STUDIES ON THE MOLECULAR BASIS OF ENVIRONMENTAL STRESS TOLERANCE IN JUTE

Ph. D. THESIS

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By

MD. SAMIUL HAQUE

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DECLARATION

I hereby declare that this thesis entitled "Studies on the Molecular Basis of Environmental Stress Tolerance in Jute" has been composed by me and all the work presented herein is my own. I further declare that this work has not been submitted anywhere for any academic degree.

Date: (Md. Samiul Haque)

CERTIFICATE

This is to certify that the research work embodying the results reported in this thesis has been carried out under our guidance and direct supervision in the experimental field and molecular biology laboratories, Bangladesh Jute Research Institute, Dhaka and Department of Biochemistry and Molecular Biology, University of Dhaka respectively. It is further certified that the work presented here is original and suitable for submission for the award of Ph.D. degree.

Selina Begum, Ph.D.
Chief Scientific Officer
B. Jute Research Institute

Prof. R.H. Sarkar, Ph.D.
Dept. of Botany
Dhaka University

Prof. Haseena Khan, Ph.D.
Dept. of Biochemistry and
Molecular Biology
Dhaka University

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Author

ABSTRACT

Jute is a principal cash crop of Bangladesh, obtained from the bark of the two cultivated species, *Corchours capsularis* (white jute) and *Corchorus olitorius* (tossa jute). Jute is a short-day plant, needs long day light for vegetative growth and short-day for flowering. The yield and quality of tossa is better than white jute. The centre of origin of white jute is said to be Indo-Burma including South China, and Africa for that of tossa.

The global demand for traditional products of jute is declining day to day due to invasion of synthetic fibre. There is therefore a golden opportunity to revive the past glory of jute through the production of paper pulp from green jute, which could solve the retting problem and ensure the bulk use.

As jute is a self-fertilized crop, its natural genetic variability is very narrow; this makes the plant breeder helpless in the development of this crop. The molecular techniques could be a suitable alternative to improve the crop. If the programme, production of paper pulp from green jute becomes a success, then at least two crops will be required in a year to meet the mill demand for green jute round the year and one crop will have to be produced in the winter season. So it is essential to develop low temperature tolerant DNA marker for marker-aided selection and finally to develop a low temperature tolerant jute variety through molecular approach.

Isolation of quality DNA from jute is very difficult because of the presence of polysaccharides and other metabolites. Two modified methods were evaluated using four types of plant material (seed, seedling, young leaf and old leaf) from two species of jute. It was observed that different methods were suitable for isolating DNA from different plant samples and seedling (grown in the dark) is the best sample for quality DNA isolation.

In DNA fingerprinting study of some jute varieties and accessions, it was observed that the two species were well differentiated but differentiation within species among the varieties/accessions was difficult to detect with limited number of

primers due to narrow genetic base. This study holds enormous prospect for the development and application of molecular markers for jute and for the generation of a jute linkage map. However, further studies with more number of primers are highly recommended to generate a linkage map that spans the entire length of the jute genome.

To develop molecular marker for low temperature tolerance, two *C. olitorius* genotypes, variety O-9897 and accession no. 1805 were selected as low temperature sensitive and tolerant, respectively, following morphological and molecular investigation. In RAPD analysis a particular band was found in tolerant accession. A cross was made between O-9897 and accession no. 1805 as seed and pollen parent, respectively, and hybrid (F_1) was produced. On germination test in low temperature stress condition, it was observed that the low temperature tolerant trait is a dominant character.

The DNA obtained from the parents and differentially grown (low temperature tolerant and sensitive) segregating F_2 populations were thoroughly evaluated with RAPD markers for tracking the expected DNA fragment associated with low temperature tolerance. It was observed that a particular band of about 1200 bp in size consistently co-migrates with a band of same size in the low temperature tolerant parent and selected F_2 populations. This band was always absent in the sensitive parent and F_2s. The observation strengthened the idea that this particular stretch of DNA is associated with low temperature tolerant character.

This co-segregating DNA fragment may be part of a low temperature tolerant gene or any fragment associated with low temperature tolerant trait. The fragment was isolated and used as probe for Southern analysis, sequenced for SCAR analysis, but failed to differentiate the parents. To study the differences between the parents at nucleotide level in the amplified products, the amplified fragments from both parents were isolated, sequenced and compared. An SNP was found between these sequences.

A bioinformatics analyses with this sequence was carried out. A strong homology (>90%) was observed at nucleotide level with the genome *Arabidopsis thaliana*. This was shown to be a part of a gene (a terminal exon). The translated product of its sequence revealed a strong homology, to that of an *Arabidopsis thaliana* expressed gene coding for low-density lipoprotein B like protein. This was a significant finding, as lipoproteins are known for its role in cold tolerance in other organisms.

Another effort was made to rescue the upstream sequence to know the total sequence of the gene using inverse PCR with SCAR primer, but no amplification was found. Further bioinformatics analysis of the sequence revealed the presence of an ORF that is shown to be expressed. This indicates that the region is present in close proximity to the locus determining low temperature tolerance. From the evidence of strong linkage observed, this ORF itself could be responsible for the trait, two possible mechanisms could be proposed, one is at there might be a combination of SNPs (both in intron and in exon) which are responsible for such differential phenotype. Second, there may alternate splicing products, which could lead to different products formed in different genotypes. It was evaluated that the gene responsible for low temperature tolerance was expressed as determined by RT-PCR.

The first and very preliminary linkage map of the jute genome was constructed with RAPD markers using two parents and their F_2 populations. Linkage analyses at a LOD score of 3.0 and a maximum distance 50 cM revealed 18 linkage groups. Among the 18 linkage groups, 15 groups (group no. 1 to 15) contained single locus and the other three groups designated as Linkage group-16, 17 and 18 contained 2, 11 and 12 loci respectively. The unique chromosome number of *Corchorus* spp. is seven (2n=14), so it was expected to obtain seven linkage groups to represent the genome. But for linkage group analysis, the effort was very limited and the total number of loci (40) was also low.

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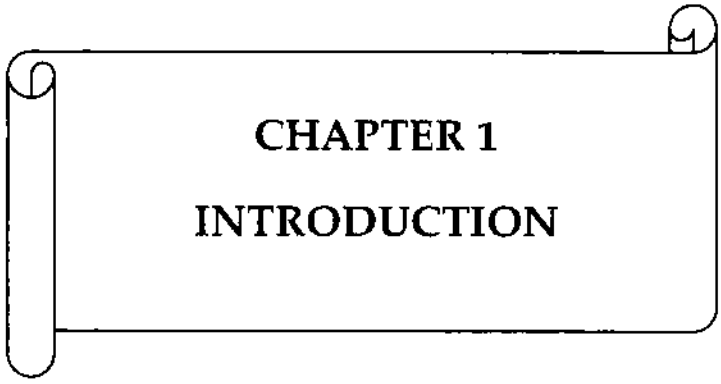


CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1. BRIEF DESCRIPTION ON JUTE

Bangladesh is a homeland of quality jute production. It is a dicotyledonous fibre yielding plant of the genus *Corchorus*, Family Tiliaceae. At the end of 18th century, Roxburg identified jute as an alternative to European hemp (*Cannabis sativa* L.) (Ghosh, 1983). Jute fibre is obtained from the bark of the two commercially important species, namely *Corchours capsularis* L. (white jute) and *Corchorus* C. *olitorius* L. (tossa jute). The yield and quality of *C. olitorius* jute is better than C. *capsularis* (Kundu, 1968). The centre of origin of white jute is said to be Indo-Burma including South China, and Africa for that of tossa (Kundu, 1951).

Jute grows under wide variation of climatic conditions and stresses of the tropics and subtropics. It is grown in Bangladesh, India, Myanmar, Nepal, China, Taiwan, Thailand, Vietnam, Cambodia, Brazil and some other countries. Bangladesh used to enjoy almost a commercial monopoly; its share in the export market was 80% in 1947-48 but in 1975-76 it fell to only 25% and currently it is further reduced due to invasion of the substitutes of jute by, poly-propylene, polyethylene, multiwalled paper bags etc. Jute fibres are used in hessians and gunnies, carpet and rugs, paper, canvas, tarpaulin, handicrafts, etc.

Jute was cultivated in ancient times in Bengal. At that time it was more or less a garden plant and its leaves were used as a vegetable and for medicinal purposes. Jute grows well where the annual rainfall is 1500 mm or more, with at least 250 mm during each of the months of March, April and May. The optimum range of temperature required is 22° to 33°C.

Jute is cultivated in the rainy season. In Bangladesh sowing usually starts at the end of February and continues up to the end of May, depending on the species and varieties. Cultivation largely depends upon pre-monsoon showers and soil moisture conditions. *C. capsularis* is more water tolerant and thus generally can be

grown in low lands, and even under water logging conditions, while C. *olitorious* is more susceptible to water logging and hence cultivated in medium to medium high lands (Kundu et al., 1959). Jute can be grown in a number of soil types, ranging from clay to sandy loam with optimum fertility and soil pH ranging from 5.0 to 8.6 (BJRI, 1996).

Jute is basically self-pollinated and has fourteen diploid chromosomes (2n=14). Jute is a short-day plant. It needs long day light for vegetative growth and short-day for flowering (ICAR, 1990). After sowing, four to five months are needed for harvesting of the crop. This is done at the flowering stage. The fibre is obtained from the bast or phloem layer of the stem (Dempsey, 1975).

Generally, jute seed is sown broadcast, about 8 to 10 kg of seed per hectare for C. *capsularis* and about 6 to 8 kg per hectare for C. *olitorius* (Hussain and Sobhan, 2001). Traditionally, farmers keep a small part of the crop area for growing seeds until the seeds mature in October/November. After harvesting, plants are bundled together with required number of plants, and kept standing for 4 to 5 days in the field for shedding off the leaves. Then the bundles are put under water. Clear slow flowing water is best for good retting. After 12 to 15 days, when proper retting is completed, the fibre is separated from the stick by hand and then washed and dried in sunlight. After drying, farmers sell the fibre in the local market.

1.2. JUTE IS THE CASH CROP OF BANGLADESH

Two species of jute, C. *capsularis* and C. *olitorius* are widely cultivated in Bangladesh. It is grown in almost all the districts of the country, Faridpur, Tangail, Jessore, Dhaka, Sirajganj, Bogra, and Jamalpur are considered the better growing areas. It is the principal cash crop. On an average Bangladesh produces 0.794 million tons of raw jute on about 0.392 million hectares of land i.e. national average production is 2.03 tons per hectare. This average production is encouraging because the fertile lands are occupied by food crops and jute is being

pushed to the marginal land. It contributes to about 6% of the total export earning and employs around 30 million peoples in different events such as cultivation, processing, carrying, marketing, research, trading, exporting etc. involving jute. Jute offers cash earnings to 5 million farm families (SYB, 2004). So, jute is really a very important cash crop of Bangladesh.

1.3. JUTE IS THE SOIL ENRICHER

Among the major cultivated crops in Bangladesh, jute is the only dicotyledonous plant, possessing a very well developed tap root system, that can penetrate 60 cm or more depth of the soil and capable of breaking plough pan (Goswami and Saha, 1969). Jute enriches soil microbial population by providing better aeration and improves soil drainage condition. It provides 1.92 tons per hectare dry leaves in a season. It contributes 36% of the dry weight as organic matter through leaves, left over roots and about half of the bark immediately as retting waste (Khandakar et al., 1995). Jute contributes 10 times more organic matter than it receives as nutrient from the soil. For breaking disease and pest cycle and also balancing the soil nutrients in upper and lower horizon of the soil, jute could be included in the cropping system to improve and maintain the soil health (Khandakar et al., 1995).

1.4. SOME UNIQUE PROPERTIES OF JUTE COMPARED TO SYNTHETIC FIBRES

- Jute is a natural product and obviously it is biodegradable and environmentally safe.
- It has a greater capacity of retaining moisture.
- It is more flexible.
- As geo jute, it has shown tremendous promise in river embankments to check soil erosion.
- Green jute can be used as a source of paper pulp for production of specialized paper.
- Jute bags have excellent stacking properties.
- Jute sticks are now extensively used for making partex and softwood in furniture.

1.5. CURRENT SITUATION

The global demand for traditional products of jute is declining day to day due to invasion of synthetic fibre. On the other hand, the food demand is increasing very sharply as a result jute is being pushed to the marginal lands where it faces various limitations thus further causing the low yield of jute. In addition to this, farmers are facing problem for retting their green jute for extracting fibre, due to lack and availability of retting water. This also contributes to lowering of the cultivated area. According to the expert's (Mandal, 2000) opinion, jute cannot survive only with its traditional use. There is a golden opportunity to revive the past glory of jute through the production of paper pulp from green jute. It would solve the retting problem and ensure the bulk use of green jute.

1.6. NECESSITY OF THE PRESENT STUDY

Bangladesh holds the largest gene bank of jute and allied fibre (JAF) crops. Bangladesh Jute Research Institute (BJRI) premises preserve 5936 accessions of jute and allied fibre germplasm including 15 species of *Corchorus*, 22 species of *Hibiscus* and 15 of allied genera yet to be characterized. Although a number of varieties have been developed experimentally from time to time from intervarietal crosses using conventional breeding techniques but none has been stable enough to be commercially released (Islam et al., 1992). As jute is a self-fertilized crop, its natural genetic variability is very narrow; this makes the plant breeder helpless in the development of this crop. The molecular techniques could be a suitable alternative to improve the crop. Jute is a new crop in the field of molecular biology. However, very little molecular information of jute or its related species is available at the gene bank, and few efforts have been undertaken in the past to develop molecular markers to study its genetic variability (Hossain et al., 2002, 2003; Basu et al., 2004; Roy et al., 2006). So any kind of molecular level information would be helpful for its improvement. As we observe and believe that jute cannot survive only of its traditional use, a bulk use must be ensured and production of

paper pulp from green jute could be a suitable alternative. If this programme becomes a success, then at least two crops will be required in a year to meet the mill demand for green jute round the year and one crop will have to be produced in the winter season. But jute is a summer season crop and its base temperature is 20°C. To produce jute in the winter season, several environmental stresses such as drought, short-day length and low temperature will need to be addressed. Low temperature below 20°C drastically reduces the physiological activities and ultimately the growth of jute plant. So it is essential to develop low temperature tolerant DNA marker for marker-aided selection and finally to develop a low temperature tolerant jute variety through molecular approach.

1.7. OBJECTIVES OF THE PRESENT STUDY

The present work was undertaken to obtain the following major objectives. A series of related experiments were conducted to support the basic work and their purposes will be mentioned later.

- To develop DNA marker(s) associated with low temperature tolerance.
- To establish a basis for marker-aided selection.
- To develop a genetic map or linkage group for jute.
- To contribute for the development of low temperature tolerant variety to meet the off-season raw material demand in pulp industry.

CHAPTER 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Environmental stresses refer to any kind of abiotic or nonpathogenic stresses such as drought, flood, low temperature, salinity, short-day etc. All abiotic stresses are not equally important for a particular crop. To improve abiotic stress tolerance ability in an organism through molecular approach is a tedious task especially when the organism is a very new in this field.

2.1. QUALITY DNA ISOLATION

The DNA of an individual is identical whether it is extracted from roots, seeds, seedlings, leaves etc. These principles of individual uniqueness and identical DNA structure within all tissues of the same organism provide the basis for DNA profiling. Many standard methods are available for isolating plant genomic DNA. However, problems arise because of contaminants. Polysaccharides are particularly problematic (Scott and Playford, 1996). For example, acidic polysaccharides inhibit *Hind* III enzyme restriction, thereby precluding classic 2-primer PCR (Demeke and Adams, 1992; Pandey et al., 1996) by inhibiting *Taq* DNA polymerase activity (Fang et al., 1992), whereas neutral polysaccharides are not inhibitory (Do and Adams, 1991). Polysaccharides can cause anomalous reassociation kinetics (Merlo and Kemp, 1976). They can also co-precipitate with DNA after alcohol addition during DNA isolation to form highly viscous solutions (Do and Adams, 1991). The DNA is unsuitable for restriction and Southern hybridization and often remains in the wells during electrophoresis. The most effective way to eliminate polysaccharide inhibition is to dilute the DNA extracts, thereby diluting the polysaccharide inhibitors (Pandey et al., 1996). However, excessive dilution of a DNA solution makes it unusable for Southern analysis.

A number of methods are available and are being developed for isolating nucleic acids from plants. Because different plants contain different amount of nucleic acids, a single nucleic acid isolation method is not likely to be suitable for all plants or even different parts of same plant (Loomis, 1974). Plant species of the

same or related genera can exhibit enormous variability in the complexity of pathways of dispensable functions. Thus, the biochemical composition in plant tissues of different species is expected to vary considerably. Chemotypic heterogeneity among species may not allow optimal DNA yields with a single protocol; thus, even closely related species may require different isolation protocols (Weishing et al., 1995). Most protocols recommend isolating DNA from fresh tissues, but sometimes the samples collected from remote and rare locations (with the aim of authenticity through DNA profiling) may consist of plant parts in dry and semidry conditions. Arun and his associates (2002) modified a procedure based on a CTAB method that was previously applied to sorghum seeds (Murray and Thompson, 1980), which contain high levels of polysaccharides (Singh and Asthier, 1988). They extended the protocol to other plant species, including chickpea, soybean, and wheat. The protocol isolates a good yield of DNA from various tissues that is suitable for PCR and restriction digestion. Points that should be considered for obtaining good quality DNA are:

- Use of lyophilized tissues offer several advantages. Dry tissue can be efficiently disrupted while the DNA is unhydrated and can be stored for several years with little loss of quality.
- The tubes should not be shaken vigorously because DNA is very vulnerable to fragmentation.
- If the DNA pellet is coloured, it should be minimized by 2-3 ethanol extractions.
- Care should be taken so that pellet is not dried excessively because over drying makes it difficult to dissolve.

Aljabani et al., (1999) have optimized a simple and rapid method for isolating high quality DNA from polysaccharide- and polyphenolic-rich tissue such as sugarcane, lettuce and strawberry. The protocol utilizes fresh tissue without making use of liquid nitrogen or freeze-drying for initial grinding of the tissue and it significantly minimizes the use of lab materials. The isolated DNA is essentially

free of polysaccharides, polyphenols, RNA and other major contaminants, as judged by: its clear colour, viscosity, ratio of the absorbance at 260 and 280 nm, digestibility with restriction enzymes, and suitability for other polymerase chain reaction (PCR) based techniques. The oxidized forms of polyphenols co-valently bind to DNA giving it a brown colour and making it useless for most research applications (Katterman and Shattuck, 1983; Guillemaut and Marechal-Drouard, 1992). One method commonly used to avoid problems with polyphenols involves freezing the tissue during or prior to homogenization (Katterman and Shattuck, 1983; Leutwiler et al., 1984).

Aljabani et al., (1999) also mentioned other precautions such as preparing all solutions involved in the isolation of DNA just before use avoid longer homogenization times as it can result in partial degraded DNA and avoid disturbing the interface during pipetting of the aqueous phase.

A rapid DNA extraction method for sugarcane and its relatives (Honeycutt et al., 1992), recently, a modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and/or polyphenol components (Porebski et al., 1997; Peterson et al., 1997) have been published but these methods are not convenient with respect to cost, simplicity or time limits.

In respect of storability, the integrity of purified DNA in solution could be compromised by nuclease, pH below 6.0 or above 9.0, heavy metals, UV light, and oxidation by free radicals. Ethylene diamine tetra acetate (EDTA) is often added to chelate divalent cations required for nuclease activity and to prevent heavy metal oxidative damage (Sibylle, 2001). Tris-based buffers will provide a safe pH of 7 to 8 and will not generate free radicals, as can occur with PBS (Miller et al., 1991; Muller and Janz, 1993). Free-radical oxidation seems to be a key player in breakdown and ethanol is the best means to control this process (Evans et al., 2000). Low temperatures are also important for long-term stability. Storage at 4°C is only recommended for short periods (days) (Krajden et al., 1999) even though some studies have shown that storage under ethanol is safe even at elevated

temperatures. Long-term storability of DNA is best obtained at -80°C (Sharova, 1977).

2.2. DNA FINGERPRINTING

DNA fingerprinting or genotyping refers to the characterization of one or more relatively rare features of an individual genome or hereditary make up. An organism's DNA contains the blueprint of its characteristics. Every human, lower animals and sexually reproduced plants has a characteristic phenotype or physical appearance because each possesses unique hereditary composition.

Since each living creature is unique, each has a unique DNA recipe. Individuals within any given species, breed or hybrid line can usually be identified by minor differences in their DNA sequences as few as one difference in a million nucleotides can be detected. Using the techniques of DNA fingerprinting and PCR (polymerase chain reaction) scientist can easily distinguish between closely related individual, map the location of specific genes along the vast length of DNA molecules in the cells or diagnose viral, or fungal infection.

However, it was supposed that genetic analysis can yield valuable information on population structure more quickly and at less cost than, for example, a tagging experiment is more reliable than morphometric studies (Hossain, 2002).

2.3. APPLICATIONS OF DNA FINGERPRINTING

The DNA fingerprints for closely related organisms are very similar. Differences in DNA sequence are observed as the presence or absence of bands. These differences are characteristic and heritable. The fingerprints can be used to establish the identities of specific DNA samples. The fingerprints can also be used as a source of DNA markers for genetic linkage maps or to identify markers linked to a specific locus. The amplification-based scanning methods that use arbitrarily amplified DNA characterize nucleic acids without prior knowledge of nucleotide sequence or cloned and characterized hybridization probes (Livak et al., 1992; Bassam et al., 1992; McClelland et al., 1996). These techniques are versatile and

universal, as demonstrated by the many applications and wide range of organisms studied, covering all domains of life (Caetano-Anolles, 1993, 1996; Rafalski and Tingey, 1993; McClelland et al., 1995; Schierwater, 1995)

The power of DNA fingerprinting was demonstrated in the very first murder case in which it was used. A man had been accused of two rape-murders committed three years apart and had made a confession. Samples of the rapist's semen were available from both attacks, and DNA fingerprinting by Alec Jeffreys showed, just as the police had surmised, that the same man was involved in both attacks. However, it was clear that the suspect was not the culprit. This result was, at first, unbelievable, and the DNA fingerprinting had to be repeated several times before he was caught, and DNA fingerprinting confirmed the identification.

