DIFFERENTIATION OF WHEAT VARIETIES THROUGH MORPHOLOGICAL AND MOLECULAR APPROACHES

A Thesis

By

SHARMIN - E – TASNUVA
Registration No. 0705046
Session: 2007
Semester: January– June, 2008

MASTER OF SCIENCE (MS)
IN
BIOTECHNOLOGY

DEPARTMENT OF GENETICS AND PLANT BREEDING
HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY
DINAJPUR

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June, 2008

DEDICATED TO

MY BELOVED PARENTS

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ABSTRACT

Genetic variation within species has been assessed by many methods and from several perspectives. In present thesis, both RAPD markers and morphology were successfully used to differentiate four cultivars of wheat named Bijoy, Prodip, Sourav and Shatabdi. Four distinct morphological characters were identified to differentiate four varieties. In lower glume beak length, at physiological maturity stage, distinctness was observed. In Bijoy it was almost rudiment (1-2mm), in Prodip its length was the highest (15-18mm), in Shatabdi it was medium 12-15mm and in Sourav it was small (8-10mm). In Zadok growth stage 25-29, a distinct difference was observed in four wheat varieties. Growth habit of Sourav was erect, Shatabdi semi-erect and Prodip intermediate. Heading days at Zadok growth stage were 68 for Sourav, 71 for Shatabdi, 63 for Bijoy and 60 for Prodip. Clum glaucosity was strong in Sourav, weak in Shatabdi and Prodip and medium in Bijoy. Among the eleven primers initially tested, two primers (OPA-02 and OPB-01) yielded comparatively higher number of amplification products with high intensity, minimal smearing and good resolutions with clear bands. All of them (100 %) were considered as polymorphic and no monomorphic band was found the primer OPA-02 produced 16 bands and the other primer OPB-01 generated 17 bands respectively. The present experiment produce 33 scoreable bands by using two primers (OPA-02 r produced 16 and OPB-01 produced 17 bands) and they were polymorphic RAPD markers. The result of RAPD marker was more distinct than the morphological marker for the differentiation of wheat varieties. Phenotypic characters were influenced by the environment but incase of molecular marker the reproducibility of RAPD markers were the same in the same reaction conditions. So RAPD marker was found as a potential, simple, rapid and reliable method to evaluate the genetic variation.

Abbreviations and definitions

WRC = Wheat Research Center

BARI = Bangladesh Agricultural Research Institute

CIMMYT = Centro Internacional Mejoramiento De Maiz Y Trigo

DUS = Distictness, Uniformaty and Stability

dNTP = Deoxy neucleotide tri-phosphate

bp = Base pair

et. al. = and his associates

M = Molar

MM = milli molar

Ng = Nano gram

Etc. = Etcetra

Taq = Thermus aquaticus

DNA = Deoxy ribonucleic acid

EDTA = Ethylene diamine tetra acetic acid

UV = Ultra Violate

ddH_2O = Double distilled water

Gel Doc = Gel documentation system

Micro liter = µl

Distinctness:

The variety shall be deemed to be distinct if it is clearly distinguishable from any other variety whose existence is a matter of common knowledge at the time of filing of the application for registration.

Uniformity:

A variety shall be deemed to be uniform if subject to variation that may be expected from particular features of its propagation, it is sufficiently uniform in the relevant characteristics.

Stability:

The variety shall be deemed to be stable if its relevant characteristics remain unchanged after repeated propagation.

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INTRODUCTION

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INTRODUCTION

1.1 Introduction

Wheat (*Triticum aestivum*) is the world's most widely cultivated food crop. It is used in various forms by more than one thousand million human beings in the world. Wheat is the second most important cereal crop after rice in Bangladesh. In Bangladesh wheat covered in 370,000 ha area, yielded 2.58 m ton/ ha (2008, AIS).

The origin of wheat is not known, as the cultivation of the crop is older than the history of man. The finding of wheat in certain mountain shows it was cultivated before the Hebrew Scriptures were written. Wheat is first mentioned in the Bible in genesis 30: 14. The lake dwellers of Switzerland cultivated wheat in the Stone Age. Chinese history shows that wheat was grown in 2700 B.C. and it was one of the five kinds of seeds sown in their annual ceremony. The many names for wheat given in the ancient languages indicate that the existence of the crop dates back to remote times.

The geographical origin of wheat is in doubt. It is thought by some that common wheat originated in southwestern Asia. According to DeCandolle, wheat once grew wild in the Euphrates and Tigris Valleys and from their spread to the reminder of the world.

The domestication process may thus be viewed as an evolutionary process, however more rapid than the slow evolution in plants under natural selection. The advent of modern plant breeding, with it s greater understanding of the genetic systems that govern genetic variability, has accelerated this process even more. Plant breeding is essentially a selection of plant material based on the existence of genetic variability. Traditionally, plant breeding aims at improving morphological

and physiological traits of the crop, but lately, traits like quality and nutritional content, and amenability for mechanical handling have become increasingly important (Sinchez-Monge 1993). Since the domestication process shows many of the same mechanisms as natural evolution, it is possible to use methods developed for evaluation of genetic variation in natural populations also for domesticated plant material and vice versa. A combination of approaches and methods, originally developed for different research areas, may therefore lead to a considerable improvement of the results obtained. The genetic structure of crop gene pools has been assessed by many methods and from several perspectives. Studies have been conducted on four hexapliod type of wheat variety, developed from wheat research center of Bangladesh that is frequently used in plant breeding. The aim of the present research is to demonstrate the morphological and molecular measures of genetic variability of four popular wheat varieties named Bijoy, Prodip, Sourav and Shatabdi.

Several morphological traits were used to differentiate the varieties. Incase of four popular wheat varieties named Sourav, Bijoy, Shatabdi, Prodip some important phenotypic traits are used to find out the differentiation of those varieties. But the morphological traits are, however, often influenced by environmental conditions (Jasienski 1997, Kercher and Sytsma 2000).

Molecular markers are the molecules that could be used to trace a desired gene(s) in test genotypes. Infact, a piece of DNA or a protein can be used as a marker. Earlier approaches that made selection of specific traits easier were based on the evolution of morphological traits (Staub *et. al.)*. However, DNA markers seem to be the best candidates for efficient evaluation and selection of plant material. Unlike protein markers, DNA markers segregate as single gene and they are not affected by the environment. Recently the concept of marker-aided selection had provided an advantage of molecular marker based approaches for crop improvement as compared to selection based solely on phenotype (Tansksley *et. al.*, 1989 ; Paterson *et. al.*, 1991).

1.2 Rationale

Wheat Research Center of Bangladesh Agricultural Research Institute has been released 24 varieties until June 2005. During approval of a variety, National Seed Board, Bangladesh is frequently demanding some marker characters of each variety and sometimes it is not possible to release a good variety due to lack of physical or morphological marker. It is really difficult for the wheat breeders to develop physical marker with other good agronomic characters and they think to do backcrosses to solve these problems, which is also time consuming and sometimes did not find any positive results. DNA finger printing might be a good solution to overcome this problem. RAPD (Random Amplified Polymorphism of DNA) markers are frequently using for DNA finger printing since 1995. It is simple and easy to analyze with a basic laboratory facilities. Wheat Research Center already started to characterize their developed wheat varieties through an advance manner. In the present study a little effort was given to characterize partially of four Wheat Research Center developed wheat varieties. Simplify marker characters will be helpful for the resource personals as well as other people to understand the differentiation of Bijoy, Prodip, Sourav, Shatabdi. The whole protocols might be used for the future developed wheat lines, so the complication of identifying characters of new lines will be eliminated.

1.3 OBJECTIVE (S)

1. Marker characterization of four popular wheat varieties named Bijoy, Prodip, Sourav and Shatabdi.
2. Identify distinct characters of Bijoy, Prodip, Sourav and Shatabdi.

