9	EC-178268	14	EC-178317	19	EC-251020
5	EC-142653	10	EC-178269	15	EC-178318	20	EC-178331

Figure 4.3 Three dimensional PCA scaling of 20 winged bean genotypes using ISSR markers

V. DISCUSSION

Winged bean [*Psophocarpus tetragonolobus* (L.) DC] is an herbaceous multipurpose legume grown in hot and humid countries. It is used as a pulse, vegetable (leaves and pods), or root tuber crop depending on local consumption preferences (Khan, 1982). In addition to its different nutrient-rich edible parts which could contribute to food and nutritional security, it is an efficient nitrogen fixer as a component of sustainable agricultural systems (Lynd *et al.*, 1983). Generating genetic resources and improved lines would help to accelerate the breeding improvement of this crop, as the lack of improved cultivars adapted to specific environments has been one of the limitations preventing wider use.

Accurate assessment of the levels and patterns of genetic diversity can be invaluable in crop breeding for diverse applications including (i) analysis of genetic variability in cultivars (Smith, 1984; Cox *et al.*, 1986), (ii) identifying diverse parental combinations to create segregating progenies with maximum genetic variability for further selection (Barrett and Kidwell, 1998), and (iii) introgressing desirable genes from diverse germplasm into the available genetic base (Thompson *et al.*, 1998).

Study of genetic diversity is the process by which variation among individuals or groups of individuals or populations is analyzed by a

specific method or a combination of methods. The data often involves numerical measurements and in many cases, combinations of different types of variables. Diverse data sets have been used by researchers to analyze genetic diversity in crop plants; most important among such data sets are pedigree data (Bernardo, 1993; Messmer *et al.*, 1993; van Hintum and Haalman, 1994), passport data, morphological data (Bar-Hen *et al.*, 1995), biochemical data obtained by analysis of isozymes (Hamrick and Godt, 1997) and recently, DNA-based marker data that allow more reliable differentiation of genotypes. Since each of these data sets provide different types of information, the choice of analytical method(s) depends on the objective(s) of the experiment, the level of resolution required, the resources and technological infrastructure available, and the operational and time constraints.

Among large category of molecular markers, inter simple sequence repeats (ISSR) was found to be useful for assessment of genetic diversity (Reddy *et al.*, 2002). ISSRs have been successfully used to estimate the extent of genetic diversity at inter- and intra-specific level in a wide range of crop species which include winged bean (Mohanty *et al.*, 2013, Chen *et al.*, 2015), Cadamom (Anjali *et al.*, 2016), cherry (Lisek and Rozpara, 2009), harmal (Zebarjadi *et al.*, 2016) and sweet potato (Huang and Sun, 2000).

In the light of above developments, the objective of the present study was to focus on the use of ISSR marker technique to evaluate genetic diversity among winged bean genotypes and establish the genetic relationship among winged bean genotypes.

5.1 ISSR analysis

Twenty winged bean genotypes were analyzed using 16 ISSR primers. Eleven primers template yielded distinct, easily detectable bands of variable intensities. Considering all primers and 20 genotypes a total scorable, amplification products were obtained 61.29% were polymorphic (Table No. 4.2). Maximum percent polymorphism was obtained using primer ISSR-8932811 (100%) while the lowest polymorphism was observed with primer ISSR-8932807 (33.33%). The maximum scorable bands (274) and maximum loci (16) were generated by primer ISSR-8932802, whereas primer ISSR-8932811 generated only 77 scorable bands. Primer ISSR-8932808 amplified minimum 7 loci, out of which 6 were found polymorphic. The size of scored bands ranged from 150bp to 2000bp.

This rate of polymorphism is low compared to values obtained with other sets of ISSR primers in *Hagenia abyssinica* (Bruce) (81.0%) (Feyissa *et al.*, 2007), *Spondias* sp. (80.0%) (Santana *et al.*, 2011), *Thujasutch uenensis* Franch. (76.1%) (Liu *et al.*, 2013), *Larix gmelinii*

(Rupr.) (98.8%) (Zhang *et al.*, 2014) and *Erythrina velutina* Willd (98.0%) (Gonçalves *et al.*, 2014).