DNA fingerprinting is now in use in many countries. In the United Kingdom, DNA fingerprinting is used to resolve family relationships in over 3000 immigration cases each year. DNA fingerprinting in the United Kingdom also has been fully accepted as a tool in forensic science, being used in several thousand forensic cases each year. In the United States, DNA fingerprinting has come under particularly close scrutiny in the courtroom.

DNA profiling is applicable in a number of areas in medicine, human forensic science, etc. such as:

- Twin zygosity testing.
- Bone marrow transplantation analysis.
- Detection of DNA changes in tumors.
- Identification of possible contamination of fetal by maternal tissue in chorionic villus analysis.
- Pathogen identification.
- Paternity testing where family studies are being performed for antenatal diagnosis of inherited diseases.
- Genetic diversity studies in flora and fauna (Hossain, 2002).

2.4. CURRENTLY USED TECHNIQUES FOR DNA FINGERPRINTING

- RAPD
- RFLP
- PCR based RFLP
- AFLP
- MINISATTELITES
- MICROSATTELITES or SSR or STMS
- ISSR
- DAMD-PCR

2.4.1. RAPD

RAPD stands for random amplified polymorphic DNA. RAPD analysis can be used for detecting genes of interest in a crossing programme. RAPD uses short primers and PCR can identify extremely large numbers of genomic polymorphism (Williams et al., 1990, 1993; Welsh and McClelland 1990).

In this technique, an arbitrary oligonucleotide primer, usually 9 to 10 bp long, that does not contain any palindromic sequences and has a G+C content of 50 to 80% is added to a sample of plant genomic DNA (Glick and Pasternak, 2003). DNA amplified products are generated for each genomic region that happens to be flanked by a pair of 10-base priming sites (in the appropriate orientation), which are within 5000 base pairs of each other. The DNA sequence between inverted primer sites is amplified, and, because the distance between primer sites can vary among individuals, different length fragments are generated. Because the primers are small and not specific to any given gene, many different fragments are produced in an amplification. Fragments present in one individual, but not in another, are defined as polymorphic markers. The amplification products are analyzed by electrophoresis. Genomic DNA from two different individuals often produces different amplification fragment patterns. A particular DNA fragment, which is generated for one individual but not for another, represents a DNA polymorphism and can be used as a genetic marker. These markers are inherited

in a Mendelian fashion. In fingerprinting studies, the banding patterns are compared directly to allow strain determination, usually without the need to correlate band differences with particular properties.

To explain RAPD, the technique of PCR has to be explained first. In PCR a thermo stable DNA polymerase and four deoxy-NTPs (dATP, dGTP, dCTP and dTTP) are used to amplify a specifically targeted segment of a longer DNA molecule. Selective amplification is achieved through the addition of two specific oligonucleotides. These anneal to denatured target DNA segments at the two ends to act as primers for DNA synthesis. The primers are present in great excess over the DNA and so annealing of the primers to the single stranded DNA is favoured over renaturation of that segment of the DNA. To anneal specifically to the desired location, the primers are usually synthesized to provide about 20 base pair (bp) of duplex DNA at each end of the segment. The three steps of the PCR reaction (template denaturation, primer annealing and elongation) must be repeated many times to achieve high levels of amplification. These three stages occur at different temperatures (typically 94°C, 37-40°C, 72°C) and repetition of these three steps, cycle about 40 times. This can amplify the target DNA segment a million fold. The number of amplified products in RAPD analysis depends upon the primer sequence, DNA concentration, type of thermocycler, geometry of the tube and the genome size.

Unlike restriction fragment length polymorphism (RFLP) analysis by Southern blotting, where it is usual for a probe to show polymorphism by hybridizing to fragments of different lengths in the DNAs being compared, RAPD primers will show polymorphism just by separating the products by gel electrophoresis. RAPD patterns can be compared for linkage to traits of interest in a breeding population.

RAPD has been used in a wide range of plant species for genetic mapping and gene tagging (Martin et al., 1991; Rafalski et al., 1996), for parentage determination and for species identification (Welsh et al., 1990). The advantage of using RAPDs in genetic analysis is cost effective. The procedure requires little DNA (Welsh et

al., 1990) and allows the use of simple procedures for the isolation of genomic DNA (Williams et al., 1990). This technique is easy and fast because a single primer may detect more than one locus; it is useful for phylogenetic studies (Smith, 1995). Finally it is applicable as and when the genome sequence is unknown (Cooke, 1999).

2.4.2. *RFLP*

RFLPs are differences in the length of some restriction fragments observed after digestion of DNA with a specific restriction endonuclease. These differences are the result of mutations occurring over time and are detected as variations (polymorphism) in the length of restriction fragments. In this technique, genomic libraries are screened for probes to variable regions and the probes are then used to detect RFLP via Southern hybridization.

When DNA is extracted from a tissue, it is digested with restriction enzymes, size fractionated on a gel using electrophoresis, and then transferred to a hybridization membrane (usually nitrocellulose or nylon) in a single stranded form. Cloned sequences of DNA (probes) are labeled (using ^{32}P, but non-radioactive labeling techniques are becoming more refined) and under appropriate conditions, the labeled single-stranded probe hybridizes to its single stranded DNA counterpart on the membrane. Unhybridized probe is then washed off and the membrane exposed to X-ray film to obtain an autoradiograph. The bands visible on an autoradiograph represent the restriction fragments in the digested plant DNA that contains the sequence homologous to the cloned sequence used as a probe.

When the size of fragments hybridizing to the probe is different in the individuals being examined, a polymorphism exists. Fragment sizes at particular sites will differ among individuals either because of base substitutions that result in the addition or the deletion of an enzymes cutting site or because of the insertion or deletion of a portion of DNA bracketed by or overlapping the cutting sites. Research on the use of RFLP for fingerprinting and cultivar identification was

documented for beets (Nagamine et at., 1989) and maize (Smith and Smith, 1991). The RFLP technique was also used to detect quantitative trait loci (QTL) associated with seed protein and oil content in soyabean (Diers et al., 1992)

2.4.3. PCR based RFLP

A PCR modification of this technique employs specifically designed primers to amplify single or low copy DNA. Williams and co-workers (1991) converted 30 mapped RFLP markers to PCR based markers by determining the end sequence of the mapped RFLP probes. They then synthesized pairs of primers based on the determined sequence. These primers were then used to amplify DNA corresponding to the region represented by the RFLP marker. They were able to show the usefulness of the technique particularly when it was coupled with digestion of the PCR products.

Hence, PCR based makers can function like RFLP markers in determining homology or by following introgression of a desired gene in a much shorter time.

2.4.4. AFLP

AFLP stands for amplified fragment length polymorphism were invented in 1995 (Vos et al., 1995) and has become widely used for mapping, tagging, and genotyping (Keim et al., 1995; Bohn et al., 1999; Corbellini et al., 2002; Hartl et al., 1999; Hazen et al., 2002; Manifesto et al., 2001). It is based on PCR amplification of a set of restriction fragments, selected from a pool of fragments, that are generated due to digestion with a pair of specific enzymes, one of them being a frequent cutter (e.g. *Mse*I), and the other being a rare cutter (e.g. *Eco*RI) (Hill et al., 1996), to facilitate designing of primers for selectioin of restriction fragments, oligonucleotide adapters, few base pairs (~20) long are ligated at the ends of these DNA fragments. With the help of these ligated adapters, the number of DNA fragments to be amplified can be restricted, since the primers are designed to bind to the following sequence: ligated adapters + the restriction site of the enzyme used for digestion + 1-3 selective bases (chosen randomly).

This method generates a large number of bands representing the amplified products from selected restriction fragments, thus facilitating the detection of polymorphism (Gupta, 2004). This technique actually detects the presence or absence of restriction fragments rather than the difference in their length. AFLP has been used for genetic mapping of plants and to distinguish among closely related species (Keim et al., 1995; Bohn et al., 1999; Corbellini et al., 2002).

2.4.5. MINISATTELITES

Minisattelites, also called hyper variable repeats (HVR) and/or variable number of tandem repeats (VNTR) is tandem repeating DNA sequences, which generally consist of 10-60 bp motifs. Regions containing minisattelites usually show high levels of RFLP. Extreme variations in the tandem repeat copy number of minisattelite loci is considered to be the source of the observed polymorphisms in humans. To-date, a number of minisattelite sequences has been identified in a variety of plant species. Most of the minisattelites share a common motif known as the core sequence. The tandemly repeated segments of a core sequence of DNA are distributed throughout the genome (i.e. at many loci) and are highly polymorphic in the number of repeats they contain, resulting in the ability to resolve genetic identity/nonidentity.

Minisattelite DNA probes have also been used to differentiate clones that have been indistinguishable using other markers including fishes (Turner et al., 1990). Although the number of loci and allelic assignments are unknown for multilocus techniques, such as DNA fingerprinting, recent theoretical advances by Lynch (1990, 1991) have provided methods for using DNA fingerprinting data (band sharing) to make estimates of population genetic parameters. These are not unbiased estimates, but provide reasonable first approximations.

2.4.6. Microsatellites or SSRs or STMS

Simple sequence repeats or SSRs are also known as short tandem repeats (STRs) or microsatellites (1-6 bases long) are ubiquitous in eukaryotic genomes and can be analyzed through PCR technology (Lagercrantz et al., 1993). The sequences flanking specific microsatellite loci in the genome are believed to be conserved within a particular species, across species within a genus and rarely even across related genera. These flanking sequences, therefore, can be used for designing primers to amplify individual microsatellite loci and the technique is described as sequence tagged microsatellite site (STMS) analysis (Beekmann and Soller, 1990). The STMS or SSR markers reveal polymorphisms due to variation in the lengths microsatellites at specific individual loci; they are, therefore, polyallelic and co-dominant in nature, thus proving to be very useful. Consequently, they have been used extensively not only for mapping SSR loci in many crop plants but also for tagging genes in a variety of organisms (Gupta and Varsheny, 2000; Roder et al., 1998; Varshney et al., 2000). Since development of SSR markers requires cloning and sequencing, initially it is very costly and labour-intensive, but once the locus specific primers become available, the approach becomes cost-effective.

Furthermore, the differences in allele size are, though, difficult to resolve on agarose gel electrophoresis with ethidium bromide staining (Heun, 1994; Huttel, 1999; Lagoda et al., 1998) high resolutions, without applying radioactivity, can be achieved through the use of polyacrylamide gels in combination with either ethidium bromide (Scrimshaw, 1992) or silver staining (Klinkicht and Tautz, 1992). Both denaturing and non-denaturing polyacrylamide gel electrophoresis (PAGE) were used to resolve size differences between alleles (Lagoda et al., 1998; Gay et al., 1999; Brondani et al., 1998). The use of fluorescent primers in combination with a semi-automated DNA sequencer has been shown to be very promising alternative (Ziegle et al., 1992; Schwengel et al., 1994) and has been actually used to assay genetic variation in soybean (Diwan and Cregan, 1997). At present RFLP and RAPD are the most widely used types of DNA markers.

Microsattelites or simple sequence repeats (SSR) or STMS combine the advantages of the co-dominantly inherited RFLP with the convenience of the PCR based method.

2.4.7. ISSR

Inter simple sequence repeats (ISSR) was developed such that no sequence knowledge was required. Primers based on a repeat sequence, such as $(CA)_n$, can be made with a degenerate 3′ -anchor, such as $(CA)_8RG$ or $(AGC)_6TY$. The resultant PCR amplifies the sequence between two SSRs, yielding a multilocus marker system useful for fingerprinting, diversity analysis and genome mapping (Zietkiewicz et al., 1994). A typical reaction yields 20-100 bands per lane depending on the species and primer (Godwin et al., 1997). ISSR markers have compared favorably with RFLP and RAPD markers with applications to DNA fingerprinting and diversity analysis in sorghum (Yang et al., 1996), finger millet (Salimath et al., 1995), maize (Kantety et al., 1995) and sweet potato (Huang and Sun, 2000).

2.4.8. DAMD PCR

Heath et al. (1993) have reported a new technique, called direct amplification of minisattelite region DNA (DAMD) that has been used to distinguish among species. This technique uses PCR to direct the amplification to regions rich in minisattelite repeat. By using the core sequence of minisattelite as a single primer, this PCR application is capable of producing RAPD like results (species specific banding pattern) for the identification of specific regions in a genome. It has been speculated that if a portion of a minisatellite array is involved in an inversion this would make PCR possible using a single minisatellite core sequence as a primer. Thus these amplified fragments can also be used to generate single-locus minisatellite probes. If inversions have taken place, then the repeats would most likely have a piece of single-copy flanking DNA inserted between them. Therefore, DAMD could also amplify sequences formerly adjacent to hyper variable minisattelite loci.

Furthermore, since minisattelite core sequences are longer than RAPD primers, DAMD-PCR can also be effectively carried out at relatively high stringency, thus yielding highly reproducible results (Heath et al., 1993).

2.5. DNA FINGERPRINTING OF PLANTS

For the study of genetic diversity, the plant scientists have used traditionally morphological as well as physiological features of plants. Several genes have been named after their physical appearance before their biochemical (DNA) nature and physical location on chromosomes were identified. The number of scorable morphological characters is very few as compared to the biologically active genes. Moreover in most cases, plant genomes have large amounts of repetitive DNA which are not expressed and do not contribute to the physiological or morphological appearance of the plants. In the case of very closely related plant varieties and species, there are very few morphological differences, which as a matter of fact do not represent the true genetic differences at the DNA level. So, there is always a need to study polymorphism at DNA level, which can be an indicative of genetic diversity.

Welsh and McClelland (1990) used arbitrary primers for fingerprinting of genomes. They also used arbitrary primers for the characterization of pathogenic microorganisms. They also reported RNA fingerprinting using arbitrary primers. The use of RAPD primers for comparative genome analysis was reported by Williams et al. (1993). Debener et al. (1996) used RAPD analysis to get genetic variation between a group of rose varieties and selected wild rose species.

The RAPD markers were used for variety identification and molecular characterization of newly bred lines of *Brassica juncea* (Fujishiro and Sasakuma, 1994). Yazaki et al. (1994) analyzed DNA polymorphism in maize inbred lines by RAPDs. Paran et al. (1991) used RFLP and RAPD markers to downy mildew resistance genes in lettuce.

Hoey and Crowe (1996) conducted a phylogenetic analysis of Pisum based on morphological characters, and allozymes and RAPD markers. Newton et al. (1994) evaluated the extent of genetic variation in mahoganies using RAPD markers.

A comparison was done with RAPD and isozyme analysis for determining the genetic relationships among *Avena sterilis* by Heun et al. (1994). Thormann and Ferrerja (1994) have done a comparison of RFLP and RAPD markers to estimate genetic relationships within and among cruciferous species.

2.6. MOLECULAR MARKERS

As long as map construction and marker aided selection depend on morphological markers alone, the pace of progress was bound to be slow because of the paucity of such markers and the improbability that any particular cross would feature any more than a few markers. The situation began to change when isozyme markers were introduced and then DNA markers. These biochemical markers have the advantage that, for the most part, their parents forms are phenotypically neutral, frequent polymorphism can therefore be obtained for biochemical markers. Because isozyme markers predated DNA markers and are easier and cheaper to use, there is a considerable literature on the use of isozyme markers (Heinz, 1969; Thom and Maretzki, 1970; Waldron and Glasziou, 1971; Fautret and Glaszmann, 1988), but DNA markers are likely to prove more important in the long term because their frequency is much greater than that of isozyme markers and it is certain that the inconvenience and cost of applying DNA markers will decline markedly over the next few years. For this reason, most scientists now prefer DNA markers.

Provided a suitable segregating population is available, it is now possible to tag a gene of interest with a DNA-marker within few weeks (Michelmore et al., 1991) and to prepare a usable map of an entire nuclear genome within a few months (Reiter et al., 1992).

With the advancement of technological improvements, the cost of DNA-based gene tagging and genome mapping are declining to a level where they can be expected to make significant contributions to plant breeding in both industrialized and developing countries.

A comparison of general features of different molecular markers and their uses (Gupta, 2004).

Principle	RFLPs	RAPDs	AFLPs	SSRs	SNPs
	Endonuclease restriction, southern blot hybridization	DNA amplification with random primers	Endonuclease restriction use of adapters selective primers	Amplification of simple sequence repeats using specific primers	Sequence analysis
Types of polymorphism detected	Base changes	Base changes	Base changes	Variation in length of repeats	Single base changes
Sequence information needed	No	No	No	Yes	Yes
Detection method (radioisotope)	Yes/no	No	Yes/no	Yes/no	No
Reproducibility	High	Low	High	High	High
No. of loci detected	1-5	1-10	>70	1-3	1 (biallelic)
Inheritance	Co-dominant	Dominant	Dominant	Co-dominant	Domi-nant
Cost	Medium	Low	Medium/ High	Low	Medium /High
Use: Varietal fingerprinting and genetic diversity	+	+	+ + +	+ + +	+
Qualitative gene tagging	+	+	+ + +	+ + +	+
QTL mapping	+	+	+ + +	+	+
MAS	+	+/-	+	+	+

2.7. UTILIZATION OF DNA MARKERS IN JUTE AND KENAF BREEDING

DNA molecular marker analysis is the method of trait analysis at DNA levels, which escape the limitation of environment influence and gene expression. Molecular markers are becoming more and more important tools for selecting plants of desired types in breeding programme (Poulsen et al., 1996).

There are two types of molecular markers: hybridization based marker (RFLP) and PCR based markers (Williams et al., 1990). Breeders can determine a trait of agronomic interest, and track the trait in genetic crosses. In other applications, molecular markers that are uniformly distributed throughout the genome are used to estimate the genetic contribution of each to each member of a segregating population.

There are many plant species where these molecular markers are easily available either through their respective genome project or sequences in publicly available DNA databases. However in jute, very little molecular work has been conducted (Hossain et al., 2002, 2003; Basu et al., 2004; Roy et al., 2006) and no real effort has been reported using sequence specific molecular markers to study its genetic variability. Only recently, some jute-specific DNA sequences have been posted in the GenBank (Ashraf et al., manuscript submitted).

Jute and kenaf cultivation has high economic significance (Mandal, 2000). The former International Jute Organization (IJO), now International Jute Study Group (IJSG) has already collected more than two thousand germplasm of jute (*Corchorus* spp. L.) and kenaf (*Hibiscus cannabinus* L.), including cultivars and related wild species. Some of these carry different kinds of beneficial genes, which had been identified and partially transferred into cultivars (Hussain and Sobhan, 2001). Much more investigation of morphological characters and agronomic traits, disease resistance and stress (salinity, drought, water logging, extreme temperature etc.) tolerance of the collected germplasm has been developed for genetic study and gene mapping (Hussain and Sobhan, 2001).

Hossain et al. (2002) conducted an experiment with 9 jute cultivars and 12 accessions; evaluated those separately using RAPD markers with 29 primers. Seven primers generated substantial differences between the different jute varieties and 6 primers gave polymorphism within the accessions. DNA fingerprinting that was obtained revealed that the cultivars both *C. capsularis* and *C. olitorius* were genetically distinct. A dendrogram was constructed, on the basis of genetic distance matrix, showed that the cultivars were well differentiated into two clusters representing two species. The genetic distance within species among the cultivars was also reflected in the dendrogram. Similar trend was also observed in case of the accessions under study. The genetic distance information would be helpful in selecting parents for conventional breeding.

In another study (Hossain et al., 2003), two PCR based fingerprinting techniques; RAPD and AFLP were applied on six jute genotypes. Two of them were low temperature sensitive and four were tolerant. Both the techniques were employed to generate polymorphism between low temperature sensitive and tolerant, because of their simplicity, and also because of the paucity of sequence information on jute. RAPD data were obtained by using 30 random primers. Five were found to give polymorphisms between genotypes. AFLP fingerprinting was generated using 25 combinations of selective amplification primers. Eight combinations gave the best results with 93 polymorphic fragments, and they were able to discriminate the two low temperature sensitive and four tolerant jute genotypes. A cluster analysis, based on RAPD and AFLP marker data, showed the trait specific grouping of individuals. This information would be useful in marker-aided selection between the traits (Hossain et al., 2003).

A similar study was made to evaluate the genetic diversity in 49 genotypes of jute using 7 SSR (*Nicotiana tabacum* L. chloroplast specific) and 10 pairs (*Eco*RI and *Mse*I) of AFLP primers by Basu et al. (2004). A total of 305 polymorphic products were detected by AFLP and two to four allelic lengths were detected with each pair of SSR primers. Results from both evaluations showed that the level of

variation between species is high but within species, among the genotypes is low. The resulting dendrogram showed common ancestral origin for many genotypes. A few major Indian cultivars of both the species, used as an internal check, were closely related to the wild accessions. The results indicate that enough diversity exists to broaden the genetic base of new jute cultivars.

Very recently, an effort was made by Roy et al. (2006) to estimate pattern of genetic diversity in jute, with 20 exotic germplasm lines and 20 commercial varieties of the two cultivated species (*Corchorus olitorius* and *C. capsularis*) and two wild relatives of jute (*C. aestuans* and *C. trilocularis*) using STMS, ISSR and RAPD markers. Result showed that, six STMS markers developed from the genomic sequence of *C. olitorius* was not fully transferable to the related species *C. capsularis*. The level of intraspecific polymorphism revealed by these markers was very low. The four ISSR and 22 RAPD primers employed in the study revealed 98.44% and 100% polymorphism, respectively, across all the species, while the level of polymorphism was significantly low within a species. The commercial varieties, particularly those of *C. capsularis*, had an extremely narrow genetic base. The germplasm accessions in both the cultivated species showed considerably higher levels of diversity and thus could be used in broadening the base of the varieties. All the accessions of *C. olitorius* together with the wild species *C. aestuans* clustered separately from those of *C. capsularis* and *C. trilocularis*, suggesting a polyphyletic origin of the two cultivated species.