CHAPTER II

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

2.1 Wheat varieties and phenotypic markers

Wheat has the broadest adaptability considering all other crops in the world (Briggle and Curtis, 1987). Bangladesh is a sub-tropical country. So it assumed that it is not a very suitable area for successful wheat production.

However, progress of tropical wheat research system Promotes Bangladesh to produce spring wheat successfully (Anon, 1990) and now the national average yield (2.58 ton/ ha) of Bangladesh is more or less equal compare to famous wheat producing countries such as Australia, India, Canada etc. The wheat varieties such as Bijoy, Prodip, Shatabdi and Sourav are excellent considering their yield, grain quality and disease resistance performance.

However, because of similar ediotype, sometimes it is confusing to differentiate the cultivars from a distance (Faruq *et. al.*, 2007); but Wheat Research Center is always trying to mark some distinct characters to differentiate these varieties during DUS (Distinctness, Uniformity and Stability) test (Naresh *et.al.*, 2007). Since its establishment, Wheat Research Center has developed 24 varieties and at present (2008) four varieties (Bijoy, Prodip, Shatabdi and Sourav) are being popularly cultivated in farmers field (Elahi *et. al.*, 2008).

2.2 Morphology and DUS characters

In many situations, the most easily obtained assessment of genetic variation is measuring of morphological or phenotypic variation. The sharing of phenotypic characters is interpreted as an indication of relatedness. Morphological traits are, however, often influenced by environmental conditions (Jasienski 1997, Kercher and Sytsma, 2000), which in turn may influence the estimation of genetic variation and relatedness. Consequently, to be really useful, morphological measurements should be accomplished on plant material that is grown in comparative trials. This may be both expensive and time consuming, and moreover, almost impossible to accomplish for some species that are very difficult to grow. However, if morphological characters are shown to be heritable, they will nevertheless reflect the genetic structure within the plant material. When studying a plant species, there are several sets of phenotypic characters that may be used for discrimination and relatedness. Agronomical important traits are valuable for a species in cultivation, and form the basis for the breeders' selection of promising plant material. Other morphological trait is used mainly for identification of genotypes and cultivars, e.g. the UPOV (International Union for the Protection of New Verities of plants) guidelines for evaluation of distinctness, homogeneity and stability. Third types of trait are used for evaluation of the genetic variation within a species, e.g. leaf shape analysis. In case of wheat, morphological characters are usually determined by DUS (Distinctness, Uniformity and Stability) test. DUS parameters are considered in Zadok Growth stages (Soller and Beckmann, 1983), where wheat life cycle is considered in 100 days.

Table 2.1: Distinctness, Uniformity and Stability traits of four wheat varieties (Anon, 2007)

Descriptors	GS	Sourav	Shatabdi	Bijoy	Prodip
Pl. Growth habit	25-29	E	SE	E	Intermediate
Leaf Spiral	40-47	Weak	Weak	Weak	Weak
FL Attitude	47-51	REC	REC	REC	REC
Heading	60	68	71	63	60
UCN hairs	55-75	Med	Few	Few	Med
Glaucosity					
- Spike	60-69	Strong	Weak	Strong	Weak
- Culm	60-69	Strong	Weak	Med	Weak
- FLS	80-92	Strong	Weak	Weak	Weak
LGB-Length	80-92	Med	Med	Short	Long
LGB-Spicules	80-92	Present	Num.	Num.	Num.
LGB-Shoulder Shape	80-92	Elevated	Elevated	Square	Elevated
LGB-Shoulder Width	80-92	Narrow	Narrow	Wide	Wide

LGB= Lower Glume Beak, E= Erect, SE= Semi erect, FL= Flag leaf
REC= Recurved, UCN= Upper culm node

2.3 Genetic markers

A genetic marker can be defined as a phenotypic character that can be assigned to a gene to mark its position on the genome or a characteristic feature of the genotype, such as a nucleotide signature, gene or heterochromatic region that can be used to track a particular individual (Hale *et. al.*, 1995). Molecular genetic markers are basically of two types, including isozyme and DNA markers, which include RFLPs, mini- and microsatellites, RAPDs, AFLPs and DALPs. Genetist has proven to be powerful tools in the assessment of genetic variation both within and between populations and in the elucidation of genetic relationships among accessions within the species. Since associations between genetic markers and the traits of interest were first reported by Sax (1923), much attention had been given to the potential applications of markers to improve plant breeding. This approach opens a multitude of new ways in crop and domestic animal breeding improvement. It provides the basis for accelerated breeding through early selection of traits. This approach could reduce the cycle time considerably and significantly increase gains per cycle in the first few generations, as had been demonstrated in corn (Stuber *et. al.*, 1987). According to Meghen(1994), there is a distinct difference between those genetic markers used in trait-based characterization and those that are used in population level characterization.

During the past decades, classical methods to evaluate genetic variation have been complimented by molecular techniques. Molecular markers consist of DNA sequences at specific positions on a chromosome, or their immediate products like enzyme molecules. These markers are inherited in a Mendelian manner and may therefore be used as landmarks for genome analysis. Biochemical markers like isozymes reveal polymorphisms at the protein level and have been used for studying genetic variation within a large number of species (Hamrick and Godt 1989, Hamrick and Godt 1996). Isozyme markers are generally co dominant, i.e. heterozygous individuals can be distinguished from homozygotes, but since these markers only detect variation in protein coding loci, they may reveal only a small amount of the variation present in the individual or population. Moreover, isozyme markers may be dependent on the developmental stage of the plant and

the environment may influence their expression. DNA markers are numerous since they potentially may cover the entire genome. They are not influenced by developmental stage or environment and allow selection of individuals already at the seedling stage that is as soon as the plants are large enough to yield sufficient DNA. There is a number of molecular techniques available for characterization of the variation at the DNA level, e.g. RFLP (restriction fragment length polymorphism), which is a hybridization based methodology using locus-specific probes, and the PCR (Polymerase chain reaction) based polymorphisms like e.g. RAPD (Random amplified polymorphic DNA), AFLP (Amplified fragment length polymorphism) and ISSR (inter simple sequence repeats). RFLP is generally considered to be a reliable method, however labor-intensive and time-consuming, and requiring large amount of DNA. Most of the PCR-based techniques are easy to perform and requires only small amounts of DNA. Furthermore, they are able to reveal a virtually unlimited number of markers. For genetic diversity studies, the RAPD technique (Williams *et.al.*, 1990) showed some important advantages. In contrast to e.g. STMS (sequence tagged microsatellite sites) analysis, prior knowledge of the DNA sequence is not needed, which makes RAPDs very suitable for investigation of species that are not well known. Unspecific primers are used to amplify non-coding as well as coding regions of the DNA. The method is fast and easy to perform but has, unfortunately, a problem with reproducibility since small changes in the PCR conditions may lead to changes in amplified fragments (Williams *et.al.*1993). This problem may, however be minimized by careful optimization and replication of reaction conditions. Competitive priming remains a more serious problem (HalldCn *et.al.* 1996). The use of longer primers in AFLP is generally believed to minimize the reproducibility problems found in RAPDs. However, reproducibility problems have been reported also in AFLP (GoulZo *et.al.* 2001). Moreover, in studies where different types of DNA analyses have been compared, RAPD has been shown to be just as efficient at estimating genetic variation as AFLP (Virk *et.al.*, 2000, Goul *et.al.*, 2001).

2.4 RAPD technology (PCR analysis using selective primers)

The introduction of PCR-based technique in 1990 by William *et. al.* termed as 'Random Amplified Polymorphic DNA (RAPD)', which has stirred up a lot of interests among researches. The technique has been applied successfully in a variety of genetic, phylogenetic and population genetic studies. It has been used to examine genetic segregation (Carlson *et.al.*, 1991; Kazan *et.al.*, 1993; Pillay and Kenny, 1996; Stott *et.al.*, 1997); individual or strain identification (Welsh *et.al.*, 1991; Yang and Quiros, 1993); information of parentage (Welsh *et.al.*, 1991; Tinker *et.al.*, 1993).