However, this does not necessarily mean that the primers evaluated for winged bean in this study amplify regions with low polymorphism. The rate of primer polymorphism may vary as a function of the population or group of individuals being evaluated, as shown by Oliveira *et al.* (2010) in populations of *Carapichea ipecacuanha* (Brot.) L. derived from different geographic regions; Qian *et al.* (2013) between populations of *Calanthe tsoongiana* Tang & F.T. Wangand; Dai *et al.* (2013) between populations of *Madhuca hainanensis* Chun & F.C. How; Yiing *et al.* (2014) in planted and natural forests of *Neolamarckia cadamba* (Roxb.) Bosser; Chen *et al.* (2015) in 45 winged bean accesssions and by Zebarjadi *et al.* (2016) on Iranian hamal.

The polymorphic information content (PIC) value provides the estimate of discriminating power of markers and it is a measure of allele diversity at a locus. The ISSR primers always produce the PIC value less than 0.5 (Ghislain *et al.*, 1999; De Riek *et al.*, 2001). This was evident in the present study, the PIC value from most of primers was range from 0.06 (primer ISSR-8932803) to 0.39 (primer ISSR-8932811). Earlier, Mohanty *et al.* (2013) also reported the PIC values in the range 0.203 to 0.354; Dos Santos *et al.* (2011) with PIC value ranged from 0.10 to 0.27;

Sadeghi *et al.* (2012) got the average PIC value of 0.37. The same PIC value also reported by Wong *et al.* (2016, 2017).

This evaluation confirms that the selected primers can be used to estimate the genetic diversity of winged bean, since ISSR primers classified as moderately informative have been used successfully in other species such as *Pongamia pinnata* (L.) Pierre (Kesari *et al.*, 2010), *Trifolium* ssp. (Aryanegad *et al.*, 2013), *Dioscosrea* spp. (Velasco-Ramírez *et al.*, 2014) and *Quercus brantii* Lindl. (Alikhani *et al.*, 2014).

The ability of an ISSR primer to distinguish all accessions was analyzed by calculating resolving power (Rp). In this study, the Rp values ranged from 0.9 in primer ISSR-8932803 to 4.7 in primer ISSR-8932811, with an average of 2.98 (Table No. 4.3). Similar results were recorded while using ISSR primers for evaluated genetic variation by Marotti *et al.* (2007) in common bean (*Phaseolus vulgaris* L.); Kayis *et al.* (2010) in marijuana (*Cannabis sativa* L.); Aruna *et al.* (2012) in sorghum (*Sorghum bicolor* (L.)) and by Ganopoulos *et al.* (2015) in barbary fig (*Opuntia ficus-indica*).

The selected ISSR primers showed informative and clear profiles; therefore, they are useful to estimate variability in the winged bean. These results agree with those obtained by Ganopoulos *et al.* (2011a, 2011b) in other species and Valadez-Moctezuma *et al.* (2014) in Opuntia using ISSR markers

5.2 Genetic diversity studies among winged bean genotypes through ISSR markers

The genetic similarity and genetic dissimilarity matrix (Table No. 4.4) revealed moderate similarity coefficient values ranging from 0.72 to 0.98 indicating the high level of genetic similarity among the genotypes studied.

Maximum similarity value of 0.98 was observed between genotypes EC-142667 and EC-27885. This shows that these two genotypes are genetically very close to each other. It was assumed that such high level of genetic similarity may be the result of biased selection of the material in the previous breeding programs, which ultimately narrowed the genetic based of winged bean; or these materials came from the same geographical region; or they are the sister line of each other'.

Genotypes EC-178310 and EC-178269 were found to be most diverse compare to other genotypes with genetic similarity value of 0.72.

Dendrogram based on unbiased measure of genetic distance by UPGMA method using NTSYS-pc formed two major clusters which group all the genotypes. Genetic dissimilarity coefficient of 0.86 was used as a standard value for classification. Out of two clusters, first cluster consisted of only single genotype EC-142666. Second cluster comprised of 2 sub-clusters, in which each sub-cluster consisted of 2 group.