To compare the relative efficiencies and to find the most suitable marker for jute diversity studies, an effort was made by Ashraf et al. (manuscript submitted) to evaluate three sequence independent DNA marker systems, RAPD, ISSR and AFLP. Their discriminating capacity was analyzed by taking five elite genotypes of each of the two cultivated species of jute, *C. olitorius* and *C. capsularis*. The level of genetic diversity among jute genotypes was scored using 62 RAPD and 135 ISSR and 749 AFLP markers.

All three-marker systems were able to discriminate between the two species, however intraspecific variation was found to be very low. For estimating genetic distance the AFLP and ISSR markers gave the most correlated results, with a correlation coefficient of r=0.86 within *C. olitorius* and r=0.71 within *C. capsularis*. The result indicated the RAPD markers had the highest polymorphic information content within the genotypes used in the study.

Some efforts were also made (Cheng et al., 2002, 2004) for molecular characterization in kenaf (*Hibiscus cannabinus* L.). With data from morphological analysis, it is difficult to identify individual varieties. A study with 14 kenaf varieties has indicated that RAPD analysis is able to identify and determine their genetic relationships to a certain extent, but the number of DNA polymorphic fragments detected was relatively low. It was also observed that genetic relationship of the kenaf varieties obtained from morpho-agronomic studies and RAPD analysis, were found very similar (Cheng et al., 2002). More molecular data needs to be accumulated to determine diversity and genetic relationships of kenaf germplasm.

Another study was made by Cheng et al. (2004) with 23 kenaf accessions and 2 accessions of its relative, roselle (*H. sabdariffa* var. *altissima*), in order to identify different genotypes and genetic relationships, using AFLP analysis. A total of 505 polymorphic markers (out of 560) were produced by six selected AFLP primer combinations. The AFLP fingerprinting was effective in identifying all kenaf accessions included in their study. Kenaf and roselle are independent species with close relationships, and great genetic diversity was also detected among the kenaf accessions with different origins, based on the analysis of AFLP markers. Unlike Ashraf et al. works on jute, AFLP analysis provided a powerful and reliable molecular tool than RAPD analysis for the identification of kenaf samples.

All molecular markers used in jute and kenaf will be useful in marker-aided selection (MAS). Jute and kenaf have a high potential tolerance to adverse conditions.

It is necessary to plant jute in the areas, such as, salinity, drought prone land, and water logged areas, through incorporating the environmental stress tolerant gene from related species to national cultivars by MAS (Wen-Jing et al., 1999).

2.8. MECHANISM FOR ENVIRONMENTAL STRESS TOLERANCE

Environmental stresses are any kind of abiotic stresses; it may be drought, waterlogging, salinity, low temperature etc. The nature of various stresses is different and their adaptive mechanisms are also different.

2.8.1. DROUGHT

Drought is one of the important environmental stresses. The whole plants respond to drought through morphological, physiological, and metabolic modifications occurring in all plant organs. The morphological adaptive mechanism for drought tolerance involves leaf characters. Leaves become serrated or modified into hair like structure, thick cuticle, sunken stomata and glossy leaf surface; that means all modifications involved to minimize evapo-transpiration loss of water (Pandey and Sinha, 1978). At the cellular level plant responses to water deficit may result from cell damage, whereas other responses may correspond to adaptive processes. Although a large number of drought-induced genes have been identified in a wide range of plant species, a molecular basis for plant tolerance to water stress remains far from being completely understood (Ingram and Bartels, 1996). The rapid translocation of abscisic acid (ABA) in shoots via xylem flux and the increase of ABA concentration in plant organs correlate with the major physiological changes that occur during plant response to drought (Zeevaart and Creelman, 1988). It is widely accepted that ABA mediates general adaptive responses to drought. However, there is evidence suggesting that additional signals are involved in this process (Munns and King, 1988; Trejo and Davies, 1991; Munns et al., 1993; Griffith and Bray, 1996).

Six cDNAs corresponding to transcripts up-regulated by water stress were isolated previously from a drought tolerant sunflower (*Helianthus annuus* L.) line

(R_1) (Ouvrard et al., 1996). Comparison of the steady-state level of transcripts between the R_1 line and a closely related drought sensitive line (S_1) has shown that three of those transcripts (*HaElip1*, *HaDhn1*, and *HaDhn2*) were differently accumulated in tolerant compared with sensitive plants during water deficit. In response to exogenous ABA in leaves of the R_1 genotype, *HaDhn1* and *HaDhn2* transcripts were up-regulated and the steady state level of *HaElip1* transcripts was not modified (Ouvrard et al., 1996). *HaDhn1* and *HaDhn2* deduced proteins belong to the dehydrin family, and *HaElip1* is a related homologue of early-light-induced protein (ELIP).

Among the water-stress-induced proteins so far identified, dehydrins, the D-11 subgroup of late-embryogenesis-abundant (LEA) proteins (Dure et al., 1989) are frequently observed, and more than 65 plant dehydrin sequences are available (Close, 1997). Dehydrins are highly abundant in desiccation tolerant seed embryos and accumulate during periods of water deficit in vegetative tissues. These proteins display particular structural features such as the highly conserved Lys-rich domain predicted to be involved in hydrophobic interaction leading to macromolecule stabilization (Close, 1996). Studies have established correlations between drought adaptation and dehydrin accumulation in wheat and poplar (Labhilili et al., 1995; Pelah et al., 1997).

Positive correlations were also reported for species tolerant to stresses that have a dehydrative component such as salt stress (Galvez et al., 1993; Moons et al., 1995) and freezing and cold stress (Arora and Wisniewski, 1994; Danyluk et al., 1994; Close, 1996; Artlip et al., 1997).

2.8.2. WATERLOGGING OR FLOODING

Flooding or excess water results in reduced absorption of water and leaf water deficit is the earliest response (Gill, 1970; Rowe and Beardsell, 1973). Later responses include leaf epinasty, stem swelling, formation of callus and adventitious roots, leaf senescence and leaf abscission.

Injury by flooding varies with timing and duration of flooding and physiochemical conditions of the floodwater (Gill, 1970; Rowe and Beardsell, 1973).

Both anatomical and physiological adaptations are important factors in tolerance to flooding (Kozlowski, 1976). Formation of aerenchyma is important in some species and production of adventitious roots appears to be one of the most important adaptations (Hook et al., 1970). The adventitious or water roots increased the absorbing surface for water and minerals. Under flooding the newly initiated roots oxidized the rhizosphere whereas unflooded roots did not (Hook et al., 1971). Oxygen entered the stem through lenticels and appeared to be transported through the cortex or phloem. The combined adaptations of increased anaerobic respiration, oxidation of the rhizosphere, and tolerance to CO_2 by the new roots appeared to account for flood tolerance of this species (Hook et al., 1972).

The mechanisms of survival during waterlogging may be important at the seed germination stage or during vegetative growth. These mechanisms may involve avoidance of waterlogging effects, e.g. by phenological development; adaptation to waterlogging, e.g. by aerenchyma or metabolic change; and recovery mechanisms following waterlogging (Greenway et. al., 1994). Plants for water-logging avoidance/tolerance may use one or all of these mechanisms. The particular mechanism used in cereals may vary depending on the crop, the growth habit and the duration of waterlogging (Setter et al., 2001). Tolerance to long term waterlogging requires plants not only to 'survive' but also to grow during the waterlogging event(s). The key strategy used for long term waterlogging is the development of aerenchyma in roots to facilitate gas diffusion (Armstrong, 1979; Blom, 1999; Jackson and Armstrong, 1999).

2.8.3. LOW TEMPERATURE OR COLD

Low temperature is an important environmental factor that limits plant distribution, survival, and crop yields. Exposure to low, nonfreezing temperatures induces genetic, morphological, and physiological changes in plants, which result

in the development of cold hardiness and the acquisition of freezing tolerance (Vasil'yev, 1961; Levitt, 1980; Guy, 1990; Thomashow, 1990; Huner et al., 1993). Biochemical and molecular analyses have demonstrated differential gene expression and the accumulation of specific proteins during the induction of freezing tolerance (Guy, 1990; Howarth and Ougham, 1993; Lee and Chen, 1993; Thomashow, 1990, 1993). Cold-induced genes have been isolated and characterized in many species, such as alfalfa *(Medicago sativa), Arabidopsis tkaliana,* barley *(Hordeum vulgare), Brassica napus,* and wheat *(Triticum aestivum)* (Houde et al., 1992; Chauvin et al., 1993, 1994; Dhindsa et al., 1993; Thomashow et al., 1993; Weretilnyk et al., 1993; Danyluk et al., 1994; Crosatti et al., 1995; Limin et al., 1995). There is a high correlation between the expression of some of these genes, which appear to be up-regulated by low temperature, and the development of freezing tolerance (Guy, 1990; Thomashow, 1990, 1993; Howarth and Ougham, 1993; Lee and Chen, 1993). In wheat cold acclimation rapidly induces a specific set of *Wcs,* which subsequently disappear upon deacclimation. *Wcs* are regulated by low temperature at the transcriptional level and winter wheat cultivars exhibit higher levels of expression than spring cultivars (Houde et al., 1992; Chauvin et al., 1993; Danyluk et al., 1994; Limin et al., 1995). *WcsZ9* is a nuclear gene specifically regulated by low temperature, but it also requires light for maximal induction (Chauvin et al., 1993). The function of these cold-stimulated genes and their encoded polypeptides have not been demonstrated fully, it is difficult to assess their direct relevance to freezing tolerance. The mechanism by which low-temperature signals are perceived and transduced into specific biochemical responses is largely unknown (Trewavas and Gilroy, 1991; Dhindsa et al., 1993; Bowler and Chua, 1994). Light, through the process of photosynthesis, provides the energy required for cold acclimation, which leads to the attainment of freezing tolerance (Dexter, 1933; Tysdall, 1933; Steponkus and Lanphear, 1968; Levitt, 1980; Griffith and McIntyre, 1993).

It was reported that tocopherols are amphipathic molecules, and in vitro studies using artificial membranes have shown that tocopherols form complexes with

specific lipid constituents and physically stabilize membranes and play crucial roles in low-temperature adaptation (Wassall et al., 1986; Stillwell et al., 1996; Wang and Quinn, 2000; Bradford et al., 2003).

The adaptation of a species to low temperature requires an increase in the flexibility of the micro-structures of protein molecules (Alexandrov, 1967). Kacperska-Palacz et al. (1977) observed protein changes during the development of cold hardiness in winter rye (*Secale cereale*). The contribution of the protein synthesized in the cold acclimated seedlings of alfalfa to tolerate low temperature, even sometimes in freezing temperature (Rajinder et al., 1988; Taiz et al. 1991).

A comparison was made between a freezing-tolerant, cold-acclimating (CA) wild potato species (*Solanum commersonii*) and a freezing-sensitive, nonacclimating (NA) cultivated species (*Solanum tuberosum*). Comparative studies allowed differentiation of plasma membrane lipid changes associated with increased freezing tolerance following CA from lipid changes that can result from metabolic adjustment to reduced temperature during CA. Following CA treatment lipid changes found in both the NA and CA species included a decrease in palmitic acid, an increase in unsaturated to saturated fatty acid ratio, an increase in free sterols, an increase in sitosterol, and a slight decrease in cerebrosides. Lipid changes detected only in the acclimating species included an increase in phosphatidylethanolamine, a decrease in sterol to phospholipid ratio, an increase in linoleic acid, a decrease in linolenic acid, and an increase in acylated steryl glycoside to steryl glycoside ratio. These changes were either absent or opposite in the NA species, suggesting an association of these lipid changes with CA. Furthermore, the lipid changes associated with increased freezing tolerance during CA were distinct from lipid differences between the two species in the NA state (Palta et al., 1993).

A wide range of studies indicates that the membrane systems of the cell are the primary site of freezing injury in plants (Levitt, 1980; Steponkus, 1984). In addition, it is well established that freeze-induced membrane damage results

primarily from the severe dehydration associated with freezing (Steponkus, 1984; Steponkus et al., 1993). As temperatures drop below 0±C, ice formation is generally initiated in the intercellular spaces due, in part, to the extracellular fluid having a higher freezing point (lower solute concentration) than the intracellular fluid. Because the chemical potential of ice is less than that of liquid water at a given temperature, the formation of extracellular ice results in a drop in water potential outside the cell. Consequently, there is movement of unfrozen water down the chemical potential gradient from inside the cell to the intercellular spaces. Thus, a key function of cold acclimation is to stabilize membranes against freezing injury. Indeed, cold acclimation prevents expansion-induced-lysis and the formation of hexagonal II phase lipids in rye and other plants (Steponkus et al., 1993). Multiple mechanisms appear to be involved in this stabilization. The best mechanisms documented are changes in lipid composition (Steponkus et al., 1993). However, the accumulation of sucrose and other simple sugars that typically occurs with cold acclimation also seems likely to contribute to the stabilization of membranes as these molecules can protect membranes against freeze-induced damage in vitro (Anchordoguy et al., 1987; Strauss and Hauser, 1986). In addition, there is emerging evidence that certain novel hydrophilic and LEA (late embryogenesis abundant) polypeptides also participate in the stabilization of membranes against freeze-induced injury. Although freezing injury is thought to result primarily from membrane lesions caused by cellular dehydration, additional factors may also contribute to freezing-induced cellular damage. There is evidence that freeze-induced production of reactive oxygen species contributes to membrane damage and that intercellular ice can form adhesions with cell walls and membranes and cause cell rupture (Olien and Smith, 1977). There is evidence that protein denaturation occurs in plants at low temperature (Guy et al., 1998), which could potentially result in cellular damage. In these cases, the enhancement of antioxidative mechanisms (McKersie and Bowley, 1997), increased levels of sugars in the apoplastic space (Livingston and Henson, 1998), and the induction of

genes encoding molecular chaperones (Guy and Li, 1998), respectively, could have protective effects.

Significant higher amount of lignin contents were observed in selected low temperature tolerant jute genotypes than sensitive one by Begum (1997). It may be mentioned that Buxton (1992) reported important role of lignin in plant defense mechanism and according to him more lignin content help plants to withstand cold stress.

2.9. DNA CLONING IN CROP IMPROVEMENT

DNA cloning has opened opportunities unimaginable just a few decades ago, including the identification and study of genes involved in almost every known biological process. These new methods are transforming basic research, in many areas particularly in agriculture such as Bt cotton, vitamin 'A' rich golden rice, transgenic tobacco etc (Gupta, 2004).

There is a continuing need to increase food production, particularly in the developing countries of Asia, Africa and Latin America. And this increase has to come from increased yields from major crops grown on existing cultivable lands. One practical means of achieving greater yields is to minimize the pest associated losses, which are estimated at 14% of the total agricultural production: 52% in wheat, 83% in rice, 59% in maize, 74% in potato, 58% in soybean and 84% in cotton (Oerke et al., 1994).

Integrated pest management (IPM) has historically placed great hopes on host plant resistance. However, conventional host-plant resistance to insects involves quantitative traits at several loci, and as a result, the progress has been slow and difficult to achieve. With the advent of genetic transformation techniques based on recombinant DNA technology, it is now possible to clone genes into the plant genome that confer resistance to insects (Bennett, 1994).

Toxin genes from bacteria such as *Bacillus thuringiensis* (Bt) and *Bacillus sphaericus* (Gill et al. 1992; Charles et al. 1996) have been the most successful group of organisms identified for use in genetic transformation of crops for pest control on a commercial scale.

Many crop species have been genetically engineered to produce Bt toxins to control the target insects. Genes conferring resistance to insects have been inserted into crop plants such as maize, cotton, potato, tobacco, rice, broccoli, lettuce, walnuts, apples, alfalfa and soybean (Bennett, 1994; Federici, 1998; Griffiths, 1998). The first transgenic tobacco plants with Bt were produced in 1987 through cloning (Barton et al. 1987; Fischoff et al. 1987; Vaeck et al. 1987).

Ingo Potrykus and Peter Beyer described the first version of **Golden Rice** in 1999 (Ye et al., 2000). It is a remarkable example of genetic transformation through gene cloning. *Agrobacterium* mediated transformation of precultured rice immature embryos were used designed so as to install the entire ß-carotene biosynthetic pathway into rice endosperm in a single transformation effort. Three vectors were constructed: pB19hpc combined the sequences for a plant phytoene synthase (*psy*) originating from daffodil (*Narcissus pseudonarcissus*; accession no. X78814) with the sequence coding for a bacterial phytoene desaturase (*crtI*) originating from *Erwinia uredovora* (accession no. D90087), the two being placed under the control of the endosperm-specific glutelin (Gt1) and the constitutive CaMV 35S promoter, respectively. To complete the ß-carotene biosynthetic pathway, cotransformations were carried out employing two vectors, one (pZPsC) carrying *psy* and *crtI*, like in pB19hpc but lacking the expression cassette for the selectable marker *aphIV*, and the other (pZCycH) providing, under glutelin promoter control, the sequence coding for the enzyme lycopene ß-cyclase, originating from *N. pseudonarcissus* (accession no. X98796) (Al-Babili et al., 1996).

Like phytoene synthase, lycopene ß-cyclase carried a functional transit peptide, allowed plastid-import (Bonk et al., 1997). The combination of both plasmids, directed ß-carotene formation in rice endosperm.

2.10. FROM MARKER TO GENE

DNA cloning has opened up opportunities for identifying genes from molecular markers. As already discussed, several efforts have been made to develop molecular marker(s) for commercially important traits in different crops and many of them were able to identify gene from the marker(s).

Poulsen et al. (1995) was able to develop RAPD markers (2.7 kb) for resistance to barley leaf rust (*Puccinia hordei*). The marker was generated by PCR with the oligonucleotide primer OPU-02 and ultimately found out the gene, located approximately 12cM from *RphQ*, using resistant and susceptible DNA bulks, constructed following the classification of F_2 plants by leaf rust infection type. Both the marker and gene will be potentially useful in the development of new barley cultivars.

Three RAPD markers closely linked to a gene for resistance to powdery mildew caused by *Blumeria graminis* in wheat has been reported by Hu et al. (1997). After performing PCR analysis, two RAPD markers, $UBC320_{420}$ and $UBC638_{550}$, were identified to be co-segregating with the disease resistance. The third RAPD marker, $OPF12_{650}$ was determined to be 5.4 to 1.9 cM from the resistance gene. Markers $UBC320_{420}$ and $UBC638_{550}$ were present in wheat powdery mildew differential lines carrying the gene *Pm1*, suggesting linkage between these markers and the *Pm1* resistance gene. Co-segregation between *Pm1* and the two markers $UBC320_{420}$ and $UBC638_{550}$ was confirmed by using wheat differential line carrying *Pm1*.

RAPD markers tightly linked to target genes may facilitate selection and enable gene pyramiding for powdery mildew resistance in wheat breeding programmes (Hu et al., 1997). Hessian fly resistance genes in wheat were identified by Dweikat et al. (1997) using RAPD markers.

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv *oryzae* (*Xoo*) is one of the most serious diseases of rice. The recessive gene *xa-13* confers resistance to Philippine race 6 of *Xoo*. To tag *xa-13* with molecular markers, RAPD analysis was conducted with the combined use of near-isogenic lines and bulked segregant analysis. From the survey of 260 arbitrary 10-nucleotide primers, one primer (OPAC05) was detected to amplify specifically a 0.9 kb band from the DNA of susceptible plants. The distance between the RAPD marker OPAC05-900 and *xa-13* was estimated to be 5.3 cM. The RAPD marker was then mapped on chromosome 8 using a mapping population of doubled haploid lines. The mapping of *xa-13* with closely linked DNA marker has provided the basis for marker-aided selection for rice improvement (Zhang et al., 1996).

The development of the industrial use of rapeseed oil, rich in erucic acid has led to increased interest in the improvement of the varieties with high-erucic-acid (50–60%) content. Two genes with additive effects control this trait. The low-erucic-acid trait was relatively easily introduced through backcrosses into various backgrounds because the zero-erucic-acid homozygotes were clearly identified in the segregating populations. To select for high erucic acid level is more difficult because of the partial overlap of the high-erucic-acid homozygous class and the intermediate one, containing heterozygotes. In order to help conventional breeding, RAPD markers were used to map the two genes involved in determining the erucic acid content in a doubled haploid progeny derived from a low x high erucic acid F_1 hybrid (Jourdren et al., 1996).

The feasibility of identifying molecular markers linked to disease resistance genes in oats was investigated (Penner et al., 1993) utilizing random primers in conjunction with PCR technology through cloning of genes involved. A pair of near-isogenic oat lines was screened for polymorphic DNA fragments linked to the stem rust resistance gene *Pg3*. Two primers were identified which amplified DNA fragments that were polymorphic between the lines analyzed. One primer (ACOpR-2) was shown to be completely linked to the *Pg3* locus; the other primer was not linked to either the ACOpR-2 or the *Pg3* loci. This type of analysis, offers an effective means of identifying useful molecular markers and of applying them to plant breeding selection strategies (Penner et al., 1993).

2.10.1. INVERSE POLYMERASE CHAIN REACTION (IPCR) AND PRIMER WALKING TO ISOLATE COMPLETE GENE SEQUENCE

Cloning of genes linked to markers has never been straightforward. A number of techniques have been utilized for cloning the complete gene. Such techniques are Inverse PCR (Ochman et al., 1988; Triglia et al., 1988, detail cited in the section 3.5.6), adapter-specific PCR or primer walking etc very useful for the amplification of uncharacterized stretches of DNA upstream or downstream of regions that have already been cloned and sequenced (Benkel and Fong, 1996).

2.10.1.1. INVERSE PCR

Inverse PCR is an effective technique to know the unknown sequence, upstream or downstream of a known sequence of a genome.