Many studies have indicated that RAPD is a powerful method for genotype identification and pedigree analysis, phylogenetic studies and genetic mapping (Welish and McClelland 1990; Martin *et.al.* 1991; Paran *et.al.* 1991; Halward *et.al.* 1992; Mazzarella, 1992; Reiter *et.al.* 1992; Vierling and Nguyen 1992; Wilde *et.al.* 1992).

The technique utilizes only one PCR primer. This single primer is short, in the range of 10 base pairs and has a random sequence. It anneals on the genome at priming sites close to each other and serves as forward and reverse primers. The products of amplification of visualized directly on a gel after electrophoratic separation of fragments and staining with ethidium bromide. Base sequence differences between individuals at the primer sites will result in polymorphisms for the presence or absence of bands. Thus, the RAPD technique is rapid, easy to perform and comparatively cheap. As it relies on universals sets of primers without any need of prior sequence information, it is immediately applicable to the analysis of most organisms. Therefore, it is suitable for evaluating species with unknown genome.

However, RAPD markers have some limitations. The presence of a band is a dominant characteristic. Thus heterozygote cannot be scored and allele frequencies cannot be estimated directly. However, Williams *et.al.* (1990) reprted that about 5% of RAPD loci tested showed co-dominant expression resulting from

differences in fragment length among the alleles. The reliability of RAPD markers has been questioned in several studies. As minor changes in reaction conditions can be significantly alter the number and intensity of the amplification products, reproducibility can be difficult to maintain. Therefore, a stringent standardization of reaction condition is required. A number of different approaches have been adopted to account for artifacts affecting the reproducibility of the analysis. An obvious solution to this problem is to carry out replicate runs and discard all non-reproducible bands (Reiter *et.al.*, 1992) or to use all bands and accept a certain error level (Stiles *et.al.*, 1993).

Thus before making extensive use of RAPD markers especially in breeding programs, the stability and genetics of these markers from parents to offspring need to be evaluated. Each locus must be examined individually for reliability in genetic studies. Only those markers that are inheritable or exhibit segregation in Mendelian manner are reliable. A few of such studies have been reported. In diploid alfalfa 76% of 37 polymorphic markers segregated as dominant Mendelian markers (Echt *et.al.*, 1992). Carlson *et.al.* (1991) found that only 70% of RAPD markers in F_1 progenies of conifers were inherited as dominant markers. In corn, about 10% of the RAPD markers were inherited ia a non-Mendelian manner (Huen, 1993), while in cocoa bean, only 68.3% of the loci segregated as expected in a Mendelian fashion (Ronning *et.al.*, 1995).

Steps involved in RAPD:

Genomic DNA

↓

Use one primer in PCR

↓

Electrophoresis

↓

Marker scoring

2.5 Correlation between morphological and molecular methods

The use of different methods to evaluate genetic diversity may reveal different patterns of variation. Phenotypic differences are not necessarily correlated with the number of underlying gene mutations, and differences in phenotypic characters are not necessarily reflections of different genetic events (Bachmann 1992). RAPDs can potentially cover the entire genome (coding as well as non-coding region), and since most of the genome is composed of non-coding DNA, it is possible that the majority of the amplified fragments are from these regions. Mutations in non-coding DNA are selectively neutral and therefore, the RAPD analysis is able to detect even small differences in DNA, which are not associated with phenotypic variation. Morphological traits are prone to selection since they often are related to fitness. However, RAPD markers have also been shown to be under selective pressure. In their analysis of Triticum *dicoccoides,* Li *et.al.* (1999) found a substantial amount of plant differentiation at the DNA level, which was associated with microclimatic stress. Moreover, quantitatively inherited characters like leaf morphology is often influenced by phenotypic plasticity (Wid *et.al.*, 1994), but see McLellan (2000). Consequently, a combination of morphological and molecular analyses may be the most useful alternative when trying to understand all aspects of genetic variation within a species (Olsson 1999).

2.6 RAPD markers to estimate genetic variation

During domestication, the genetic variation in crop plants has narrowed due to continuous selection for particular traits like high yield and disease resistance, it is extremely important to study the genetic composition of the germplasm of existing cultivars with their ancestors and related species for comparison.

Mailer and May (1999) demonstrated heterogenecity of RAPD sequences in individual seedlings and bulked samples of four cultivars of *B. napus.* Using four different RAPD primers, a qualitative and quantitative assessment was made of the level of DNA sequence heterogenecity presents in the seedlings of four representative Australian rapeseed cultivars. It was found that, depending upon the

primer/ cultivar combination, the seedling diverged from total homogenecity to almost complete homogenecity. The increase or decrease of sample specific RAPD sequences was evaluated in proportional mixtures Of DNA from individual seedlings. These results were then compared with those obtained from bulked DNA samples containing DNA from all the seedlings of a cultivar. From these comparisons, it was found that for a specific RAPD to be detectable in a bulked sample, the particular polymorphism had to be present in at least 15% of the individual seedlings. Even so, the bulked samples produced cultivar specific RAPD banding patterns with all four primers, showing that any of these primers could be used to identify the different rapeseed cultivars.

Genetic variation among nine cultivars of *B. campestris* by employing RAPD markers was assessed by Das *et. al.* (1999). RAPDs generated a total of 125 bands using 13 decamer primers (an average of 9.6 bands per assay) of which nearly 80% were polymorphic. The present polymorphism ranged from 60-100%. Genetic similarity matrix, based on Jaccard's index detected coefficients ranging from 0.42 to 0.73 for RAPD and cluster analyses using data, clearly separated the yellow seeded, self-incompatible cultivars.

Thormann *et.al.* (1994) evaluated the genetic relationships of crop germplasm using RFLP and RAPD markers and concluded that those two markers could result in different estimates of genetic relationships among some accessions. Hybridization tests of RAPD fragments with similar molecular weights demonstrated that some fragments, scored as identical not homologous the differences occurred at the interspecific level. The results suggest the RAPD data may be less reliable than RFLP data when estimating genetic relationships of accessions from more than one species.

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHIODS

3.1 Materials

Four hexaploid wheat varieties named Bijoy, Prodip, Sourav and Shatabdi of Bangladesh Agricultural Research Institute were used in the study.

Table 3.1 Characteristics of four wheat varieties used in the study and their sources

Varieties	Species	Status	1000 grain weight	Days to heading	Features of flag leaf	Source
Bijoy	*T**. *aestivum*	HYV**	50	63	Semi-erect	WRC***, BARI
Prodip	*T. aestivum*	HYV	56	60	Semi-erect	WRC, BARI
Sourav	*T. aestivum*	HYV	49	68	Semi-dropy	WRC, BARI
Shatabdi	*T. aestivum*	HYV	46	70	Dropy	WRC, BARI

T*= *Triticum*, HYV**= High Yielding Variety, WRC***, BARI=Wheat Research Center, Bangladesh Agricultural Research Institute

3.2 Methods of Distinctness, Uniformity and Stability (DUS) Test

►A plot containing 500 normal wheat plants were grown at commercial seed rate and spacing in research block I of wheat research center. There was another set either 15 days interval in planting or grown in research block II of Wheat Research center in December 15, 2007. This year can be considered as test period as UPOV suggested.

Test Method

- ► UPOV published guidelines and instructions were followed to conduct DUS test.
- ► Characters were routinely recorded and verified throughout the growing period
- ► Consequently, partial descriptions of varieties were prepared.

Year 1: Laboratory and field observation tests

Distinctness

Descriptive reports were verified from submitted and collected materials and from harvested materials. The differences were recorded and a provisional descriptor have prepared.