The above investigation was accordance with the study of Chen *et al.* (2015), in which winged bean genotypes were grouped into 5 clusters on the basis of genetic distance revealed by ISSR markers, showed some differences among the accessions of winged bean, however there was no significant correlation between the genetic distance and land under cultivation. Koshy *et al.* (2013) also reported in winged bean, the cluster analysis is a standard method for analyzing the relatedness of individuals and hence grouping them, from measured data. In the UPGMA dendrogram, the regenerants were grouped into a single main cluster along with the control, suggesting a close relatedness of the regenerants with the parent plant.

The principal component analysis (PCA) along with UPGMA dendrogram analysis delineated all genotypes as four group. The PCA graphs exhibit the dispersal distribution of genotypes.

Furthermore, Mohanty *et al.* (2013) and Chen *et al.* (2015)also pointed out that ISSR markers analysis for diversity can provide practical information for the management of available genetic resources in winged bean breeding programme.

The results of genetic distance and genetic identity between the accessions of studied winged bean indicated that it had closed relationship and narrow genetic background.

VI. SUMMARY AND CONCLUSIONS

The winged bean is a potential vegetable crop with very high content of protein, phenolic and polyphenolic compounds. Winged bean is cultivated in various countries *viz*. India, Burma, Sri Lanka, Thailand, Philippine and Vietnam. But up to now, there was only some studies on the diversity of winged bean both in molecular, morphological and chemical characterization.

The present study entitled "Characterization of winged bean [*Psophocarpus tetragonolobus* (L.) DC.] genotypes based on molecular markers" was carried out at State Level Biotechnology Center, Mahatma Phule Krishi Vidyapeeth, Rahuri, Dist. Ahmednagar during the year 2016-2017.

6.1 Summary

In the present investigation, 16 ISSR primers were used to evaluate genetic diversity of 20 winged bean genotypes, out of which, 11 primers successful amplified. The results getting from Dice similarity coefficient, UPGMA dendrogram and principal component analysis all indicate the high level of genetic similarity and close relationship between the winged bean genotypes studied. All analysis methods showed the genotypes studied in 2 main clusters and 4 groups with narrow genetic background.

6.2 Conclusions

This investigation was conducted with the objective to estimate the genetic diversity and establish a genetic relationship among 20 winged bean genotypes.

1. Eleven ISSR primers amplified and showed the polymorphism in winged bean genotypes. A total of 124 loci were generated by amplification with the size ranging from 150bp to 2000bp. Out of them, 76 loci were polymorphic with 61.3% polymorphism. Each primer produced on an average of 11.27 loci in the twenty winged bean genotypes.
2. Only one primer (ISSR-8932811) showed 100% polymorphism.
3. Maximum PIC value (0.39) was observed in primer ISSR-8932811. Minimum PIC value (0.06) was observed in primer ISSR-8932803.
4. The highest Rp value was observed in primer ISSR-8932811 (4.7), while minimum value was recorded in primer ISSR-8932903 (0.9)
5. Maximum number of loci (16) was produced by primer ISSR-8932802
6. The highest mean number of bands/locus was shown by ISSR-8932803 (18.2), however, highest number of bands/accession was recorded with primer ISSR-8932802 (13.7).
7. Among 124 loci amplified, 3 were unique produced by primers ISSR 8932802, ISSR-8932807, ISSR-8932812 and were presented in

genotypes EC-142661, EC-178269 and EC-38821 respectively.

8. The Dice similarity coefficient values based on ISSR primers analysis ranged from 0.72 to 0.98. Maximum similarity value of 0.98 was recorded between the two genotype EC-142667 and EC-27885. Minimum similarity value of 0.72 was observed between winged bean genotype EC-178310 and EC-178269.
9. The dendrogram constructed by using NTSYSpc 2.1 software revealed all 20 genotypes as separate clusters in 2 major cluster with 4 groups, indicating the high level of genetic similarity among the genotypes studied.
10. Two and three-dimension principle component analysis (PCA) based on ISSR data showed similar clustering of the 20 winged bean as evident from cluster tree analysis (Figure 13 and Figure 14).
11. Two genotypes (EC-178310 and EC-178269) with similarity index 0.72 can be used for further breeding program.
12. Further studies should be carried out to evaluate the relationship between genetic diversity and heterosis; morphological and biochemical characterization analysis can be done for fully evaluation of genetic diversity of the present winged bean genotypes studied.

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