Total sequence of the promoter of fungal resistant gene (*HMG I/Y*) of pea was determined by Druffel et al. (2005) using inverse PCR. Pea genomic DNA was cleaved with the *Taq* I (4 bp cutter) had the specificity to cleave the site located within the 3' end of HMG I/Y gene open reading frame and various sites upstream or down-stream of the gene of interest.

The digested DNA was purified and ligated to itself (using T4 DNA ligase) to create a series of small circular DNAs representing the genome, including the HMG-I/Y gene and its adjacent sequence. A primary PCR was performed. The product was then diluted to 1:10 in preparation for secondary PCR. A secondary PCR was done. This yielded a ~780 bp segment, which was cloned and sequenced. The two new sequences generated from the clone were recognized as being within the region 5' of the HMG I/Y start site. The length of this sequence (780 bp) was insufficient for the complete analysis of a HMG I/Y 5' region but contained an *Alu* I site. Thus the pea DNA was cut with *Alu* I and the DNA was again circularized by ligation to obtain additional upstream sequence. Two new sets of primers were designed and used for the primary and then secondary PCR reaction. The subsequent PCR product was cloned and sequenced as before and increased the total sequence upstream of the HMG-I/Y gene to 1748 bp. To verify the sequence 1748 bp upstream of the HMG-I/Y gene, a primer set was used to amplify an approximate 2500 bp segment off of uncut genomic DNA. The PCR product was cloned and completely sequenced to confirm that the inverse sequence reactions were assembled correctly.

2.10.1.2. PRIMER WALKING

Primer walking is also an efficient and reliable procedure that enables the identification of unknown regions flanking a known DNA sequence based on PCR amplification.

An effort was made by Ashoub and Abdalla (2006) based on the annealing and ligation of single stranded DNA primers with 3′ sequences complementary to the tetra-nucleotide overhangs generated by DNA digestion with restriction endonucleases (*Apa* I, *Pst* I, *Sac* I and *Sph* I) that form 3′ overhangs. The ligation depends on the phosphorus group presented at the 5′ nucleotide of digested DNA. After ligation, a DNA library with 5′ overhangs at both sites was formed.

These 5′ overhangs served as sites for adaptor primers and nested primers for PCR amplification in combination with the gene-specific primers and finally able to rescue the complete DNA sequence of potato leafroll virus. This strategy was verified by the amplification of full-length virus sequence clone. The procedure could be adapted to target any upstream and downstream regions flanking known sequences within the plant genome.

2.11. FROM MARKERS TO GENETIC MAPS OR LINKAGE GROUPS

A genetic or linkage map is a linear map of the relative positions of genes along a chromosome. Distances are established by linkage analysis, which determines the frequency at which two gene loci become separated during chromosomal recombinations. Genetic maps are based on the relative order and distances of genetic markers. The position of genes on a chromosome can be located via its linkage with these DNA markers. A genetic map can be built up by using enough linked pairs of markers lying at close intervals to each other (Paterson et al., 1991).

A number of PCR-based marker technologies have expedited the construction of high-density linkage maps; facilitated genetic analysis and map based cloning (Yong et al., 2002). These techniques include RFLP (McCouch et al., 1988), RAPD (Williams et al., 1990), AFPL (Vos et al., 1995), SSR (Tautz, 1989) etc.

A mapping population is a prerequisite for the construction of a linkage map. Mapping population can be derived by selfing the F_1 to produce an F_2, which is then scored for segregation of the markers or backcross the F_1 to one of the parents and observing the segregation in the first backcross generation. It is better to use an F_2 population if this is possible, as more information can be gained from this than from a backcross population of comparable size (Chawla, 2002). A mapping population of about 50 F_2 or backcross plants is sufficient for a fairly detailed map (Chawla, 2002).

Remnant seeds from previous crosses are also suitable for mapping populations. Recombination frequencies can also be estimated from doubled haploids (DH) derived from the pollen of F_1 plants. At first there will be many more linkage groups than the basic number of chromosomes, but the numbers will tend to converge as more markers are added (Chawla, 2002).

The first RELP map in rice was developed by McCouch et al. (1988). The map was based on the F_2 mapping population derived from the cross of indica and japonica cultivars. It consisted of 135 RELP markers and covered 1398 cM. Similar RFLP linkage maps for maize and tomato were developed in 1986 (Helentjaris et al., 1986).

A second RELP map was constructed by Saito et al. (1991) from 144 F_2 plants of indica type "Kasalath" crossed with japonica FL134. Utilizing 322 RELP markers, and morphological and biochemical markers, the map spanned 1836 cM, with 347 loci, and exceeds the first RELP map by 32.2% in rice.

A more saturated molecular map of rice was constructed by Causse et al. (1994) from an interspecific backeross population of *Ozyza sativa* X wild African relative *O. longistaminata.* The 726 markers used included RELPs (238), rice cDNA (250), oat cDNA (112), barley cDNA (20), maize genomic clones (2), microsatellite markers (11), telomere markers (3), isozymes (11), cloned genes (26), RAPD (6), and mutant phenotypes (47). The map covers 1491 cM, with one marker approximately every 2.1 cM.

As more markers were located on genetic maps, it became possible to detect single genetic loci associated with the quantitative trait loci (QTL). Furthermore, the chromosomal location of the QTL was more precisely estimated, and their linkage relationships with other genes could be accurately determined (Paterson, et at., 1991; Gregorio, 1997).

QTL mapping in rice was first reported by Wang et al. (1994). Genes for complete and partial blast resistance were mapped using F_7 recombinant inbred lines between a durably resistant japonica (Moroberekan) and highly susceptible indica (CO39) cultivars.

The RAPD technique (Welsh and McClelland, *1990*; Williams et al., *1990*) has widely been used in plants for the construction of genetic maps in species such as *Arabidopsis* (Reiter et al., 1992), bananas (Faure et al., 1993) and slash pine (Nelson et al., 1993).

A high-density genetic linkage map of *Arabidopsis thaliana* was constructed by Reiter et al. (1992) using a population of recombinant inbred lines. A total of 252 RAPD markers were imposed on previously constructed map based on RFLP markers. The final map contains 320 marker loci with an average distance of 2 cM between markers. This map has a total length of 630 cM, slightly greater than previous RFLP maps.

A partial molecular linkage map of banana (*Musa acuminata*) was constructed based on 58 RFLP, four isozyme and 28 RAPD markers segregating in an F_2 population of 92 individuals. A total of 90 loci was detected, 77 of which were placed on 15 linkage groups while 13 segregated independently. Segregation distortions were shown by 36% of all loci, mostly favoring the male parent. Chromosome structural rearrangements were believed to be one of the main causes of these distortions. The use of genetic linkage data help to improve for designing of breeding strategies (Faure et al., 1993).

AFLP technique (Vos et al., 1995) is becoming important in fine-mapping of genes through positional cloning and in mapping traits controlled by major genes and QTLs. As many as 208 polymorphic AFLP markers were mapped from only 20 primer combinations in rice.

Previously mapped RELP markers were used as anchors in constructing the linkage map. The map spanned 3058 cM, which is 1247 cM longer than the map generated by RELP alone (Huang et al., 1994). AFLP markers filled the gaps between the RELP markers, reducing the distance from 13.4 cM to 8.9 cM. Furthermore, the AFLPs extended the map towards the telomeric region of most chromosomes. AFLP markers have been utilized for construction of genetic maps in barley (Becker et al., 1995).

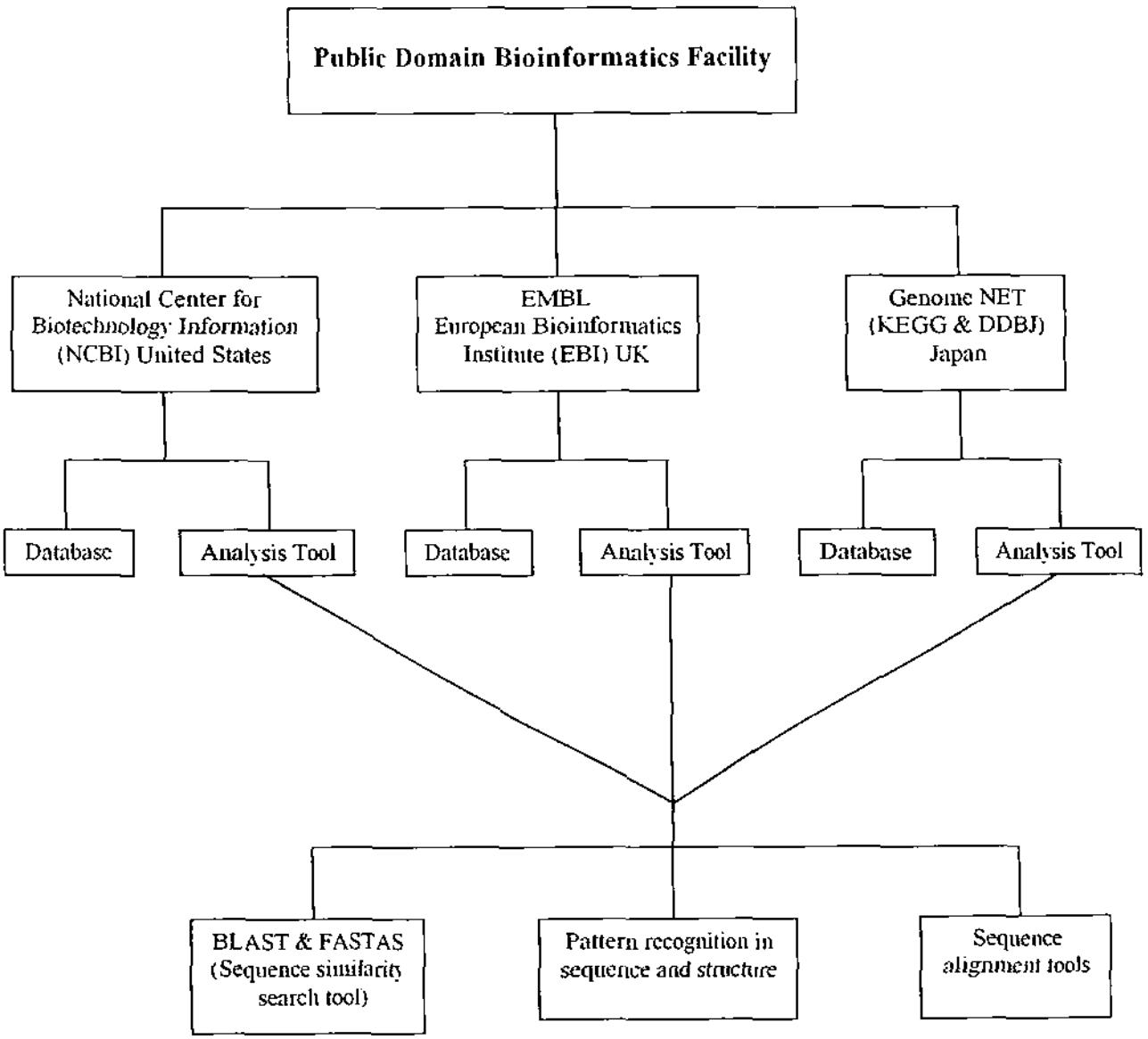
2.12. BIOINFORMATICS

Bioinformatics is the field of science in which biology, computer science, and information technology merge into a single discipline. The ultimate goal of the field is to enable the discovery of new biological insights as well as to create a global perspective from which unifying principles in biology can be discerned (Gibas et al., 2001).

Bioinformatics or computational biology is the use of techniques from applied mathematics, informatics, statistics, and computer science to solve biological problems. Research in computational biology often overlaps with systems biology. Major research efforts in the field include sequence alignment, gene finding, genome assembly, protein structure alignment, protein structure prediction, prediction of gene expression, protein-protein interactions, and the modeling of evolution. The terms bioinformatics and computational biology are often used interchangeably, although the latter typically focuses on algorithm development and specific computational methods. A common thread in projects in bioinformatics and computational biology is the use of mathematical tools to extract useful information from noisy data produced by high-throughput biological techniques.

2.12.1. Web Services as Bioinformatics Tools

The bioinformatics tools that are currently used for molecular biology and biotechnology have been described below (Gupta, 2004).



Major publicly available database and data mining tools
(Courtesy: Gupta, 2004).

2.12.2. ALIGNMENT TOOLS

Local Sequence Alignment Tools

These tools allow us to find out the location and identity of a query (input) sequence in the database. They have a very wide spectrum of uses including basic studies like 'taxonomy and evolution' to applications in 'crop breeding' and 'human health care'.

FASTA Family: FASTA is used for an appropriate format, for data exploration and transaction (i.e. as software).

Pat Match: Pat Match allows users to analyze a given sequence against all or a number of selected sequence datasets available in the databases.

BLAST (earlier version): The most popular tool for searching sequence databases is a package called BLAST (basic local alignment search tool) (Altschul, 1990).

The following programmes can be used for more detailed analyses:

BLASTp compares an amino acid query sequence against a protein sequence database; **BLASTn** compares a nucleotide query sequence against a nucleotide sequence database; **BLASTx** compares a nucleotide query sequence translated in all reading frames against a protein sequence database; **tBLASTn** compares a protein query sequence against a nucleotide database dynamically translated in all reading frames; **tBLASTx** compares the six frame translations of a nucleotide query sequence database; **BLASTz** compares long stretches of nucleotides >2kb; **BLASTbgp** allows use of two BLAST modes, the first is **PHI BLAST**, which uses protein motifs, and the second is **PSI BLAST**, which uses an interactive alignment procedure to detect weak pattern matches, **bl²seq** allows a comparison of two sequences using the BLASTp and BLASTn programmes.

BLAST (later version 2.1): These tools include the following:

VecScreen gives an output, which lists all segments of the query that closely match any of the sequence in the Univac database including sequences of

plasmids, phage, cosmids, BACs, PACs, and YACs. **IgBLAST** facilitates analysis of immunoglobulin sequences in GenBank. **MegaBLAST** is a multiple alignment tool, which compares a set of ESTs with a set of genes. **SNP BLAST** gives an output, which lists all high confidence SNPs available in the SNP database in the sequences that correspond the query sequence. **PowerBLAST** is an application for automatic analysis of genomic sequences.

2.12.3. MULTIPLE SEQUENCE ALIGNMENT TOOLS

Multiple sequence alignment technique is used to study group of related genes or proteins to infer evolutionary relationships between genes and to discover pattern that are shared among groups of functionally or structurally related sequences. Following are some tools used in multiple sequence alignments.

CLUSTALW: This is a programme for progressive multiple sequence alignment and is used for phylogenetic analysis.

PHYLIP: It contains 30 programmes that implement different phylogenetic analysis algorithms.

BioNumeric Version 2.0: This programme can handle experimental data available for a genetic similarity/diversity study.

2.12.4. SOME IMPORTANT WEB SITES COMMONLY USED FOR BIOINFORMATICS

Subject	Source	Link
Nucleic acid sequence	GenBank	http://www.ncbi.nih.gov:80/enterz/query/fcgi?bd=Nucleotide
Genome sequence	SRS at EMBL/FBI Entrez Genome	http://srs.cbiac.uk
	TIGR database	http://www.ncbi.nlm.nin.gov:80/enterz/query/fcgi?bd=genome
Protein sequence	GenBank	http://www.tigr.org/tbl/
	SWISS-PORT at ExPASY	http://www.ncbi.nlm.nin.gov:80/enterz/query/fcgi?bd=protein
	PIR	http://www.expasy.ch/spro/
Protein structure	Protein database	http://www.ndrf.georgetown.edu
Post translational modifications	RESID	http://www.resb.org/pdb/
Biochemical and biophysical information	ENZYME	http://www.ndrf.georgetown.edu /pirwww/search/textresid.html
	BIND	http://www.expasy.ch/enzyme
Biochemical pathways	Path DB	http://www.ncbi.nlm.nih.gov:80/enterz/query/fcgi?db=structure
	KEGG	http:www//ncgr.org/software/pathdb
	WIT	http://www.genome.ad.jp/kegg/
Microarry	Gene Expression Links	http://www.wit.mcs.anl.gov/WTT2/
Other interesting sites	European Bioinformatics Institute	http://www.ebi.ac.uk
	DNA Database of Japan	http://www.nig.oc.jp/home/html

Courtesy: Gupta, 2004.

CHAPTER 3

MATERIALS AND METHODS

MATERIALS AND METHODS

A series of related experiments were conducted to support the main objective of the work (experiment no. 5) and the details of the experiments are described independently. The field experiments were conducted in the green house premises of Bangladesh Jute Research Institute (BJRI) and the laboratory experiments were carried out in the Department of Biochemistry and Molecular Biology, University of Dhaka, during the period between July 2001 to December 2005.

3.1. EXPERIMENT 1 : METHOD FOR QUALITY DNA ISOLATION FROM DIFFERENT PARTS OF JUTE PLANT

3.1.1. Plant materials

The fundamental plant materials were the seeds of the two cultivated species of jute varieties D-154 and O-9897 from *Corchorus capsularis* and *Corchorus olitorius,* respectively. The seeds were obtained from Bangladesh Jute Research Institute (BJRI). The other plant materials were derived from the seeds, which were seedlings (4 days old germinated seedlings on moist filter paper in petri dishes at 30°C in the dark), young leaves (3 to 7 days old) and old leaves (35 to 40 days old) for both the species.

3.1.2. Solutions and Reagents

For the modified Dellaporta et al., (1983) protocol (mentioned as **Method-I**): Cetyltrimethylammonium bromide (CTAB), 2-mercaptoethanol (2-ME), CTAB extraction solution, 24:1 (v/v) Chloroform/ Isoamyl alcohol (CI), CTAB precipitation solution, High salt Tris EDTA (TE) buffer, 80% ethanol, TE buffer, DNase free RNase, mortar and pestle and screw cap glass tubes etc. For modified Doyle and Doyle's (1990) protocol (mentioned as **Method-II**): The additional reagents were Phenol, Isopropanol and 3M Na-acetate.

3.1.3. PROTOCOL FOR DNA ISOLATION

3.1.3.1. Method-I

One gram of each of the 4 fresh samples (seeds, seedlings, young and old leaves) was ground in liquid nitrogen to a fine powder with a mortar and pestle. Each of these samples were transferred to a 15 ml screw cap glass tube containing 5 ml preheated (65°C) CTAB extraction solution and mixed thoroughly before incubating for 30 minutes at 65°C in a water bath with occasional mixing. The homogenate was extracted with an equal volume of 24:1 Chloroform/Isoamyl alcohol (CI). After centrifugation for 20 minutes at 4000 rpm at room temperature, the top (aqueous) phase was recovered into a fresh screw cap tube. This step was repeated three more times for seed and seedling and four more times for young and old leaf samples with equal volume of CI mixture. One-tenth ($1/10^{th}$) volume of 65°C CTAB/NaCl solution was added to the recovered aqueous phase and mixed well by inversion before extracting with an equal volume of CI. The tubes were mixed well, centrifuged (4000 rpm) and the top phase recovered. Exactly one volume of CTAB precipitation solution was added, mixed well by inversion. If a precipitate was visible then proceeded to next step, if not, the mixture was incubated for 30 minutes at 65°C. The solution was then centrifuged for 10 minutes at 4000 rpm at room temperature. The pellet was collected and resuspended in high-salt TE buffer (2-3 ml per gram of starting material). The suspended extract was treated with DNase free RNase ($1/100^{th}$ volume) and incubated for 20 minutes at 37°C. The suspension was transferred to micro centrifuge tubes (1.5 ml) and the DNA was precipitated by adding 0.6 volume of ice-cold isopropanol. After mixing well by inverting slowly and then centrifuging for 10 minutes at 10000 rpm at 4°C, the pellet was washed with 80% ethanol, dried and resuspended in a minimal volume of TE (Tris 10mM, EDTA 1 mM) buffer (usually 100-500µl per gram of starting material) and stored at -20°C.

3.1.3.2. Method-II

In this method the 1st step is similar to the previous one. Then one volume of 25:24:1 Phenol/Chloroform/Isoamyl alcohol (PCI) was added. The mixture was mixed and centrifuged at 4000 rpm for 20 minutes at room temperature. The supernatant was transferred to a new tube; the PCI extraction was repeated 3 to 4 times or more, depending on samples, more purifications were required, respectively for seedlings, seeds, young leaves and old leaves) until a clear supernatant was obtained (Haque at el., 2004). The DNA was precipitated with ice-cold isopropanol. The DNA pellet was washed in 80% ethanol (ice-cold), dried and dissolved in TE buffer. After RNase treatment, the DNA was treated with one volume of PCI. The mixture was then centrifuged at 10000 rpm for 10 min at 4°C. The DNA was precipitated with 1/10th volume of 3M Na-acetate and double volume of ice-cold 99% ethanol. After washing with 80% ethanol, the DNA was dried, dissolved in TE buffer and stored at -20°C.

3.1.3.3. Quantification of DNA

Two methods were followed for quantification of DNA which were: (a) *Spectrophotometric method*: The optical density (OD) of the isolated DNA was measured at 260 and 280 nm wavelength (Specord 50, Analytikjena) to assess the quality and quantity. (b) *Gel analysis*: The genomic DNAs along with λ DNA were run in a 0.8% agarose gel, the gel was stained by ethidium bromide solution and visualized using UV illumination and documented by Kodak Bio doc system. The DNAs were quantitated by comparing with the intensities of known concentration of λDNA.

3.2. EXPERIMENT 2 : DNA FINGERPRINTING OF SOME VARIETIES AND ACCESSIONS OF JUTE

3.2.1. Plant materials

A total of 18 jute genotypes (Table 3.1) including six varieties and five accessions from *Corchorus capsularis* and four varieties and three accessions from *Corchorus olitorius* were used for fingerprinting analysis.

Table 3.1: List of 18 genotypes with their origin and unique characters.