Uniformity

Plants from submitted or collected materials need to comply with uniformity standard (TG ½ of UPOV). Off-type plants were detected. Suspected off-types and majority plants were harvested and threshed and grown in the following appropriate season to confirm the characters.

Stability

No specific test was needed but inferred from the plots by noting variation and segregation within the plots grown from the collected or submitted samples.

Year 2: Laboratory and field observation tests

Distinctness

Data obtained from 1st year observation were compared with the data of 2nd year's plots of the same variety. In addition, descriptive records were prepared to supplement the 1st year's provisional report.

Uniformity

The observations made in the 2nd year were used to confirm the provisional report prepared in the 1st year.

3.3 DNA extraction

A plant DNA extraction Kit (Genei ^{TM}) which was designed as quick single tube method for DNA extraction only 10 to 15 mm long tender wheat plant tissue (10 days aged wheat seedling). Phenol extractions did not involve with this protocol, so the extracted DNAwere directly used for PCR amplification.

Materials required for this protocol

1.5 ml tubes (eppendorf), ethanol, vortex, speed vacuum, centrifuge and dry i

Protocol

1. The entire leaf was washed with autoclaved water and it was rinsed with ethanol. Around 10 to 15 mm long young leaf was taken and placed it in 1.5 ml sterile tube. Kept it in dry ice for 20 minutes and crush the leaf but into smaller pieces with sterile tip.

2. It was placed agfain in dry ice while I was working with the next sample.

3. The samples were removed from dry ice and 20 ml of precooled solution A was added. Again crushed, the samples were kept on dry ice till I had finished processing all the samples.

4. The vials were transferred into a water bath maintained at 65-70° C temperatures. Incubated in water bath for 5-7 minutes.

5. The vials were removed and vacuum infiltrated (Speed vacuum) for 5-7 minutes. Removed quickly and immediately placed in 65-70° C hot water bath for 30 minutes. Green soup was observed at this stage, which indicated cell lysis.

6. Vortex was made for 5 seconds and spined at 10000 rpm for 7 minutes at a room temperature.

7. The supernatant was taken and added 200 µl of cold ethanol plus 20 µl sodium acetate pH 5.2. Gently mixed by inverting and it was kept in dry ice for 10 minutes till the supernatant turned viscous.

8. Centrifuged at 14-20 minutes at room temperature and removed the surernatant. Particulated matter sticking to side of the tube was observed at this stage.
9. Blot dried and added 20 µl of solution B from the top of the tube so that the buffer slides down the side where DNA has precipitated.

10. The tube was kept at 60 to 65° C for 3 minutes for complete solubilization.

11. Spined at high speed for sometimes to remove any insoluble materials and collect the sample in a sterile tube.

12. The collected DNA was used for PCR amplification directly, the remaining DNA was stored for future use at -20° C.

3.3.1 Conformation of DNA

Five µl 2X loading dye was mixed with 3 µl DNA samples. Then these DNA samples (8 µl) were loaded in the 0.8% agarose gel on the gel tank. The electrophoresis machine was run for 1.0 h at 120 V. Two colors appeared after few minutes. The separation was monitored by the migration of the dye in the gel. When the first dye (bromophenol blue) had reached two-third of the gel length, then the power supply was cut off. After electrophoresis the gel was stained by ethidium bromide for atleast 15 min and the bands were visualized under UV light Gel Doc system(Biometra, Japan).

3.3.2 DNA quantification

It is crucial to estimate the concentration of DNA. Optimization of DNA used in microsatellite analysis was essential to distinguish the bands. Below a certain critical concentration of DNA, microsatellite markers are no longer visible. On the other hand, excessive or too much DNA concentration might produce poor resolution or "smears" resulting in a lack of distinct bands in the gel. Two methods were applied for checking DNA concentration more precisely.

3.3.2.1 λ(lamda) DNA (concentration marker)

λ DNA was used for quantification of DNA concentration as 0.5 µl, 1.0 µl and 2.0 µl. It was consequential that 0.5 µl λ DNA contains 25 ng/ µl. DNA. 5 µl. 2X loading dye was mixed with the 3 µl. DNA sample of each genotype. Then 8 µl. DNA sample loaded to the 0.8% agarose gel on the gel tank. λ DNA was loaded in first 3 wells as known DNA concentration marker. The electrophoresis machine was run for 2 h at 100 volts. Two colors (dye) were taking apart after few minutes by the migration of the dye in the gel. When the first dye (bromophenol blue) had reached two-third of the gel length, then the power supply was switched off and the gel was stained with ethidium bromide solution. After staining, the gel was placed in gel doc

DNA bands were visualized by UV light. Care should be taken during carry out ethidium bromide and UV light.

3.3.2.2 Spectrophotometer

Spectrophotometer was used to quantify DNA more precisely. DNA quantification was carried out a 260 mm. First a cuvette was carefully washed and filled with 2 ml (2000 µl) deionized water to use as a blank and the reading was adjusted to zero. Other cuvettes (the "test"cuvette) were filled with 2 µl of deionized water and 2 µl of each DNA sample and mixed homogeneously with the help of micropipette. Then the optical density of each sample was measured at 260 nm. The cuvettes were rinsed again with deionized water and wiped with tissue paper and absorbance readings for the other samples were taken correspondingly and data recorded. The original samples concentrations were determined from the absorbance reading along with the following formula:

$$DNA\,conc.(\mu g / \mu l) = absorbance \times \frac{Volume\,of\,deionized\,water\,(\mu l)}{Amount\,of\,DNA(\mu l)} \times conversion$$

3.3.3 Preparation of working solution of DNA samples

Optimization of DNA concentration is the prerequisite to molecular marker analysis. Before PCR amplification of DNA concentrations were adjusted to 25 ng/ µl using the following formula:

$S1 \times V1 = S2 \times V2$

Where, S1= Initial strength (ng/ µl),
V1= Initial volume (µl),
S2= Final strength (ng/ µl) and
V2= Final volume (µl).

Preparation of 0.8% Agarose gel (200ml)

200 ml 0.5X TBE (electrophoresis buffer) was taken in a flask.

1.6 g agarose was added to it.

The mixture was then cooked for 5 minutes in an oven to dissolve it.

The gel so produced was left in room temperature for some time to be cool down at tolerable level.

The gel solution was then poured into gel mould carefully.

Meanwhile two combs were placed on the gel.

The gel was solidified within 30 minutes.

The end casters were removed after gel has polymerized.

The gel was submerged into 0.5 X TBE in the gel tank.

The combs were removed from the gel.

The gel was then ready for loading the DNA samples.

3.4 Amplification of RAPD markers by Polymerase chain reaction (PCR)

3.4.1 Principles of the amplification of RAPD markers

A target DNA sequence is exponentially amplified with the help of arbitrary primers, a thermo stable DNA polymerase, deoxynucleotide triphosphates, magnesium chloride and reaction buffer. The reaction involves repeated cycles, each consisting of a denaturation, a primer annealing and elongation step. In the first step, the DNA is made single stranded by raising the temperatue to 94° C (denaturation). In the second step, lowering of the temperature to about an optimal annealing temperature, the primer binds to their target sequences on the template DNA (annealing). In the third cycle, temperature is chosen as where the activity of the thermo stable *Taq* DNA Polymerase is optimal i.e. usually 72° C. The Polymerase then extended the three ends of the DNA primer hybrids to the other primer-binding sites. Since this happens at both primers annealing sites on both the DNA strands, the target fragment is completely replicated. Reapeting these three cycles 40 to 50 times results in the exponential amplification of the target between the 5 ends of the two primer binding sites. Amplification products are separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

3.4.2 Primer selection

Eleven primers of random sequence (Operon Technologies, Inc., Alameda, California, USA) were screened on a sub sample of two randomly chosen individuals from four different varieties to evaluate their suitability for amplification of their DNA sequences, which could be scored accurately. Primers are evaluated based on intensity of bands, consistency within individuals, presence of smearing, and potential for discrimination. The details of primers are given in table 3.2. A final subset of two primers (OPB-01 and OPA-02) exhibiting good quality banding patterns and sufficient variability were selected for analysis.

Table 3.2 Parameter of the random primers used in the present study for Screening

Primer code	Sequence (5´-3´)	GC content(%)
OPB-01*	GTTTCGCTCC	60
62AB10C2	GGACCCAACC	70
63AB10A3	GTCGCCGTCA	70
64AB10G4	TCTGGTGAGG	60
66AB10G6	ACCTGAACGG	60
68AB10A8	GTGTGCCCCA	70
OPA-02*	TGCCGAGCTG	70
70AB10C10	GGTCTACACC	60
71AB10G11	AGCGCCATTG	60
72AB10G12	AGGGCGTAAG	60
73AB10T13	CTGGGGACTT	60

***Primer selected for RAPD analysis for all samples of the cultivars**

3.4.3 Preparation reaction mixture to perform polymerase chain reaction (PCR)

PCR reactions were performed on each DNA samplee in a 10 µl reaction mix containing the following reagents:

Reagents	**Amount**
Ampli *taq* polymerase buffer (10X)	1 µl
Primer (10 µM)	2.5 µl
dNTPs(250 µM)	1 µl
Ampli *Taq* DNA poymerase	0.2 µl
Genomic DNA (25 µg / µl)	2 µl
Sterile deionized water	3.3 µl
Total	**10** µl

During the experiment, PCR buffer, dNTPs, and primer solutions were thawed from frozen stocks, missed by vortexing and placed on ice. DNA samples were alco thawed out and mixed gently. The primers were pipetted first into 0.2 ml PCR tubes. For each DNA sample being tested, a pre-mix was then prepared including the following order: buffer, dNTPs, DNA template and sterile distilled water. Taq polymerase enzyme was

DNA then added to the tubes containing primers. Samples were added and the tubes were then sealed and placed in a thermocycler and the cycling was started immediately.

Thermal profile

DNA amplification was performed in an oil-free thermal cycler. The reaction mix was preheated at 94°C for 3 minutes follower by 40 cycles of 1 min denaturation at 94°C, 1 min annealing at 54°C and elongation or extension at 72°C for 2 minutes. After the ladt cycle, a final step for 7 minutes at 72°C to allow complete extension of all amplified fragments. After completion of cycling program, reactions were held at 4°C.

3.4.4 Electrophoresis of the amplified products:

Agarose gel (1.5%) was prepared and poured into platform carefully when the gel solution cooled to 55°C. Let the gel polymerize for at least 30 miinutes before removing the combs. After removing the casters, gel with platform was placed at the tank and poured 0.5X TBE buffer into the tank to submerge the gel. Then the combs were removed cautiously that gel slots were not injured.

Then the PCR products were mixed with 5 ul of 2X gel loading dye. 15 ul of the mixture was loaded slowly per well on the gel in the gel tank allow them to sink in the bottom of the wells. The tank was covered and all connections were checked. Electrophoresis machine run for 1-1.5 hr at 100 volts. The separation process was

monitored by the migration of the dyes in the loading buffer. When the bromophenol blue dye had reached about three- fourths of the gel length, the electrophoresis was stopped.

3.4.5 Ethidium bromide staining:

After completion of electrophores the gel was soaked in ethidium bromide (10mg/ml) solution for 15-20 min.

10mg / ml Ethidium bromide (10ml) preparation:

- 0.1g Ethidium bromide was added in 10 ml ddH_2O.
- Then the mixture was stirred vigorously for several hours.
- The container was then wrapped in aluminum foil.
- The container was then stored at room temperature.

3.4.6 Documentation of the DNA samples:

After staining the gel was taken out carefully from the staining tray and placed on high performance ultraviolet light box (UV transilluminator) of gel dic for checking the DNA bands. The DNA was observed as band and saved the records.

3.5 RAPD data analysis:

Since RAPD markers are dominant, we assumed thet each band represented the phenotype at a single allelic locus (Williams *et al.,* 1990). All distinct bands or fragments (RAPD markers) were thereby given identification numbers according to their position on gel and scored visually on the vasis of their presence (1) or absence (0), separately for each individual and each primer.

3.6 Methods and statistical measures for assessing genetic variation

3.6.1 Statistics

The use of RAPD markers presents some practical problems since they do not allow differentiation between homozygotes and heterozygotes. Consequently, many statistical procedures normally used in population genetics are not easily applicable to RAPD data. However, there are statistical analyses developed also for dominant markers, based on the assumption that each locus can be treated as a two-allele system, i.e. presence or absence of a band.

3.6.2 Genetic relationships

Once the morphological traits or the generated molecular marker profiles have been evaluated, there are different strategies how to estimate the similarity between the analyzed individuals. Similarity indices measure the amount of closeness between two individuals, the larger the value the more similar is the two individuals. There is a variety of alternative measures for expressing similarity, like Jaccard's coefficient of similarity which can be used for binary data and often is applied in RAPD-based studies. This coefficient is based on number of positive matches between two individuals where as joint absences are excluded. Dissimilarity coefficients instead estimate the distance or unlikeness of two individuals, the larger the values the more different are the two individuals. The Euclidean distance and the squared Euclidean distance are two commonly used measures of dissimilarity in both morphological and molecular analyses.

Matrices of similarities or dissimilarities between pairs of individuals may then be used as a starting point for statistical procedures such as cluster analyses, multidimensional scaling (MDS), or principal coordinate analyses (PCO). In a cluster analyses relatively homogeneous groups of individual cluster together in a hierarchical way and this clustering is visually displayed in a dendrogram. MDS and PCO summarize dissimilarity data between individuals, or groups of

individuals, in a non- hierarchical manner and then display the variation in an ordination plot.

3.6.3 Partitioning of variation

When a set of populations is investigated, the amount of genetic variability can be expressed at different hierarchical levels, e.g. between regions, between populations within regions and within populations.

Partitioning of variation in morphometric characters may be performed using canonical variates analysis (CVA). CVA provides a measure of the proportion of the total variation that is due to within-group variation, referred to as Wilk's lamda (γ). The proportion of the total variation due to variation between groups is then given by 1- γ. For molecular data, the AMOVA (analysis of molecular variance) procedure developed by Excoffier *et.al.* (1992) has been widely used (Bussel 1999, Nybom and Bartish 2000). This method was originally developed for haplotype data, but has recently become widely used also for dominant markers like RAPDs. AMOVA has been applied mostly for estimation of population structure in diploid organisms, but has also been shown to give good estimation of population variability in tetraploid individuals (Jenezewski *et.al.* 1999). AMOVA is based on squared Euclidean distances among individuals and alternative approach to AMOVA is to calculate hierarchical components of the Shannon's diversity index (Bussell 1999). This index did not require any assumption about Hardy-Weinberg equilibrium.

CHAPTER IV

RESULTS AND DISCUSSION

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Morphological characterization

Morphological traits are, however, often influenced by environmental conditions (Jasienski 1997, Kercher & Sytsma 2000), which in turn may influence the estimation of genetic variation and relatedness. Agronomical important traits are valuable for a species in cultivation, and form the basis for the breeder's selection of promising plant material. Other morphological traits are used mainly for identification of genotypes and cultivars, e.g. the UPOV (International Union for the Protection of New Varieties of Plants) guidelines for evaluation of the genetic variation within a species, e.g. leaf shape analysis. In case of wheat morphological characters are usually determined by DUS (Distinctness, Uniformity and Stability) test. DUS parameters are considered in Zadok growth stages (Soller and Beckmann, 1983), where wheat lifecycle is usually considered in 100 days. In the present study, in Zadok growth stage 25-29 a distinct different was observed in four wheat varieties. Sourav habit was erect, Shatabdi was semi-erect and Prodip was intermediate. Heading days were (Zadok growth stage 60) 68 days for Sourav, 71 days was for Shatabdi, 63 days was for Bijoy and 60 for Prodip. Culm Glaucosity was observed strong in Sourav, weak in Shatabdi and Prodip, medium in Bijoy. Lower glume beak length was medium in Sourav and Shatabdi, short in Bijoy and very long in Prodip.