Sl. #	Genotypes	Origin	Unique characters
01	Var. D-154	Local collection, PLS	Stem green, petiole upper side dull coppery red, ovate leaves.
02	Var. CVL-1	-do-	Full green, leaves lanceolate.
03	Var. CVE-3	Thailand, PLS	Stem green, petiole upper side bright coppery red.
04	Var. CC-45	Egypt	Stem green, petiole upper side light coppery red. Photo insensitive.
05	Var. BJC-83	CVL-1 X Fuleshori	Full green, leaf blade wavy, leaves tip pointed.
06	Var.BJC-7370	PLS	Stem green, petiole upper side light coppery red, broader leaves.
07	Acc. No. 905	Japan	Higher harvest index.
08	Acc. No. 1505	Local collection	Full green, blue seeded.
09	Acc. No. 1515	Nepal	Short field duration.
10	Acc. No. 1833	Local collection	Short field duration (40-60 days).
11	Acc. No. 2470	Local collection	Stem red, water logging tolerant.
12	Var. O-4	Local collection	Full green, leaves long & broad, photosensitive
13	Var. O-9897	O-5 X BZ-5	Full green, leaves lanceolate, short day tolerant.
14	Var. OM-1	Uganda PLS	Full green, ovate glossy leaves.
15	Var. O-72	(O-9897 X O-2012) X O-9897	Full green, ovate non-glossy leaves.
16	Acc. No. 1333	Japan	Full green.
17	Acc. No. 1805	Egypt	Base of stem & stipule red, low temperature tolerant.
18	Acc. No. 1749	Local collection	Full green and early maturing.

Note: PLS means Pure Line Selection.

From these genotypes, seedlings (obtained from seed germination in petri dishes at 30°C in the dark) were used for DNA isolation.

3.2.2. DNA extraction

DNA was extracted from each sample following **Method-I** (cited in section 3.1.3.1).

3.2.3. Method: Random Amplified Polymorphic DNA (RAPD)

The basic principle involved in this technique assumes the presence of same random sequence in inverse orientation within an amplified distance. So that the same sequence works as forward and reverse primers at multiple loci. In RAPD system usually 10 nucleotides long primers are used (Glick and Pasternak, 2003).

3.2.4. Amplification conditions

DNA samples from 18 jute genotypes were amplified with 40 arbitrary decamer primers (Appendix I) from Operon Technologies, USA; 9 primers from set OPAB (01, 03, 05, 06, 08, 10, 12, 18 and 20); 10 primers from set OPG (03, 05, 06, 07, 08, 09, 11, 13, 15, and 16); 10 primers from set OPH (02, 03, 04, 05, 07, 10, 12, 13, 14 and 15); 8 primers from set OPQ (01, 05, 06, 07, 08, 16, 17 and 20); 1 primer from set OPE (01) and 2 primers from set OPN (02 and 03). Out of 40 primers 18 primers had 70% and the rest 22 primers 60% GC nucleotides. The reaction mixture (25 ml) contained the following: 1X reaction buffer (20mM Tris-HCl pH 8.4, 50 mM KCl), 0.2 mM dNTPs, 2 mM $MgCl_2$, 60 ng primer, 1.0 unit of *Taq* DNA polymerase, and 25-40 ng genomic DNA. DNA was amplified in a thermal cycler (Eppendorf Mastercycler Gradient) that was programmed as follows: after preheating for 5 minutes at 95°C; 40 cycles of 1 minute at 95°C (denaturation), 1 minute at 40°C (annealing) and 2 minutes at 72°C (extension), and a final extension at 72°C for 7 minutes that was followed by cooling to 4°C. Before amplification, all the PCR chemicals and amplification conditions were optimized using different concentration of the ingredients and condition of the PCR machine.

3.2.5. Visualizing the PCR products

DNA dye (Xylene cyanol: 0.25%, Bromophenol blue: 0.25% and Glycerol: 30%) was added to the amplified PCR product and mixed well with gentle agitation. After a momentary spin the PCR products were loaded in the wells of 2% agarose gel. Electrophoresis was accomplished at 80 volts. Then the gel was visualized under UV transilluminator followed by staining in ethidium bromide (0.5 μg/ml) for 20-30 minutes and destaining in distilled water (Sambrook et al., 1989).

3.2.6. Scoring and analyses of data

The amplified products were scored for further analysis. During scoring, only intense clearly resolved amplification products that were reproducible in multiple runs were considered for further analysis. The DNA fragments that were amplified by a given primer were scored as '1' for presence or '0' for absence of a particular locus for all of the genotypes that were studied. A cluster analysis was accomplished using the software STATISTICA version 3.0 (Stat Soft, 1994).

Study of sequences of simple sequence repeats (SSR) enriched clones commercially developed using the genome of *Corchorus olitorius* variety O-4 was undertaken. The raw sequences were edited and used for designing forward and reverse primers on the basis of the flanking region of the repeats (Appendix II). A total of 16 genotypes of which varieties D-154, CVL-1, CVE-3, CC-45, BJC-83, BJC-7370 from *Corchorus capsularis* and varieties O-4, O-9897, OM-1, O-72 and accession no. 1805 from *Corchorus olitorius* (described in Table 3.1) was used for SSR amplification. The characteristics of the 5 remaining genotypes such as BJC-2142 (*C. capsularis*), SDLT-1, SDLT-2, SDLT-3 and advanced breeding line 7/95 (*C. olitorius*) are cited in Table 3.2.

Table 3.2: List of jute varieties and accessions used for SSR analysis

Genotypes	Species	Origin	Unique characteristics
SDLT-1	*Olitorious*	Exotic (PLS)	Full green, blue seeded, short day tolerant
SDLT-2	*Olitorious*	O-4 X Uganda red	Brown seeded, red, photo insensitive
SDLT-3	*Olitorious*	O-4 X Uganda red	Brown seeded, red, less photosensitive
Line 7/95	*Olitorious*	Advanced breeding Line	Short day tolerant
BJC-2142	*Capsularis*	CC-45 XS-718	Blue seeded, snow white fibre, early

DNA isolation, quantification, PCR analysis, visualization of PCR products, scoring of gel and finally analysis the data were almost similar to that of the RAPD system. There was little difference in PCR conditions and visualizing the amplified products in the gel system. As the product size ranged from 150 to 300 bp, high resolution polyacrylamide gels were used instead of agarose gel in this system.

3.2.7. Thermal cycling profile used in PCR with SSR primers

The thermal cycling profiles that were programmed to amplify the 16 genomes by Polymerase Chain Reaction (PCR) for 35 cycles were as follows:

	Temperature	Time	No. of cycles
Initial denaturation	95°C	5 minutes	1(First)
Denaturation	95°C	30 seconds	35
Annealing	58°C	40 seconds	35
Elongation	72°C	30 seconds	35
Final elongation	72°C	5 minutes	1

For different primers, different annealing temperatures were employed. For SSR primers the optimum annealing temperature used was in the range of 55° to 62°C.

3.3. EXPERIMENT 3 : DEVELOPMENT OF MOLECULAR MARKER FOR LEAF SHAPE AND LEAF GLOSSINESS CHARACTER OF JUTE

This experiment was actually designed to develop molecular marker(s) for waterlogging tolerance (an important environmental stress) in *C. olitorius* jute because, the yield and quality of *C. olitorius* jute is better than *C. capsularis* but highly sensitive to waterlogged condition. No *C. olitorius* genotypes were reported as waterlogging tolerant earlier; in the recently passed, a genotype (*Corchorus olitorius* var. Incisifolius) was reported as flood tolerant (Newaz, 1983). A cross was made between *C. olitorius* flood sensitive variety O-4 and tolerant genotype Incisifolius. The F_2 populations were raised by selfing F_1 in earthen pots and normally grown for 60 days with their parents following BJRI standard cultivation procedure (BJRI, 1996). A concrete tank was prepared to accommodate the pots with parents and F_2 plants. The water height in the tank was increased gradually for creating artificial waterlogged condition with slight current made possible by using water pump. All possible efforts were made to create an artificial flooding condition, which was very close to the natural one. The waterlogged condition was maintained for 30 days with close observation of the plant characteristics. During waterlogged condition, profuse adventitious roots were formed and the plants looked healthy. After 30 days, water was receded gradually until dry. However, no plants including even the tolerant parent survived. This experiment was repeated two more times, but the result was same. This may have been due to the seed lot and the different screening procedures. It was not thought to be wise to proceed with this material for the development of flood tolerant molecular marker. However, two important leaf characters of the parent Incisifolius were observed: (a) leaf texture i.e. leaf glossiness and (b) leaf structure i.e. leaf serration. These two leaf characters support the plants for thriving drought situation (Kulwal et al., 2003). In xerophytes, the adaptive mechanism for drought tolerance is mostly involves in leaf characters.

Leaves become serrated or modified into hair like structure, thick cuticle, sunken stomata and glossy leaf surface that means all modifications involved to minimize evapo-transpiration loss of water (Pandey and Sinha, 1978). Then the experiment was further advanced for the development of DNA marker for leaf characters. The yield performance of the advanced populations was also evaluated.

3.3.1. Plant materials

Four homozygous lines (F_8) were developed with special reference to leaf shape (entire and serrated) and leaf texture (matte and glossy) for the development of their molecular markers. Jute is a short-day crop. So it was possible to grow two generations in a year. The seeds were sown in early February; the plants flowered after minimum vegetative growth and set some fruits. The seeds were collected and bulked on the basis of leaf characters after ripening of the fruits. These seeds were sown in early September and harvested in December. The yield contributing characters were also evaluated in comparison with their parents at F_8 level.

3.3.2. The working materials

Six genotypes composed of 2 parents (variety O-4 and Incisifolius) and 4 homozygous lines (F_8). Their identities were as follows:

1. P_1 (parent-1, non-glossy and entire leaves). **2.** P_2 (parent-2, glossy and serrated leaves). **3.** P_1 type, **4.** P_2 type. **5.** P_1 + glossy leaf type and **6.** P_2 + non-glossy leaf type (Figure 3.3).

3.3.3. DNA isolation

Seedlings were raised from 6 seed samples through germination of the seeds on moist filter paper for 3 days at 30°C in dark. DNA was isolated from seedlings using modified Dellapotra at el. (1983) method (detailed in section 3.1.3.1.).

Bulking the 6 samples such as formed four more working DNA samples:

7. Bulk-1: Entire leaf (1+ 3+ 5) 8. Bulk-2: Serrated leaf (2 + 4 + 6)

9. Bulk-3: Glossy leaf & (2 + 4 + 5) 10. Bulk-4: Non-glossy leaf (1 + 3+ 6)

Now 10 working samples, where 2 parents, 4 homozygous lines and 4 bulks for desired traits were formed.

3.3.4. Marker development techniques

Various PCR based marker techniques such as: (a) Random Amplified Polymorphic DNA (RAPD); (b) Inter Simple Sequence Repeats (ISSR) and (c) Sequence Tagged Microsatellite Sites (STMS) or Simple Sequence Repeats (SSR) were used. Forty RAPD (Appendix I), 15 ISSR (Appendix III) and 6 SSR (Appendix IV) primers were used for amplification of the 10 working DNA samples. The amplified DNA fragments were visualized in 2% agarose gel under UV transilluminator for RAPD and ISSR amplification and nondenaturing polyacrylamide gels (6%) were used for SSR amplification. The PCR products were scored as '1' or '0' for presence or absence of a particular band respectively and analyzed by software STATISTICA version 3.0.

3.4. EXPERIMENT 4 : STUDY OF THE YIELD PERFORMANCE OF FOUR HOMOZYGOUS LINES OF TOSSA JUTE IN COMPARISON WITH THEIR PARENTS

3.4.1. Plant materials: Six genotypes composed of 2 parents (variety O-4 and Incisifolius) and four homozygous lines (F_8) obtained from the experiment no. 3 (cited in section 3.3.1) were used in this study. The major objective of this experiment was to evaluate the yield performance of the homozygous lines in comparison with their parents.

3.4.2. Experimental design

The experiment was conducted following Randomize Complete Block Design (RCBD) composed of 6 treatments (genotypes) and 6 replications.

The following data were recorded:

1. Plant height (PH) in cm.
2. Basal diameter (BD) in mm.
3. Leaf area per plant (cm^2).
4. Leaf dry weight per plant (g).
5. Stem dry weight per plant (g).
6. Bark dry weight per plant (g).
7. Flowering (%) at 60 days after sowing (DAS).
8. Flowering (%) at 120 DAS.
9. Plants not flowering (%) at 120 DAS.
10. Harvest Index (%)

3.4.3. Procedure

Seeds of the six genotypes were sown in the Green house premises of BJRI. The unit plot size was 6 m^2 (3m x 2m). As the experimental design was RCBD; a complete set of treatments i.e. 6 genotypes were employed in the 6 blocks. The treatment in each plot within a block was randomly selected. After germination, different cultural practices were carried out as per BJRI recommendations (BJRI, 1996). All the above data were recorded at the time of harvest i.e. 120 days after sowing except one; which was the flowering data taken at 60 DAS. Six plants were randomly selected; after measuring the plant height and basal diameter; the leaves, stem and bark were separated from each plant and dried in an oven at 70°C for about 72 hours until constant weight was determined. Leaf area was measured by portable leaf area meter (Area Meter AM100, ADC Bioscientific Ltd.) and Harvest Index (HI) was calculated using the following formula:

Harvest Index (%) = Economic yield (here bark)/Biological yield (excluding root) x 100. The collected data were analyzed statistically using MSTAT programme.

3.5. EXPERIMENT 5 : DEVELOPMENT OF DNA MARKER FOR LOW TEMPERATURE TOLERANCE IN JUTE

3.5.1. Plant materials

Seeds of 6 *Corchorus olitorius* L. genotypes (Table 3.3) composed of 2 low temperature sensitive varieties (O-4 and O-9897) and 4 low temperature tolerant accessions (Acc. no. 1540 1805, 1852 and 2015) and F_1 and F_2 seeds and plants were used in this study.

Table 3.3: List of six genotypes, F_1 and F_2 seeds and plants.

Sl. no.	Species	Genotypes	Source
1	*Corchorus olitorius* L.	Variety O-4	Bangladesh. Pure line selection
2	*Corchorus olitorius* L.	Variety O-9897	Hybridization between var. O-4 and Brazilian var. BZ-5
3	*Corchorus olitorius* L.	Acc. no. 1540	India
4	*Corchorus olitorius* L.	Acc. no. 1805	Egypt
5	*Corchorus olitorius* L.	Acc. no. 1852	Bangladesh
6	*Corchorus olitorius* L.	Acc. no. 2015	India
7	F_1 and F_2 seeds and plants.		

3.5.2. Development of F_1 and F_2 seeds and plants

Two low temperature sensitive cultivars and 4 tolerant accessions reported by Begum et al. (1998) were preliminarily selected as study materials. Finally two genotypes such as cultivar O-9897 and accession no. 1805 were selected for detailed study as low temperature sensitive and tolerant parents respectively. A cross was made between these two parents where cultivar O-9897 was the seed parent. The hybrid (F_1) seeds were produced and evaluated by germination in an incubator in normal and low temperature (16°C) stress condition.

The F_2 seeds were obtained by selfing F_1. A good number of segregating F_2 populations was raised by differentiating the cold tolerant (16°C) and sensitive in an Envoron Air Growth Cabinet. The environmental conditions in the cabinet were 16°C temperature, 70-75% relative humidity and a light period of 12.5 hours having light intensity of around 30,000 lux.

3.5.3. DNA extraction

Genomic DNA was extracted from young leaves of the parents, sensitive and tolerant F_2 plants following the modified protocol from *Dellaporta et al.* (1983, detailed in section 3.1.3.1.). A total number of 114 DNA samples composed of 2 parents, 50 cold sensitive and 62 cold tolerant F_2 were taken for marker development studies. The DNA was RNase-treated and subsequently quantified on 0.8% agarose gel by comparison with known concentration of standard lambda DNA.

3.5.4. Techniques for marker studies: In this study, 4 PCR based molecular marker systems were employed, which were:

1. **RAPD**: Random Amplified Polymorphic DNA. Single decamer 40 random primers (Appendix I) were used for detecting polymorphism among the study materials.
2. **ISSR**: *Inter Simple Sequence Repeats.* Single 15 primers (Appendix III) were used in this experiment, where 10 primers were single or di-nucleotide anchored at 5' end of the SSR motif and 5 primers were single nucleotide anchored at 3' end.
3. **AFLP**: Amplified Fragment Length Polymorphism. Two adapter ligated 29 primer combinations (Appendix V) were used.

4. SSR: Simple Sequence Repeats or Sequence Tagged Microsatellite Sites (STMS). Six specific forward and reverse primers (Appendix II; designed by considering the SSR flanking region) were used. Another 47 SSR primers were obtained but could not be used for analyzing the segregating populations because of the degradation of the isolated F_2 DNAs. A mapping population is being developed using recombinant inbred lines through single seed descent (SSD) technique for future use.

3.5.5. Cloning, sequencing and designing of SCAR primer

The DNA fragment, associated with low temperature tolerance was isolated from gel, cloned in *Escherichia coli* (strain dH5α) using vector pUC18 and sequenced. From the sequence, forward (F) and reverse (R) oligonucleotide sequence characterized amplified region (SCAR) primers were designed, such that the original 3' end of the RAPD primer was now positioned at the 5' end of the SCAR primer (Guo et al., 1997). This included 10 bases of the original RAPD primer, plus the next ten to fifteen 3' bases of the genomic sequence. On the basis of sequence information the designed SCAR primers were used.

3.5.6. Inverse PCR

To know the upstream sequence of the DNA fragment associated with low temperature tolerance, a plan was designed to rescue the total gene sequence using inverse PCR (Figure 3.1). DNA isolated from both the parents was completely digested with enzyme Bam-HI. The fractionated DNA of about 1 to 6 kb size was isolated from the gel and self ligated (using T4 DNA ligase) at different DNA concentration and temperature. PCR analysis was performed with the ligated products with SCAR primers.

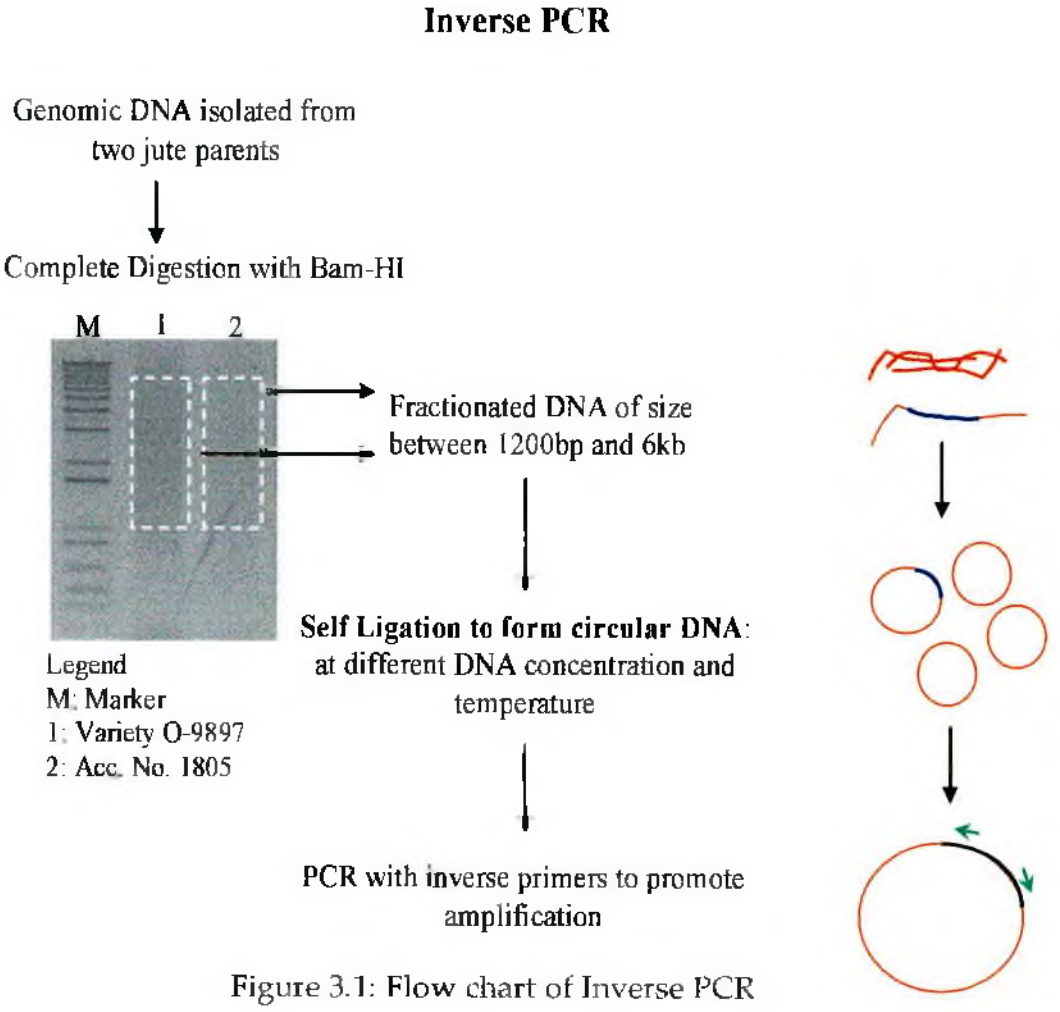


Figure 3.1: Flow chart of Inverse PCR

3.5.7. Bioinformatics analysis of the DNA sequence associated with cold tolerance

Sequences were cleaned of vector and ambiguous data and analyzed with gene prediction software to find open reading frames (ORF) and possible exon/intron boundaries. Sequences were also submitted in BLAST programs (BLASTn and tBLASTx) to search for conserved domains and similar sequences present in the DNA data bank.