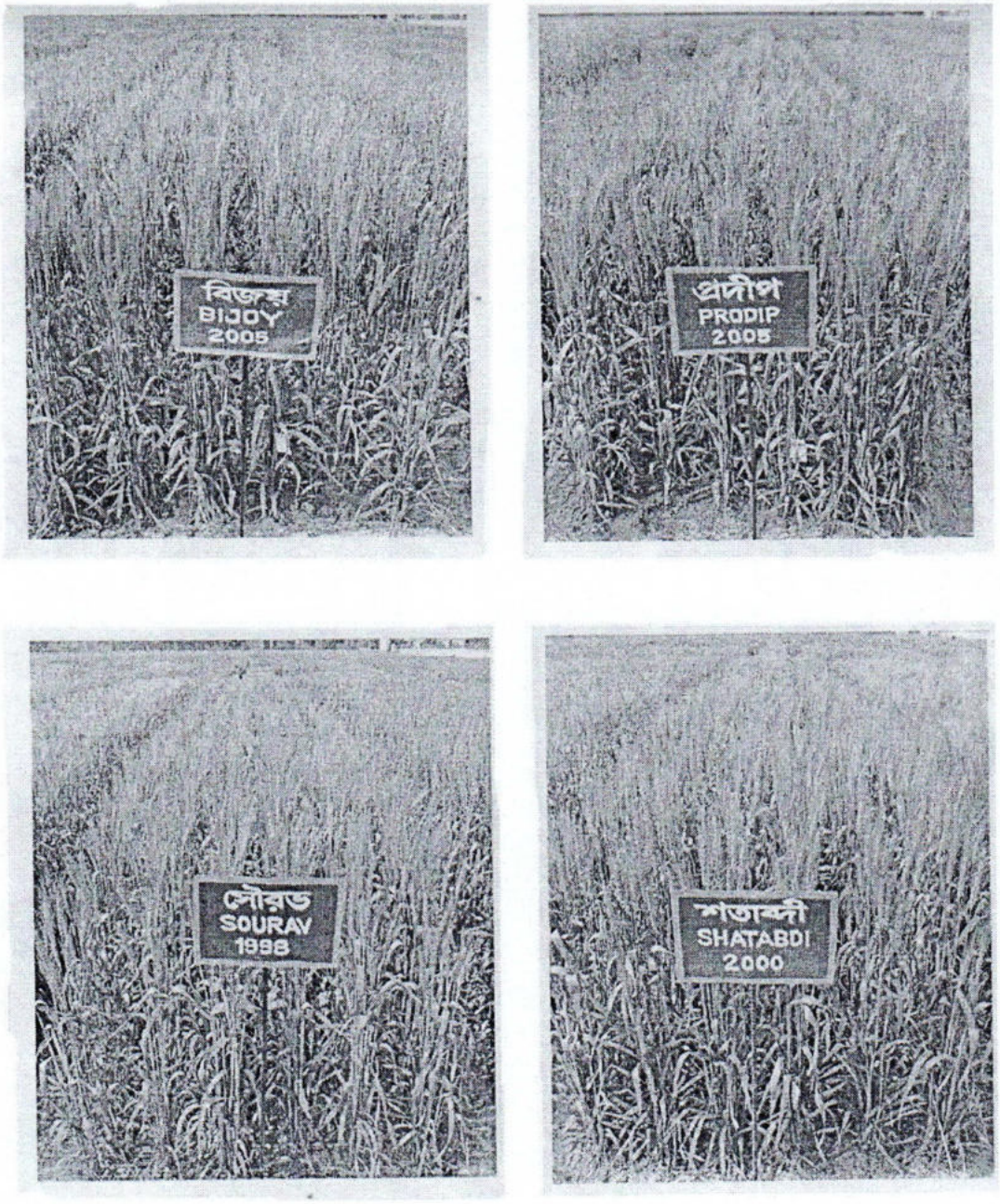

Figure 4.1 Four wheat varieties named Sourav, Shatabdi, Bijoy and Prodip

91
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4.2 Identifying distinct phenotypic marker

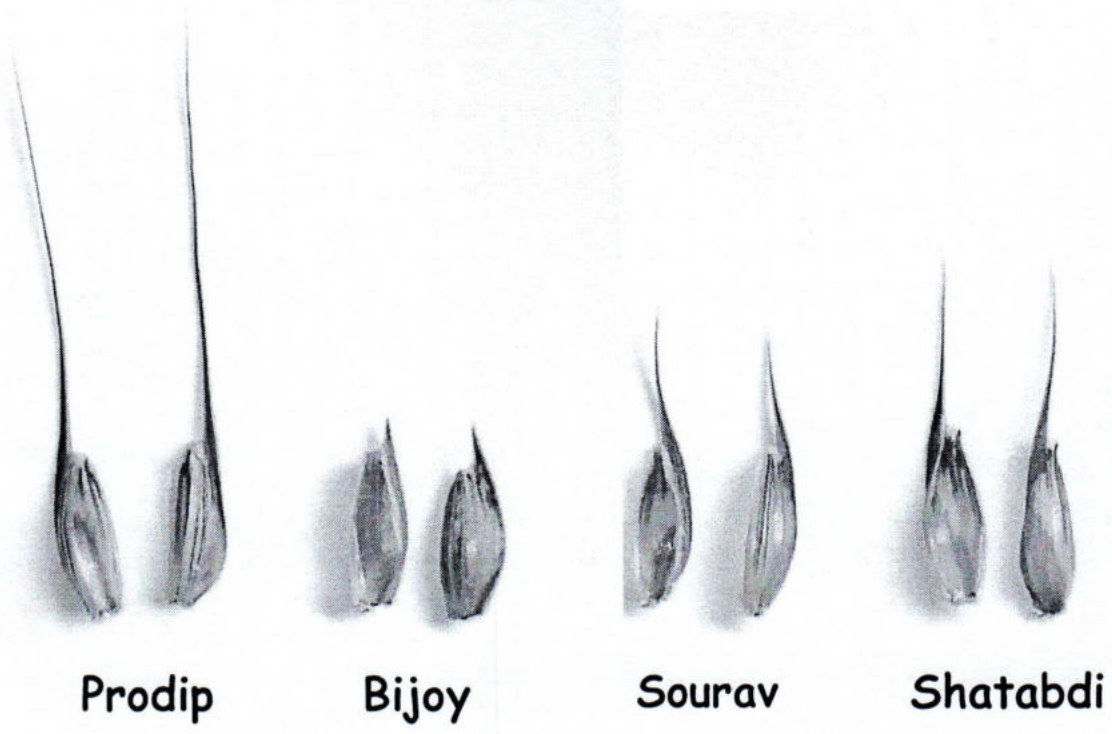


Figure 4.2 Distinct phenotypic markers (lower glume beak length) of four wheat varieties

Four wheat varieties were raised up in Field I, WRC, BARI, Dinajpur and Field II, WRC, BARI,Dinjpur. No significant differences were observed in lower glume beak length in same variety in the both fields. However, significant differences were observed among the varieties in the two fields. Observed results have been presented in Table 4.1 and Table 4.2

Table 4.1 Differences in lower glume beak length in same varieties in two different location.