3.5.8. RNA Isolation and RT-PCR

Total RNA was isolated with Trizol (GibcoBRL) from three days old seedlings grown in the dark. The integrity of RNA was checked in formaldehyde gel and was accepted when their ratio of optical density at 260 nm and 280 nm was between 1.9 and 2.0. The mRNA was extracted using genElute (Sigma). Reverse transcription (RT) was carried out with Superscript III (Invitrogen) using Oligo dT-20 in a thermocycler following the instruction manual. The PCR was carried out under standard conditions with 4 µl of the RT product in a 25 µl reaction volume containing specific primers, GAGAGCACTTTAAAGTTG GG and TCAAGAATC TGATCTGGTG as forward and reverse, respectively, both designed from the ORF region. The amplification was carried out in a thermocycler programmed at 94°C for 5 minutes; followed by 30 cycles of 30 seconds at 94°C (denaturation), 30 seconds for 60°C (annealing) and 60 seconds at 72°C (extension) and the final extension at 72°C for 5 minutes.

3.6. BIOINFORMATICS ANALYSES

Bioinformatics analyses were done with the sequence (Appendix VI) obtained from the DNA fragment associated with low temperature tolerance. Basic local alignment search tool (BLAST) searches (Altschul et al., 1990) against GenBank databases were performed using the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov). The exon-intron distribution and orientation was determined by "NetGene2" (Hebsgaard, et al., 1996) at CBS, Technical University of Denmark server (http://www.cbs.dtu.dk/ services/NetGene2/) and by "Splice Predictor" (Brendel et al., 1998) at Iowa State University server (http://deepc2.psi. iastate.edu/cgi-bin/sp.cgi).

BLASTn (version 2.2.11): A nucleotide query was compared against a nucleotide sequence in the database. **BLASTx**: Compared nucleotides query sequence

translated in all reading frames against a protein sequence database. **tBLASTx:** Compares the six frame translations of a nucleotide sequence database (it is used to identify clusters of genes in large duplicated segments).

PSI-BLAST (version 2.2.11): Position specific iterated (PSI) BLAST is a more powerful version of BLAST that is used to uncover several new and interesting members of the family.

3.7. CONSTRUCTION OF DENDROGRAM AND LINKAGE GROUP

The amplified PCR products were analyzed running through different percentage of agarose and polyacrylamide gel and scored as '1' or '0' for presence or absence of a particular band respectively. The data were analyzed for dendrogram and linkage group formation using software STATISTICA version 3.0 and MAPMAKER version 3.0 (LOD >3.0 and maximum distance <50 cM) respectively.

CHAPTER 4

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

As reviewed jute is a new crop in the field of molecular biology and quite different from other agricultural crops. The desired part of most of the agricultural crops is fruit or seed. But the fibre, obtained from jute is the phloem tissue. The development of this crop through molecular approach is unlike other crops; therefore, it is necessary to standardize every step for the study of this crop. The results are presented experiment wise and discussed separately.

4.1. EXPERIMENT 1: METHOD FOR QUALITY DNA ISOLATION FROM DIFFERENT PARTS OF JUTE PLANT

4.1.1. RESULTS AND DISCUSSION

In any molecular biology work, the quality of DNA is more important than its quantity for digestion, ligation, cloning and sequencing. But isolation of quality DNA from jute is very difficult because of the presence of polysaccharides and other metabolites. In this study, two modified methods (cited here as **Method-I** and **Method-II**, Dellaporta et al., 1983 and Doyle and Doyle, 1990 respectively), and four types of plant materials (seed, seedling, young leaf and old leaf) from two species of jute (*Corchorus capsularis* variety D-154 and *Corchorus olitorius* variety O-9897) were used. The basic difference between the two methods is the usage of phenol. DNA extraction following **Method-I** (without phenol) showed (Table 4.1) that, the ratio of the optical density (OD) measured at 260 and 280 nm ranged from 1.71 to 2.11. These values were very close to the ratio 1.8 indicating that it was good quality DNA. It is interesting to note that no DNA was found from seed samples from both the species. It was suspected that the CTAB extraction buffer failed to disrupt the cell walls of the compact seed tissue to release DNA.

To confirm this, the experiment was repeated with increasing lyses period from one to four hours but the results remain unchanged. The amount of DNA obtained ranged from 58.76 to 98.97 µg per g of tissue. The quality and quantity of the isolated DNA was also reflected on 0.8% agarose gel (Figure 4.1). The DNA isolated following **Method-II** (with phenol) showed (Table 4.1) that the ratio $OD_{260/280}$ nm ranged from 1.27 to 1.78, indicating a wide range of quality.

Table 4.1: Quality and quantity assessment of DNA isolated from different plant parts and methods.

Genotype and Method	Samples	OD 260/280	Total DNA (µg/g of tissue)
Corchorus capsularis Variety D-154 **Method-I**	Seeds	No DNA	No DNA
	Seedlings	1.75	68.23
	Young leaves	2.11	58.76
	Old leaves	2.06	66.91
Corchorus olitorius Variety O-9897 **Method-I**	Seeds	No DNA	No DNA
	Seedlings	1.72	68.67
	Young leaves	1.89	88.23
	Old leaves	1.71	98.97
Corchorus capsularis Variety D-154 **Method-II**	Seeds	1.73	97.30
	Seedlings	1.78	86.80
	Young leaves	1.35	47.47
	Old leaves	1.30	50.20
Corchorus olitorius Variety O-9897 **Method-II**	Seeds	1.60	110.00
	Seedlings	1.68	63.38
	Young leaves	1.27	45.50
	Old leaves	1.29	49.00

The ratio of the DNA isolated from seeds and seedlings from both species was very close (1.60 to 1.78) to 1.8, but DNA obtained from young and old leaves were poor in quality, which was also reflected on the agarose gel (Figure 4.1). It was observed that the DNA in the lanes 11, 12, 15 and 16 failed to migrate in the gel at the same rate as the others. This could be due to the presence of other impurities like protein. Proteins associated with the isolated DNA could not be removed with phenol. The presence of such proteins may have retarded the migration rate decreasing the quality of the isolated DNA. The yield of DNA ranged from 45.50 to 110.00 µg per g of tissue (Table 4.1). The quantity of DNA is also dependent on the amount of ground tissue harvested and collection of the supernatant.

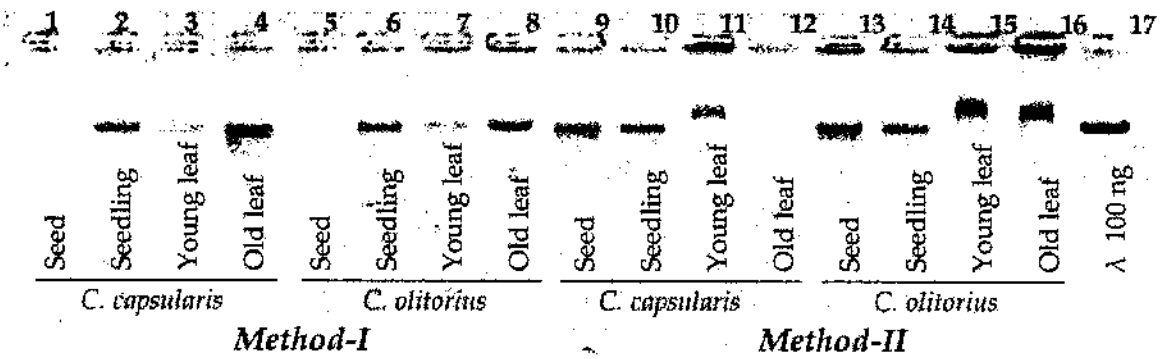


Figure 4.1: Genomic DNA in 0.8% agarose gel.

From the study, it may be concluded that **Method-I** is suitable for isolating DNA from seedlings and leaves but not from the seeds. On the other hand, **Method-II** is effective for all samples, however, compromising on the quality of DNA isolated from leaf samples. If the sample is limiting, as in the case of dead seeds, then **Method-II** is likely to be more suitable.

4.2. EXPERIMENT 2 : DNA FINGERPRINTING OF SOME VARIETIES AND ACCESSIONS OF JUTE

DNA fingerprinting or molecular identity of jute genotypes, particularly for the varieties, is very important. In most of the cases there is no way to differentiate the varieties of same species at seed level. A jute breeder may also become confused to identify a particular variety at seed level. As a result, the unfair seed dealers use this weakness to cheat the innocent farmers by supplying poor quality seeds in the name of good varieties. So DNA fingerprinting is necessary to protect such biopiracy.

4.2.1. RESULTS

Eighteen genotypes of two species of jute, *Corchorus capsularis* L. and *Corchorus olitorius* L. were used in this study. Amplification of the isolated genomic DNA from each of the 11 jute varieties and 7 accessions using all 40 primers, revealed a variety of RAPD patterns (Figure 4.2, 4.3, 4.4 and 4.5). A total of 140 scorable loci, DNA fragments or RAPD markers were observed, of which 19 or 13.57% loci did not show polymorphism, 50 or 35.71% loci were able to show interspecific polymorphism, 50 or 35.71% loci showed polymorphism among the *C. capsularis* genotypes, 20 or 14.28% loci were found polymorphic among the *C. olitorius* genotypes and the remaining 71 or 50.71% loci showed polymorphism among the genotypes of both the species. Among these 40 primers, 25 produced amplification but failed to differentiate the species or varieties, 3 failed to amplify and only 12 were able to generate genetic variability between different cultivars and accessions of jute. They were OPAB-03, OPAB-08, OPAB-18, OPG-03, OPG-05, OPG-08, OPG-10, OPH-04, OPH-12, OPH-19, OPQ-16 and OPQ-17 (Table 4.2). The Tables 4.2 and 4.3 show the number of amplified fragments against genotypes and primers and number of band difference obtained by the RAPD primers and square Euclidean distances between genotypes, respectively.

The amplified products were scored and used for construction of a dendrogram (Figure 4.6) as well as for determining their genetic distances. Table 4.3 shows two triangular sections. The upper portion of triangle shows the number of band differences between the pairs of jute samples, and the lower triangle shows the distances between the jute genotypes, respectively. From these data, distinguish between the *C. olitorius* and *C. capsularis* genotypes (varieties and accessions) could easily be illustrated.

SSR analyses was performed in the same way as RAPD analyses and results shown in Figure 4.7 was very similar to that of RAPD analyses (a little modification mentioned in section 3.5.4).

The polymorphisms obtained from SSRs were used to estimate the genetic distance of the 16 genotypes using the unweighted pair-group average (UPGA). The genetic distance was estimated and organized in matrices and the associations among the 16 jute genotypes were revealed by cluster analyses (Figure 4.8) applying the unweighted pair-group method with arithmetic averages (UPGMA). Among the 47 SSR primers screened, 28 of them produced good amplification products that detected polymorphism between the species (*C. capsularis* and *C. olitorius*). The amplification products generated 197 bands from 28 SSR primers. Most of the polymorphic bands (184) distinguished between the two jute species. A very low percentage of polymorphic bands were observed among the genotypes within species. These primers generated the highest percentage of polymorphism within each species: 40.60% for *C. olitorius* and 6.60% for *C. capsularis*.

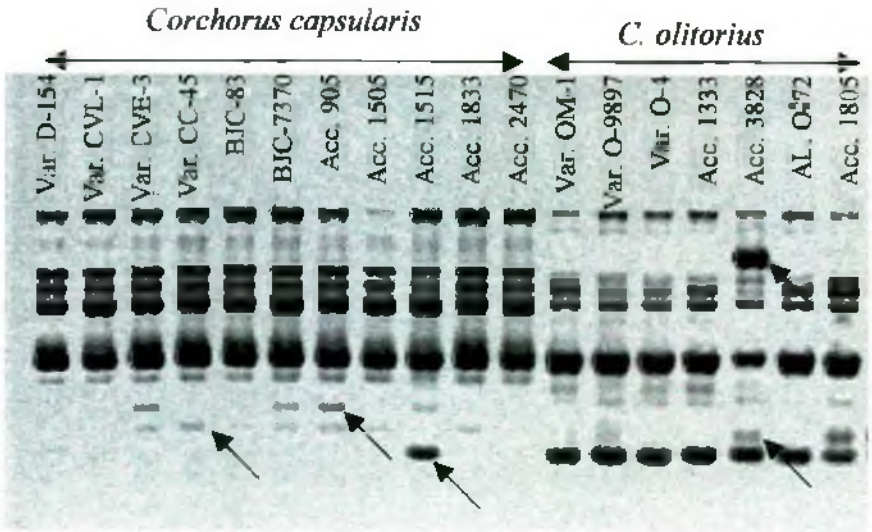


Figure 4.2: RAPD profile amplified with primer OPH-12
Arrow indicates inter and intra specific polymorphism

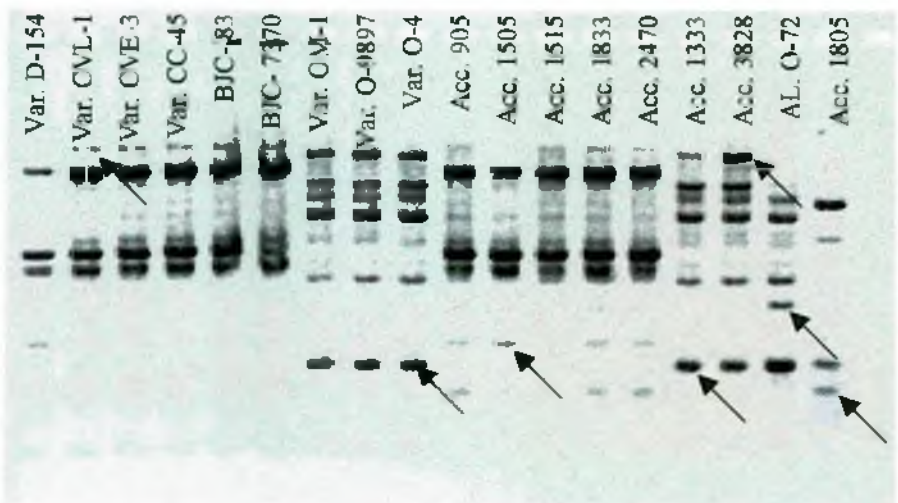


Figure 4.3: Polymorphism amplified with RAPD primer OPH-04
Arrow indicates inter and intra specific polymorphism

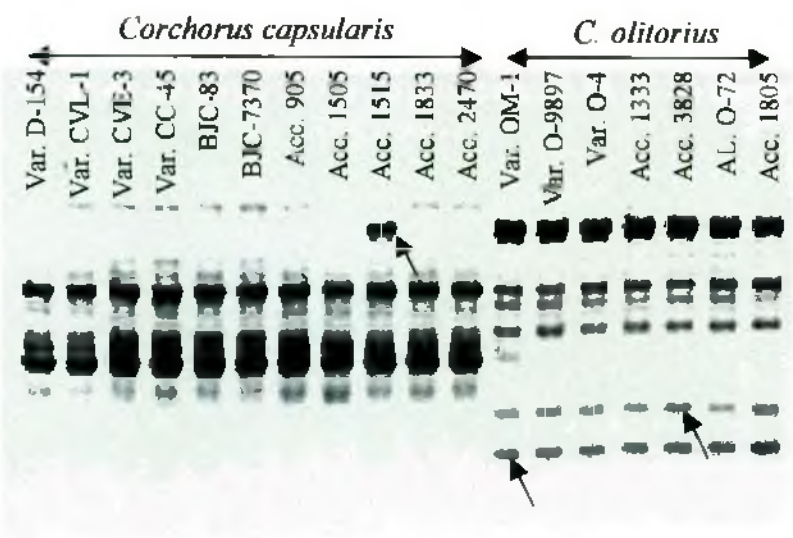


Figure 4.4: Polymorphism amplified with RAPD primer OPAB-08
Arrow indicates inter and intra specific polymorphism

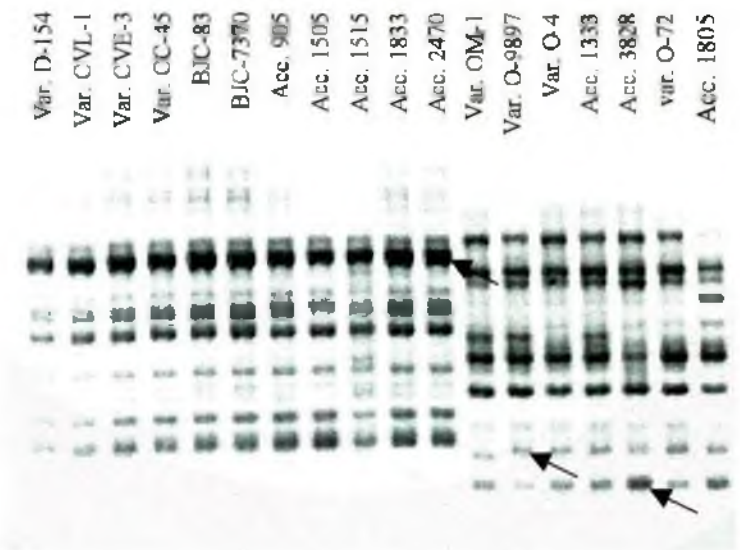


Figure 4.5: RAPD profile amplified with primer OPG-05, showing interspecific polymorphisms
Arrow indicates interspecific polymorphism

Table 4.2: Number of amplified fragments scored against genotypes and primers

Genotypes	OPAB-03	OPAB-08	OPAB-18	OPG-03	OPG-05	OPG-08	OPG-10	OPH-04	OPH-12	OPH-19	OPQ-16	OPQ-17	Total band
D-154	4	8	6	4	7	3	6	4	8	2	9	6	67
CVL-1	4	9	6	4	9	5	6	7	8	2	9	6	75
CVE-3	4	10	6	4	11	5	5	8	10	2	9	6	80
CC-45	4	10	6	4	13	5	6	8	9	4	9	6	84
BJC-83	4	10	6	4	13	5	5	8	9	6	9	6	85
BJC-7370	4	10	7	4	13	5	4	8	10	7	9	6	87
Acc. 905	4	10	6	4	12	5	7	8	10	5	9	6	86
Acc. 1505	5	8	6	4	9	5	5	8	9	4	9	6	78
Acc. 1515	5	11	6	5	9	5	7	8	10	5	9	6	86
Acc. 1833	5	10	7	4	13	5	6	10	9	6	9	6	90
Acc. 2470	5	9	6	5	13	4	6	7	8	7	9	6	85
OM-1	4	8	4	13	9	4	6	8	8	4	6	5	79
O-9897	3	7	3	5	8	3	6	11	9	6	6	5	72
O-4	3	7	3	6	8	3	7	10	8	7	6	5	73
Acc. 1333	4	7	3	6	9	4	4	8	8	5	6	3	67
Acc. 3828	3	7	3	5	9	4	7	8	10	4	6	4	70
O-72	3	7	3	5	8	1	6	8	6	7	6	5	65
Acc. 1805	3	7	3	5	8	3	5	4	8	4	4	5	59
Total band	71	155	90	91	181	74	104	141	157	87	139	98	1388

Table 4.3: Number of band difference and Square Euclidean Distances between jute genotypes

Number of Band Differences																		
	D-154	CVL-1	CVE-3	CC-45	BJC-83	BJC-7370	Acc. 905	Acc. 1505	Acc. 1515	Acc. 1833	Acc. 2470	OM-1	O-9897	O-4	Acc. 1333	Acc. 3828	O-72	Acc. 1805
D-154	0	8	13	17	18	20	19	11	19	23	18	12	5	6	0	3	2	8
CVL-1	12	0	5	9	10	12	11	3	11	15	10	4	3	2	8	5	10	16
CVE-3	17	9	0	4	5	7	6	2	6	10	5	1	8	7	13	10	15	21
CC-45	19	11	6	0	1	3	2	6	2	6	1	5	12	11	17	14	19	25
BJC-83	22	14	7	3	0	2	1	7	1	15	0	6	13	12	18	15	20	26
BJC-7370	28	16	11	9	6	0	1	9	1	3	2	8	15	14	20	17	22	28
Acc. 905	24	16	13	11	10	12	0	8	0	4	1	7	14	13	19	16	21	27
Acc. 1505	20	16	19	17	16	18	10	0	8	12	7	1	6	5	11	8	13	19
Acc. 1515	24	20	21	21	22	22	12	12	0	4	1	7	14	13	19	16	21	27
Acc. 1833	24	14	13	7	6	8	12	16	22	0	5	11	18	17	23	20	25	31
Acc. 2470	26	24	27	21	20	20	24	26	30	16	0	6	13	12	18	15	20	26
OM-1	84	84	87	87	90	88	94	88	84	90	82	0	7	6	12	9	14	20
O-9897	80	82	85	85	86	84	90	84	82	84	82	18	0	1	5	2	7	13
O-4	79	83	84	84	85	85	89	87	81	85	79	17	7	0	6	3	8	14
Acc. 1333	87	89	90	92	91	89	87	81	79	93	83	21	17	16	0	3	2	8
Acc. 3828	84	88	87	87	90	90	84	82	78	94	88	20	16	17	11	0	5	11
O-72	79	81	86	86	87	85	83	79	75	89	85	27	13	18	16	17	0	6
Acc. 1805	72	78	85	85	88	88	86	80	76	90	84	26	20	23	19	20	19	0
	Square Euclidean Distances																	

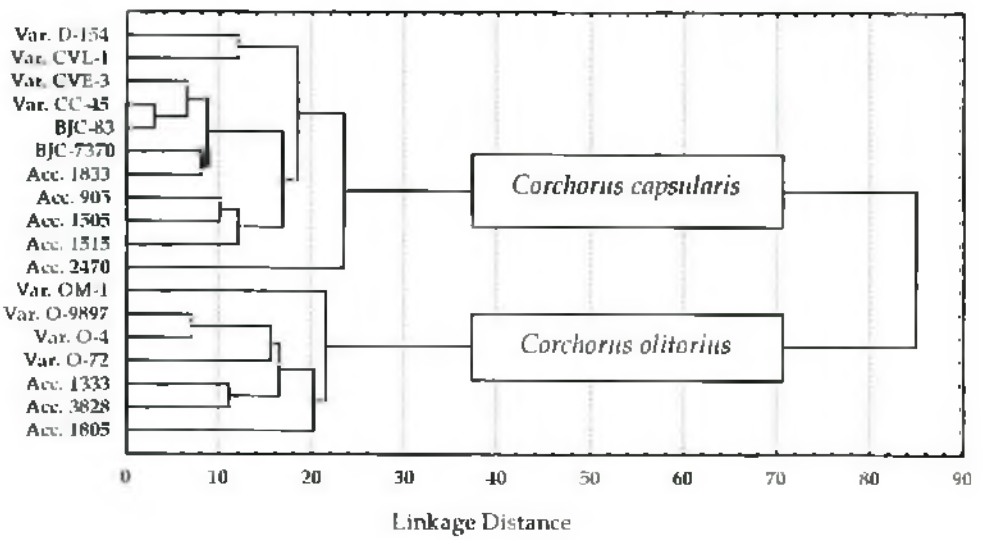


Figure 4.6: A dendrogram using RAPD data with cultivars and accessions of jute

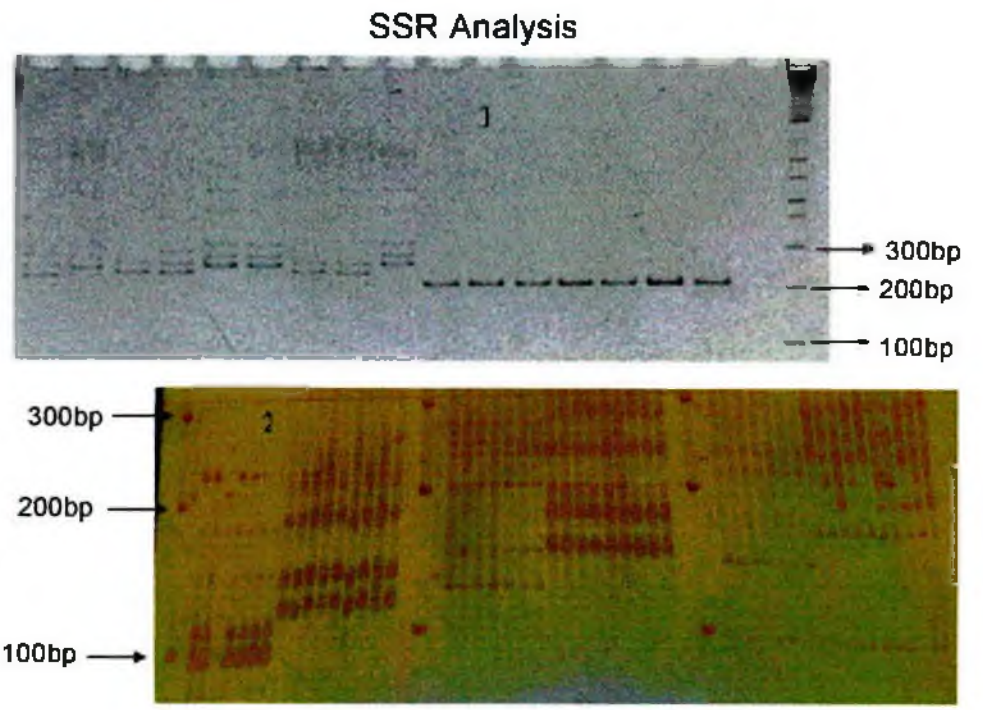


Figure 4.7: Jute genotypes (16) amplified with SSR primers in PAGE

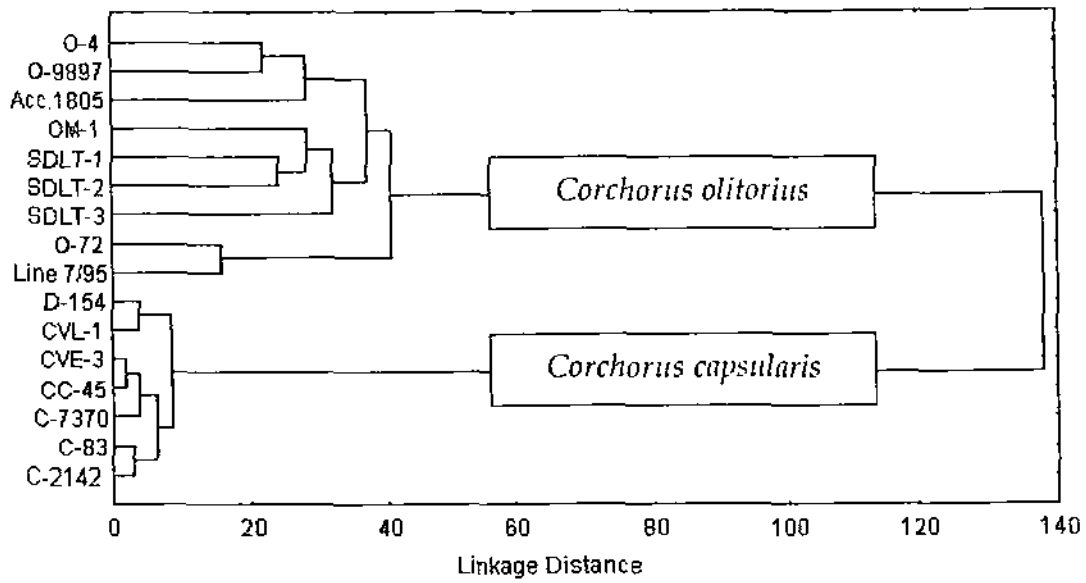


Figure 4.8: A dendrogram for 16 strains of jute using SSR markers

4.2.2. DISCUSSION

From the data presented, the molecular markers that were generated can identify DNA of jute varieties and accessions amplified with 12 RAPD primers. The experiment was primarily based on 40 different 10-mer oligonucleotide primers that were scored in 10 jute varieties and 8 accessions. Among the 40 primers, 12 generated substantial differences between inter and intra specific polymorphism. Only fragments reproducible in the subsequent attempts were considered for documentation. Weak and spurious bands were not included in the analysis. Table 4.2 shows that the primer OPG-05 is able to produce a total of 181 bands (average 10.06 bands per genotype) in 18 genotypes and the highest number (13 bands) was recorded in 5 C. *capsularis* genotypes. On the other hand the lowest number (71 or 3.94 bands per genotype) was recorded in primer OPAB-03.

From the calculation it was also observed that the average number of bands per genotype per primer was higher (6.84) in *C. capsularis* than in *C. olitorius* (5.77).

The highest number of band difference (31) was found between *C. capsularis* accession no. 1833 and *C. olitorius* accession no. 1805 (Table 4.3). The average genetic distance among the *C. olitorius* genotypes (18 units) was higher than *C. capsularis* (16.40 units) and the distance between the species was 86.10 units. DNA fingerprints that were obtained by RAPD analysis revealed that all the 18 genotypes from both species (namely D-154, CVL-1, CVE-3, CC-45, BJC-83, BJC-7370, acc.905, acc.1505, acc.1515, acc.1833, acc.2470, OM-1, O-9897, O-4, acc.1333, acc.3828, O-72. and acc.1805) were genetically distinct. A dendrogram (Figure 4.6) was constructed using the programme STATISTICA version 3.0 (StatSoft, 1994), based on the genetic distance matrix (Table 4.3). The relationship that is portrayed by this clustering also agreed with the available pedigree information (Hossain et al., 2002). Two major clusters representing two species were resolved among the genotypes that were examined in the study. All *C. capsularis* genotypes belonged to one and the *C. olitorius* genotypes to the other cluster, here mentioned as *C. capsularis* and *C. olitorius* cluster. The study also revealed that the two jute species are distantly related with high level of similarity within species, thus confirming previous reports. On the basis of cluster analysis, pair wise genetic distances, reported by Basu et al. (2004) that the two jute species are widely separated. Presence of distinct patterns of diversity between the two species was also reported by Palit et al. (1996). Such distinction corroborates with previous hypothesis that the two species originated from two different geographical locations: *C. capsularis* originated from the Indo-Burma region and *C. olitorius* from Africa (Kundu 1951). The results agree with Basu et al. (2004), suggesting that the two species are indeed allopatric, sharing certain common alleles. This could be related to the strong sexual incompatibility barrier between these two cultivated jute species, which do not cross-fertilize (Patel and Datta, 1960; Swaminathan et

al., 1961). Similar results were obtained by Hossain et al. (2002, 2003) and Basu et al. (2004) in jute.

The genetic distance (from distance matrix shown in Table 4.3) among the genotypes within *C. capsularis* cluster ranged from 3 to 30 units. The minimum distance (3 units) was observed between variety CC-45 and BJC-83 and was maximum (30 units) between acc. no. 2470 and 1515. On the other hand, the genetic distance among the genotypes within *C. olitorius* cluster ranged from 7 to 27 units. The minimum distance (7 units) was recorded between variety O-4 and O-9897 and was maximum (27 units) between variety OM-1 and O-72. These data correlated with the field observation in case of *C. olitorius* with little discrimination in *C. capsularis*. In *C. olitorius*, variety O-4 and O-9897 are phenotypically very close. The basic difference is the photosensitivity (var. O-9897 is short-day tolerant). But the genetic distance should be wider between the *C. capsularis* variety CC-45 and BJC-83 according to their phenotypic characters.

This genetic distance information could be useful in breeding programmes in order to introduce agronomically important genes. However, more extensive molecular data is needed in order to make a more general conclusion about the relationship between jute genotypes. Despite the strong homology that is exhibited by the closely related genotypes, the work demonstrates that it is now possible to differentiate between them. Furthermore, most of the cultivars that were studied can be individually characterized with cultivar specific RAPD markers. The results show that if such an analysis were extended to additional members of the jute germplasm, particularly to lines with unknown pedigree information or from diverse sources, it would be possible to obtain a detailed picture of their genetic relationship.

SSR primers have been used to analyze diverse jute germplasm and were compared with morphological and physiological data. Once SSR primers have

been evaluated for their effectiveness in determining genetic diversity, they would provide a very cost-effective molecular tool that could be used in various breeding programmes or DNA fingerprinting of a variety of cultivars.

In this study, the first batch of 47 SSR primer pairs were synthesized and used for scoring genetic diversity in 16 genotypes of jute. Twenty-eight out of the forty-seven pairs of primers generated suitable polymorphic products in the jute genotypes. Although the primers were *C. olitorius* specific, some showed sharp bands in *C. capsularis*. The primers revealed a high percentage of polymorphism between the two species. The polymorphisms within *C. olitorius* were greater than *C. capsularis;* 184 polymorphic bands were found between the two species, 80 between *C. olitorius,* and only 13 between *C. capsularis*. The result was able to partition the genetic diversity at both inter and intra-specific levels. Although the SSR markers were best at discriminating between the species, interspecific diversity was also determined with many of the primers. Through this study, not only were the presence of jute specific SSRs established but also polymorphic repeat motifs were found within species. The di- and tri- nucleotide motifs rendered a high number of highly polymorphic bands. Best results of PCR amplification reactions were obtained with the poly (CT, GT, CA, GA, TC, and TG) dinucleotide repeats.

A dendrogram (Figure 4.8) was constructed using the polymorphic data showing the genetic relation between the different jute genotypes. It shows separate clusters of distinct genotypes from a particular geographical location such as variety D-154 and CVL-1 of *C. capsularis,* which are both of Bangladesh origin, indicating that these are genetically more related. Similarly variety O-72 and Line 7/95, two genotypes of *C. olitorius* from the same region, were grouped together. The cultivar O-4 and O-9897, previously shown to be cold sensitive (Begum, 1998) has also been grouped in the same cluster.

Thus comparisons of molecular to morphological and physiological data produced generally similar conclusions of relatedness among accessions, confirming the utility of molecular analysis. Similar result was observed by Basu et al. (2004) in jute. As already mentioned in literature review (section 2.7).

The present study revealed that the two jute species are distantly related. Presence of distinct patterns of diversity between the two species was also reported by Palit et al. (1996). The results thus provide support to the earlier belief that the two species originated from two different geographical locations: *C. capsularis* originated from the Indo-Burma region and *C. olitorius* from Africa (Kundu, 1951).

The marker system demonstrated a lower percentage of polymorphism within *C. capsularis* than *C. olitorius* species. From the distance matrix, it was observed that the average genetic distance among the genotypes of *C. capsularis* was only 6.86 units when it was 35.50 units in *C. olitorius*. These findings revealed that the SSR primers were more effective in *C. olitorius* than *C. capsularis*. This may have happened because the primers had been developed from *C. olitorius* genome O-4. The result could be correlated with the lower percentage (3 to 4%) of out crossing in *C. capsularis* as opposed to 8 to 12% natural cross pollination within *C. olitorius* (Ghose and Gupta, 1945). In the flower of *C. capsularis*, the pollen receptive period of the stigma is short and is usually fertilized before or immediately after the petal opens (Kundu et al., 1959). This decreases the chances of cross-fertilization thereby decreasing the genetic variability in *C. capsularis*. In *C. olitorius* flower, the stigma is receptive to pollens for a longer period of time after the petal opens. This increases the likelihood of cross-fertilization in *C. olitorius* and hence a greater genetic variability than *C. capsularis*.

It has been further observed that some of the wild jute accessions of the two species, in spite of containing diverse morphological characters, originated from a very closely related common ancestor. It should also be kept in mind that marker-based genomic analyses do not adequately indicate the full picture of the genetic variability present in the expressed portions of the crop genome. Attempts to break the sexual barrier for genetic introgression by technologies as somatic hybridization, chromosome doubling, and embryo rescue and then by searching for suitable homologous recombination in the hybrid plants may bear fruit. Alternatively, transfer of genes of interest from genetic resource collections by genetic transformation techniques may provide new ways to generate desired plant types with suitable agronomic traits (Ghosh et al., 2002).

In conclusion, this study holds enormous prospect for the development and application of molecular markers for jute.

4.3. EXPERIMENT 3 : DEVELOPMENT OF MOLECULAR MARKER FOR LEAF SHAPE AND GLOSSINESS CHARACTERS OF JUTE

4.3.1. RESULTS

An experiment was conducted for the development of molecular marker/s for waterlogging tolerance in tossa (*Corchorus olitorius* L.) jute. It is a very important agronomic trait for tossa jute. Accordingly a cross was made between a recommended variety O-4 (sensitive) as seed parent and a well reported waterlogging tolerant *C. olitorius* variety Incisifolius (Newaz, 1983) as pollen parent (Figure 4.9). After collecting F_1, segregating F_2 populations (Figure 4.10) were raised with their parents by selfing F_1. The populations along with parents were screened for waterlogging tolerance (Figure 4.12 and 4.13) but the result was not encouraging. No plants were found to be tolerant to waterlogging. The experiment was repeated and the result remained unchanged. Actually the seed lot was not same as examined earlier. The parent Incisifolius has been collected from Egypt and O-4 is local. It was observed that the leaf shape and texture of Incisifolius were serrated and glossy, respectively, which supported plant characteristics for drought tolerance (Pandey and Sinha, 1978). With this in mind, four homozygous lines (F_8) were developed through selection for the development of DNA marker/s for those leaf characters. The segregating pattern of leaf texture and structure is presented in Figure 4.11. The DNA from 2 parents and 4 homozygous lines (F_8 generation) were isolated and evaluated for their quality and quantity (Figure 4.14). A total of 10 DNA samples were formed consisting of 2 parents, 4 homozygous lines and 4 bulks for leaf characters such as entire, serrated, matte and glossy leaf. The 10 DNA samples were evaluated using RAPD, ISSR and STMS primers (gel picture presented in Figure 4.15 and 4.16).

The bands were scored and analyzed for the detection of DNA marker/s associated with the traits and for genetic diversity. A dendrogram was constructed (Figure 4.17). The percent polymorphism and genetic distance (from distance matrix) were also presented in Table 4.4.

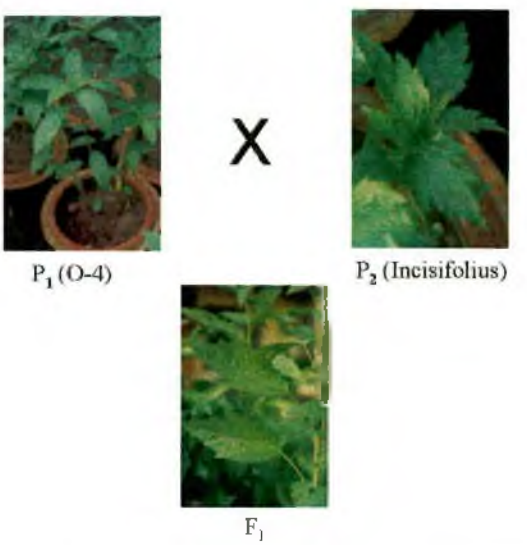


Figure 4.9: Two parents and their hybrids (F_1)

Figure 4.10: Segregating F_2 population in the pots

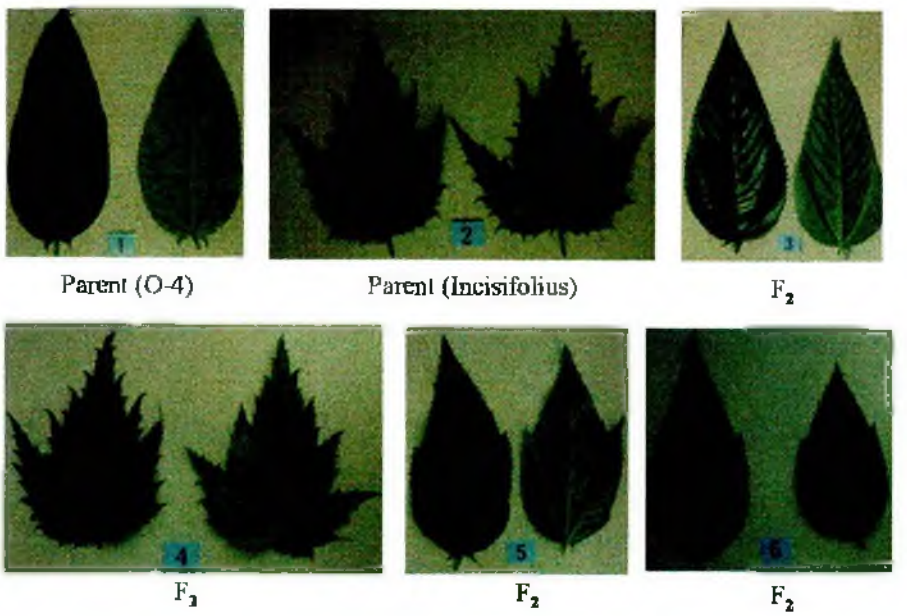


Figure 4.11: Different leaf shape and texture of F_2 populations

Figure 4.12: Artificial waterlogged condition

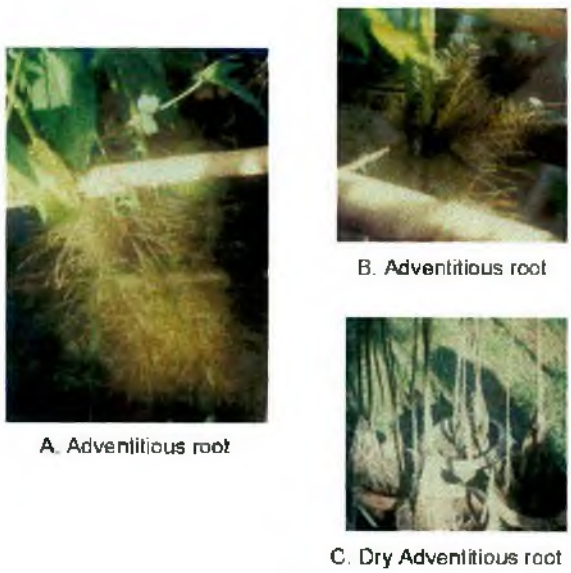


Figure 4.13: Adventitious roots in wet and dry condition

Figure 4.14: Genomic DNA in 0.8% agarose gel

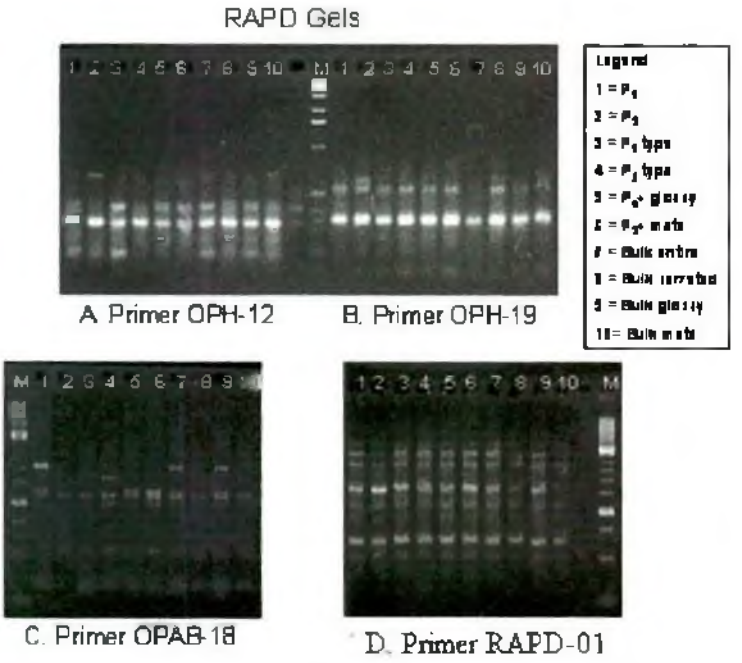


Figure 4.15: Amplification with RAPD primers

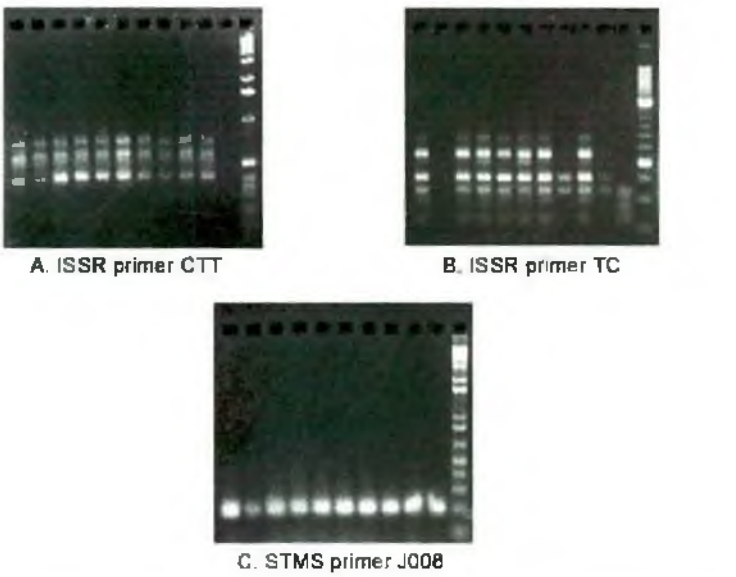


Figure 4.16: Amplification with ISSR and STMS primers

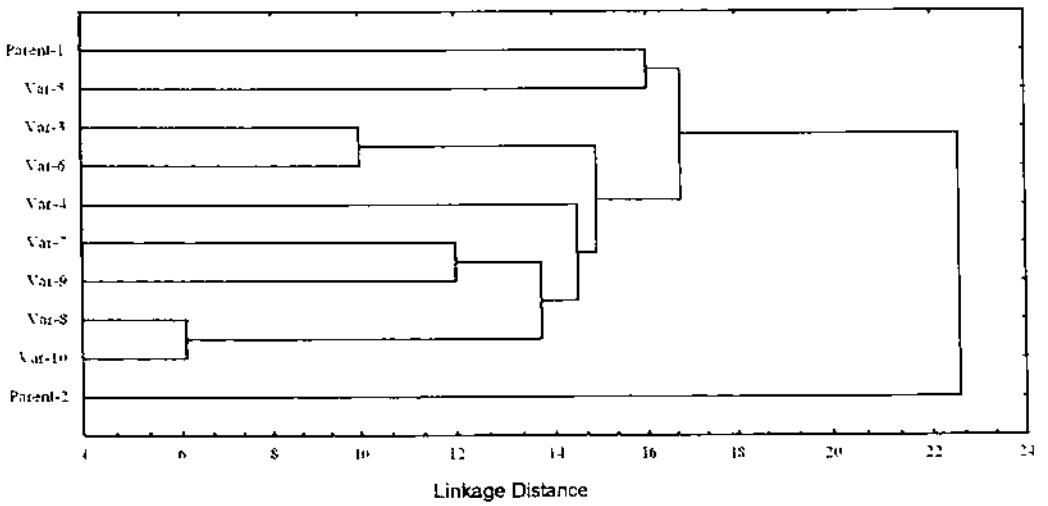


Figure 4.17: A dendrogram of 10 variables (2 parents and 8 F_8 populations)