Location	Beak length (mm)			
	Bijoy	Prodip	Sourav	Shatabdi
Field I, WRC, BARI, Dinajpur	1.2	15	8.0	10
Field II, WRC, BARI, Dinajpur	1.3	16	8.5	10
LSD (0.05)	NS	NS	NS	NS

Table 4.2 Differences in lower glume beak length in four varieties in different locations

Variety	**Lower glume beak length(mm)**	
	Field I	**Field II**
Bijoy	1.2	1.3
Prodip	15	16
Sourav	8.0	8.5
Shatabdi	10	10
% CV	**2.5**	**1.9**
LSD (0.05)	**4.73**	**3.08**

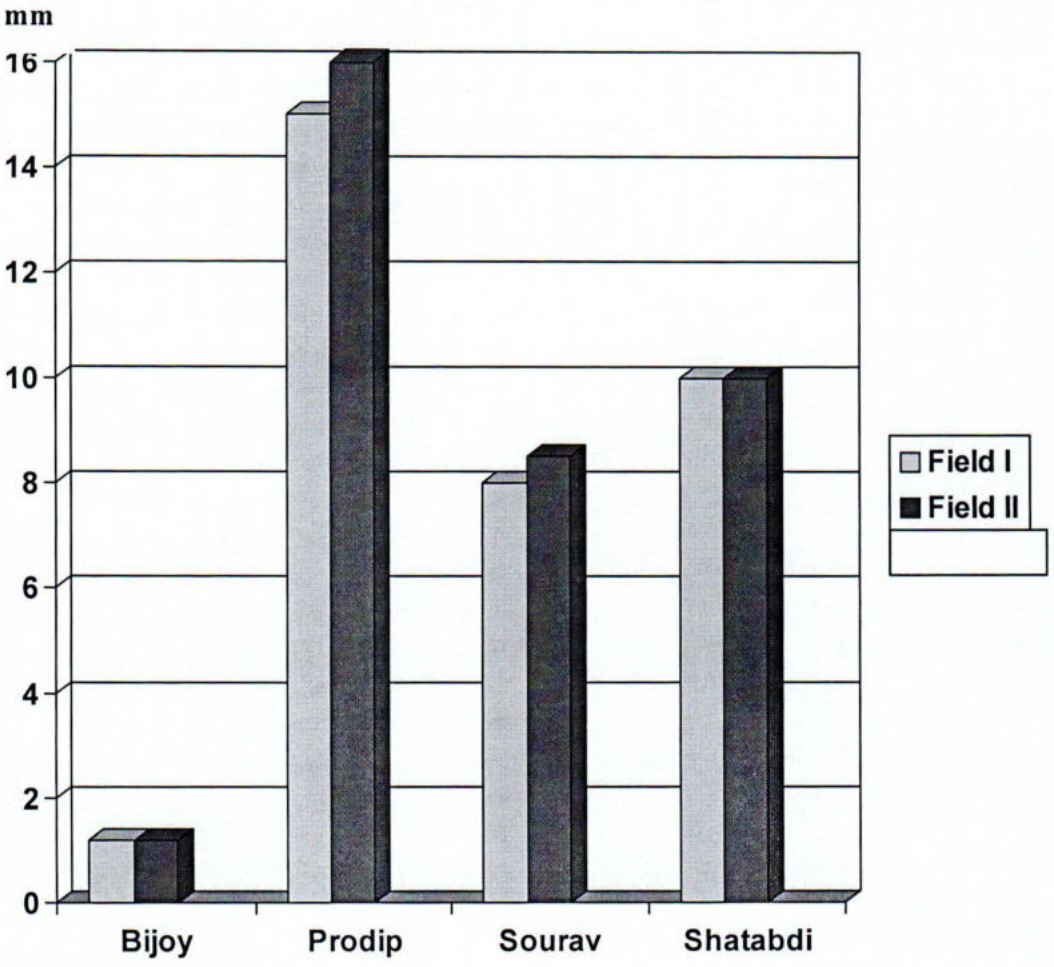


Figure 4.3 Differences in lower glume beak length in same varieties in two different locations

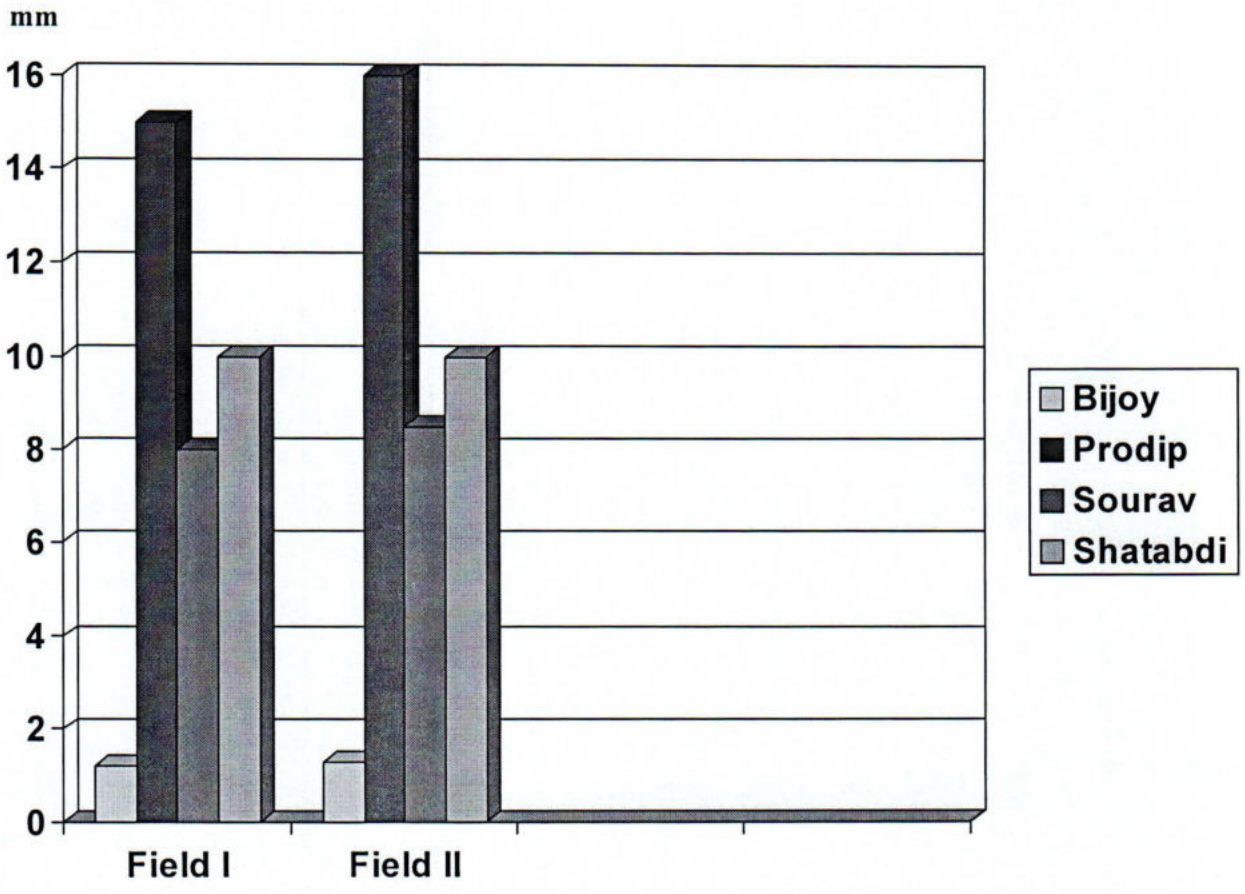


Figure 4.4 Differences in lower glume beak length in four varieties (Bijoy, Prodip, Sourav, Shatabdi) in two different locations

4.3 Primer selection and RAPD profiles

Among the eleven primers initially tested, two primers (OPA-02 and OPB-01) yielded comparatively higher number of amplification products with high intensity, minimal smearing and good resolutions with clear bands. Two random primers (OPA-02 and OPB-01) produced RAPDs with varied patterns. The two primers generated 33 distinct bands. All of them (100%) were considered as polymorphic and no monomorphic band was found. The primer OPA-02 produced 16 bands and the other primer OPB-01 generated 17 bands respectively.

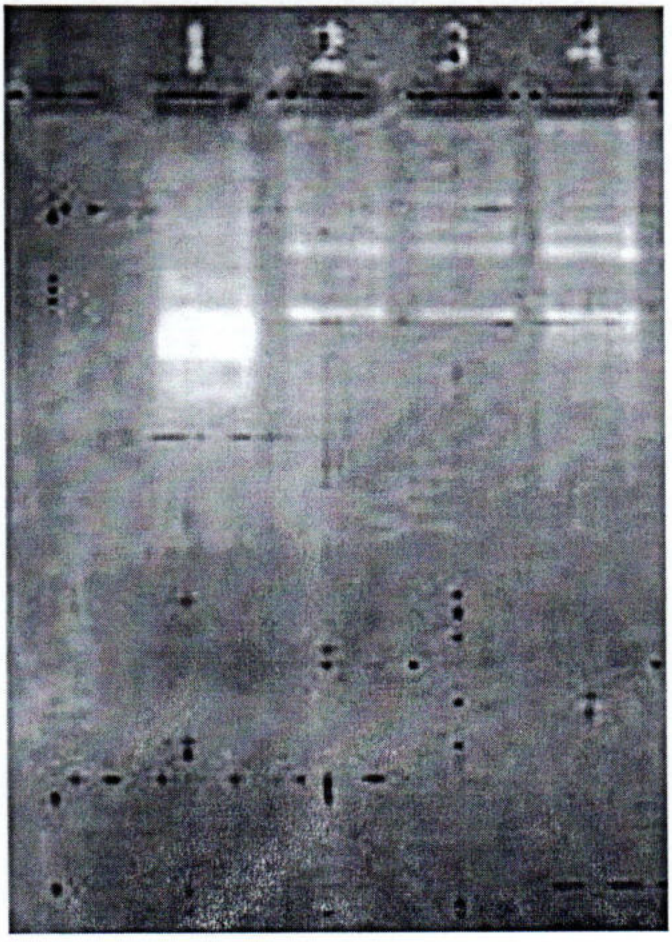
Table 4.3 Total scoreable bands and polymorphic bands amplified by two RAPD primers in studied four wheat cultivars

Primer	Sequences (5′-3′)	Scored band	Polymorphic band
OPB-01	GTTTCGCTCC	17	17
OPA-02	TGCCGAGCTG	16	16

The level of polymorphism (100%) indicated the effectiveness of RAPD technique to study substantial amount of polymorphisms among the different varies of *Triticum aestivum*. This proportion of polymorphism higher compared to some previous RAPD analysis in *Triticum aestivum*, e.g. 71% in Maxican wheat (Castgna *et.al.*, 1997), 80% in Chinese (Sun *et.al.*, 1998) wheat cultivars. This difference can be attributed to the primers used and genotypes evaluated.

The present experiment produced total 33 scoreable bands by using two primers (OPA-02 produced 16 and OPB-01 produced 17 bands) and they were polymorphic RAPD markers. This high level of polymorphism detected by the arbitrary primers was almost similar to the previous reports in other RAPD studies on *Triticum aestivum* cultivars, such as 31 scored per primers in Mexico.

The banding patterns of four wheat cultivars using two different primers are shown in figure 4.5 and 4.6



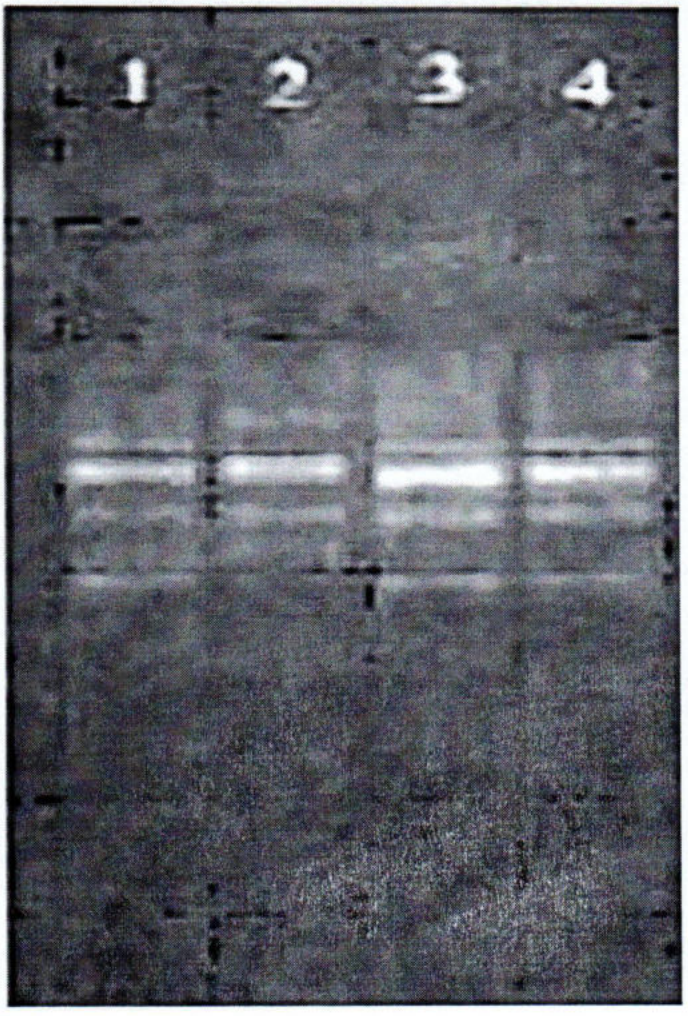
Lane 1= Prodip

Lane 2= Bijoy

Lane 3= Shatabdi

Lane 4= Sourav

Figure 4.5 Random amplified polymorphic DNA (RAPD) patterns found in four wheat genotypes using primer OPA-02



Lane 1= Prodip

Lane 2= Bijoy

Lane 3= Shatabdi

Lane 4= Sourav

Figure 4.6 Random amplified polymorphic DNA (RAPD) patterns found in four wheat genotypes using primer OPB-01

CHAPTER V

SUMMARY AND CONCLUSION

CHAPTER V

SUMMARY AND CONCLUSION

The morphological characters might be changed depends on environmental factors, such as droughtness, high nitrogen application (micro environment), prolong winter. As a result some variation was found in our research also. Lower glume beak lengths were observed in Bijoy 1-2 mm, in Prodip 15-18 mm, in Shatabdi 12-15 mm and in Sourav 8-10 mm respectively. But it is cannot say this information is standard; these may farther change in other environment. In favorable environment the lower glumme beak length of Bijoy might be turned into 3-4 mm and in stressed condition the lower glumme beak length of Prodip might be reduced in to 12-15 mm. But in same environment the lower glumme beak length of all these four varieties will be perform proportionally significant difference. In the case of molecular markers it is expected that the reproducibility of RAPD markers will be the same in the same reaction conditions. But it is needed to do in a developed analytical environment and it is expensive. So it is concluded that besides development of molecular markers, distinct morphological marker identification is also very important to differentiate the wheat varieties. In the present study, it was attempted and developed distinct morphological markers besides development of RAPD markers, which will differentiate the four popular wheat varieties Bijoy, Prodip, Shatabdi and Sourav.

It was not managed to test the two-time reproducibility of bands, because of inadequate laboratory facilities, specially, a lot of working load. However, the whole works were done in intensive care with the help of very skilled researcher of BARI and BRRI.

CHAPTER VI

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