Parent-1: variety O-4
Parent-2: variety Incisifolius
Variable-3: P_1 type
Variable-4: P_2 type
Variable-5: P_1 + glossy leaf type
Variable-6: P_2 + nonglossy leaf type
Variable-7: Bulk for matte type
Variable-8: Bulk for glossy leaf type
Variable-9: Bulk for lanceolate leaf type
Variable-10: Bulk for serrated leaf type

Table 4.4: Percent polymorphism and genetic distance between variables

	Percent polymorphism (upper triangle)									
	P_1	P_2	Var. 3	Var. 4	Var. 5	Var. 6	Var. 7	Var. 8	Var. 9	Var.10
P_1	**0**	**24**	15	17	13	8	16	18	12	10
P_2	**27**	**0**	20	21	21	19	20	14	16	18
Var. 3	17	22	**0**	15	16	7	13	16	13	9
Var. 4	19	24	16	**0**	16	12	12	13	15	11
Var. 5	16	23	17	17	**0**	14	15	20	14	14
Var. 6	13	22	10	14	15	**0**	14	15	12	8
Var. 7	17	22	16	14	17	14	**0**	14	11	10
Var. 8	20	23	17	15	20	17	15	**0**	12	4
Var. 9	15	18	16	16	15	14	12	13	**0**	9
Var.10	13	22	12	13	19	14	13	**6**	14	**0**
Square Euclidean Distance (lower triangle)										

4.3.2. DISCUSSION

The yield and quality of *C. olitorius* jute is better than *C. capsularis* but very sensitive to waterlogged condition. That is why the cultivation of tossa jute is limited in the upland. Developing a waterlogging tolerant DNA marker is very important but in the present study it was not possible to proceed with these materials. However, it was decided to develop DNA markers for leaf structure (serrated versus entire leaf) and texture (matte versus glossy leaf). The serrated and glossy leaf characters are important for supporting drought tolerance (Pandey and Sinha, 1978). From the segregating pattern it was observed that the serrated leaf character is incompletely dominant over entire leaf and matte character is dominant over glossy character (Figure 4.11). From the DNA analysis with limited number of RAPD, ISSR and STMS primers, no consistent marker associated with the desired traits were observed. From the dendrogram (Figure 4.17) it was observed that two major clusters were formed representing the two parents where the seed parent and all other homozygous lines and bulks were in one cluster and only the pollen parent in the other cluster.

A number of researchers reported hybridization between the two species *Corchorus capsularis* and *C. olitorius*, to be unsuccessful (Finlow, 1917, 1923; Banerjee and Datta, 1960; Patel and Datta, 1960). But many scientists (Islam and Rashid, 1961; Swaminathan and Iyer, 1961; Choudhuri and Mia, 1962; Mia and Shaikh, 1967) have claimed to have succeeded in producing hybrids. However all those putative hybrids showed dominance of the female parents in F_1 and F_2 generations. Raut and Naik (1983) claimed to have followed the hybrid progenies up to F_3 generation but there was no further report of exploitation of those hybrids at later generation. This is probably because of the fact that in the later generation the entire population resembled the female parent.

From the DNA analyses of the two parents and 8 homozygous lines (F_8 and bulks), it was found that all the advanced population were skewed to the seed parent (variety O-4).

It is a molecular evidence of the finding of the conventional breeders. From the distance matrix table (Table 4.4), the highest genetic distance (27 units) was observed between the two parents and was lowest between variables 8 and 10. As it was known, the higher the polymorphism, the higher the genetic distance. So, the highest percentage of polymorphism was found between the parents (24%) and was lowest between the variables 8 and 10 (4%).

4.4. EXPERIMENT 4: STUDY OF THE YIELD PERFORMANCE OF FOUR HOMOZYGOUS LINES OF TOSSA JUTE IN COMPARISON WITH THEIR PARENTS

4.4.1. RESULTS

An effort was made to determine the yield performance of 4 homozygous lines along with their parents. The availability of the data (obtained from the experiment, mentioned in section 4.3) led to this study. The data such as plant height (PH in cm), base diameter (BD in mm), leaf area per plant (cm^2), dry weight of different plant parts (leaf, stem and bark) per plant (g), flowering records such as flowering at 60 and 120 days after sowing (DAS in %), number of plants not flowered at harvest time (%) and harvest index (HI in %) were recorded, analyzed statistically and the means were compared by Duncan's multiple range test (DMRT). The results are presented in Table 4.5, 4.6, 4.7 and 4.8.

Table 4.5: Mean Sum of Square (MSS) for 10 parameters in six genotypes of jute.

Sl. #	Characters	CV (%)	MSS
01	Plant height (cm)	09.17	1685.993**
02	Basal diameter (mm)	15.24	9.930*
03	Leaf area/plant (cm^2)	19.79	358892.933**
04	Stem dry weight/plant (g)	18.10	60.756*
05	Bark dry weight/plant (g)	16.27	25.108*
06	Leaf dry weight/plant (g)	15.58	12.449**
07	Flowering (%) at 60 DAS	25.19	1609.072**
08	Flowering (%) at 120 DAS	16.68	430.257**
09	Plant not flowered (%) at 120 DAS	09.72	1494.144**
10	Harvest Index (%)	09.21	8.272 NS

Design-RCBD, Replication-6, Treatment-6.

* Significant at 5% and ** Significant at 1% level of probability, NS means not significant

Table 4.6: Growth performance with respect to plant height, base diameter and leaf area of six genotypes of jute.

Genotypes	Plant height (cm)	Basal diameter (mm)	Leaf area (cm^2)
Variety O-4 (T_1)	228.70 A	11.67 A	1159.00 A
Var. Incisifolius (T_2)	175.20 C	8.74 C	489.00 C
O-4 type (T_3)	193.30 BC	9.14 C	627.20 BC
Incisifolius type (T_4)	195.70 BC	9.32 BC	532.80 C
O-4 glossy type (T_5)	206.30 AB	11.81 A	982.60 AB
Incisifolius matte type (T_6)	213.00 AB	11.32 AB	661.40 BC

Note: Same letter indicates not significant and T_1 to T_6 indicates treatments

Table 4.7: Growth performance with respect to stem, bark and leaf dry weight of six genotypes of jute.

Genotypes	Stem dry wt./ plant (g)	Bark dry wt./plant (g)	Leaf dry wt./plant (g)
Variety O-4 (T_1)	15.620 A	9.902 A	6.048 A
Var. Incisifolius (T_2)	5.634 C	3.728 C	2.180 B
O-4 type (T_3)	8.802 BC	5.990 BC	3.170 B
Incisifolius type (T_4)	7.608 BC	5.206 BC	2.374 B
O-4 glossy type (T_5)	11.740 AB	8.508 AB	5.128 A
Incisifolius mate type (T_6)	9.764 BC	6.500 ABC	2.946 B

Note: Same letter indicates not significant and T_1 to T_6 indicates treatments.

Table 4.8: Flowering response at different stages of growth among six genotypes of jute.

Genotypes	Flowering (%) at 60 DAS	Flowering (%) at 120 DAS	Plant (%) not yet flowered
Variety O-4 (T_1)	03.43 D	10.52 D	86.05 A
Var. Incisifolius (T_2)	03.37 D	17.60 B	78.99 A
O-4 type (T_3)	17.57 C	33.31 A	49.59 B
Incisifolius type (T_4)	39.45 AB	14.30 BC	45.97 B
O-4 glossy type (T_5)	44.45 A	06.54 E	49.00 B
Incisifolius mate type (T_6)	32.03 B	13.60 CD	54.37 B

Note: Same letter indicates not significant, T_1 to T_6 indicate treatments and DAS means days after sowing.

4.4.2. DISCUSSION

The result presented in Table 4.5 show that among the 10 parameters, 3 treatments: base diameter, stem and bark dry weight were significant at 0.05 level and 6 treatments: plant height, leaf area, leaf dry weight, flowering (%) at 60 DAS, flowering (%) at 120 DAS and percentage of plants not flowered at harvesting time were highly significant (0.01). Only one treatment, harvest index, was found insignificant. Though the bark dry weight represents the fibre yield (about 50% of the bark is fibre, Khandakar et al., 1995) and was found highly significant, the harvest index was insignificant as it is a ratio. From the Table 4.6 it was observed that plant height, base diameter and leaf area were significantly different among the treatments (T_1 to T_6). The highest and lowest plant height was found in the treatments T_1 (seed parent O-4) and T_2 (pollen parent Incisifolius), respectively but T_1T_5, $T_3T_4T_5T_6$ and $T_2T_3T_4T_6$ were statistically similar. Considering the analyzed data presented in Table 4.6, 4.7 and 4.8, it was observed that the performance of the variety O-4 was better than all other treatments. It may be concluded that the variety developed by Bangladesh Jute Research Institute is a rigid one and it is not so easy to supersede this.

4.5. EXPERIMENT 5: DEVELOPMENT OF DNA MARKER FOR LOW TEMPERATURE TOLERANCE IN JUTE

4.5.1. RESULTS

For the selection of low temperature tolerant genotype, the well-reported four low temperature tolerant accessions and two sensitive varieties were reevaluated in the growth chamber (Figure 4.18) and in the low temperature incubator in small pots (Figure not shown).

Figure 4.18: Germination and growth of selected accessions and varieties in growth cabinet at 16°C temperature (at 35 days after emergence).

Before selecting the parents, two varieties (O-4 and O-9897) and 4 low temperature tolerant accessions (accession no. 1540, 1805, 1852 and 2015) were analyzed through DNA profiling using several RAPD markers to identify whether the four accessions were genetically similar or not (as those are phenotypically similar). From the analysis a clear distinction was observed between low temperature tolerant and sensitive genotypes when amplified with RAPD primer OPG-05. A specific amplified product was present in all the low emperature tolerant accessions and absent in two sensitive genotypes (Figure 4.19).

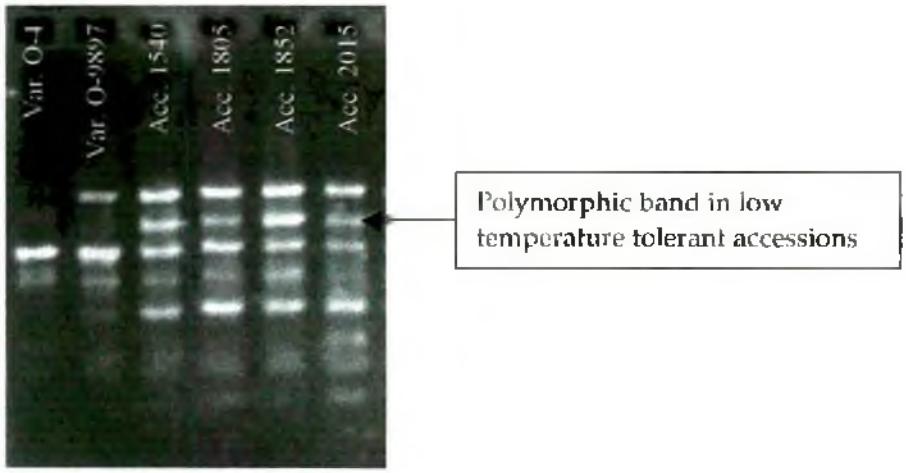


Figure 4.19: Polymorphic band in low temperature tolerant accessions in RAPD amplified with primer OPG-05 in agarose gel.

A different DNA profile was observed when the same materials were amplified with RAPD primer OPAB-18, where a particular band was found in the sensitive genotypes and absent in the tolerant accessions (Figure 4.20).

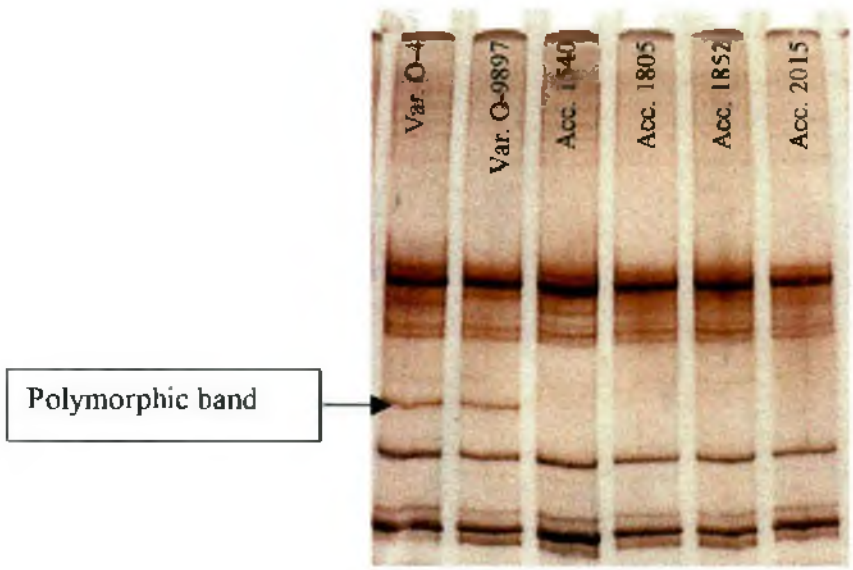


Figure 4.20: Band pattern of low temperature sensitive and tolerant genotypes of jute amplified with RAPD primer OPAB-18 in PAGE.

DNA of the six genotypes was thoroughly evaluated using RAPD and AFLP (Figure 4.21) and two independent dendrograms were constructed (Figure 4.22 and Figure 4.23).

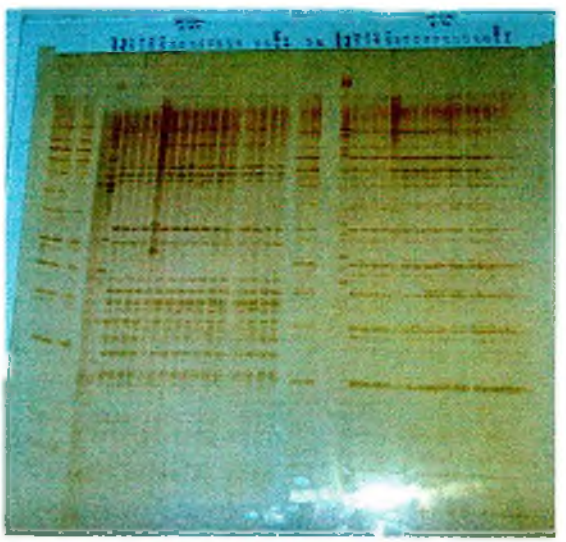


Figure 4.21: An AFLP gel amplified with different primer combinations.

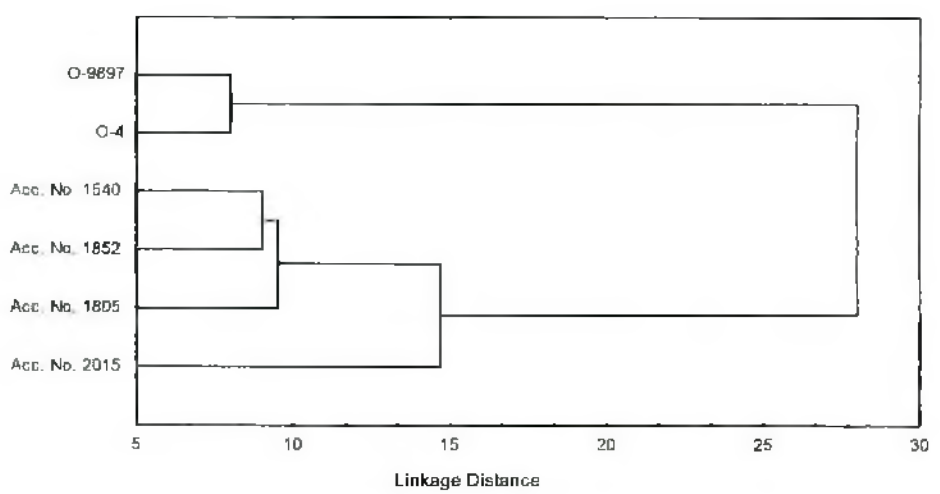


Figure 4.22: A dendrogram of six genotypes obtained with RAPD data.

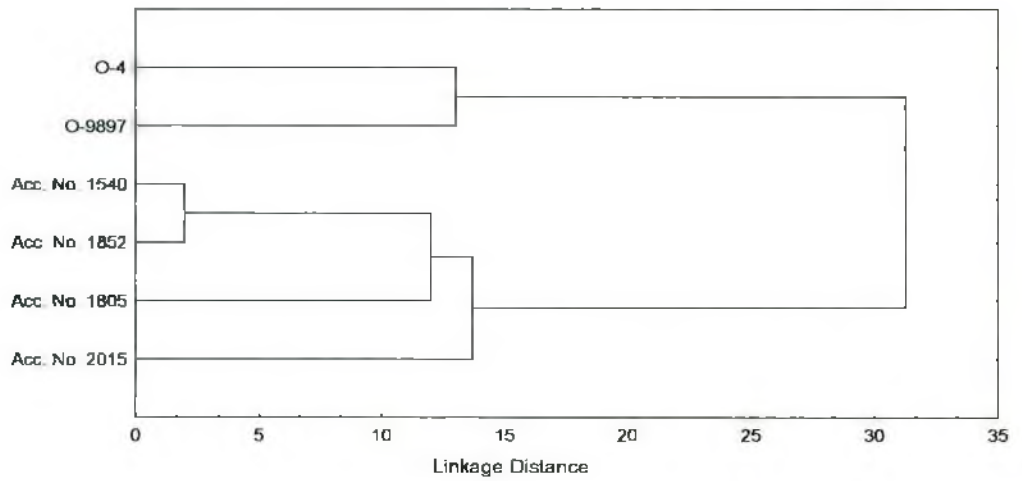


Figure 4.23: A dendrogram of six genotypes obtained with AFLP data.

From the evaluation, variety O-9897 and accession no. 1805 were selected as seed and pollen parents (Figure 4.24) respectively.

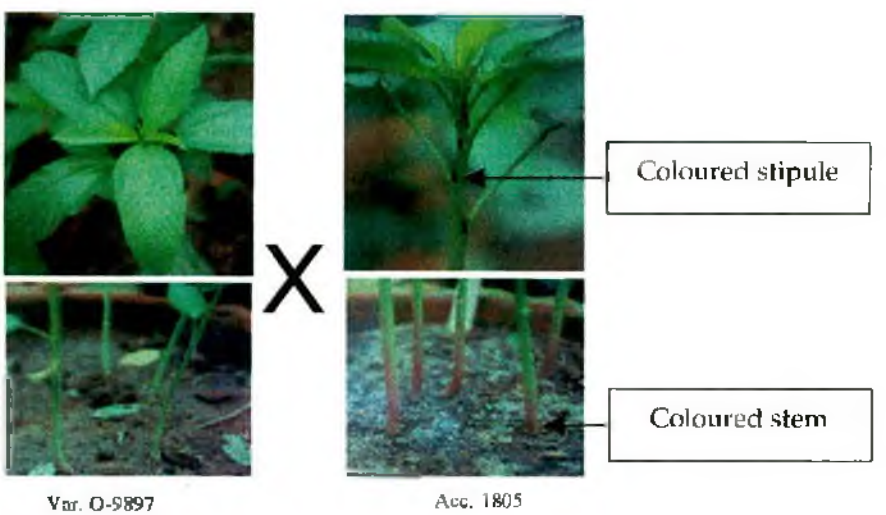


Figure 4.24: Low temperature sensitive and tolerant parents showing difference in stem and stipule colour

A cross was made between the selected parents. The germination test was performed using the seeds of two parents and their hybrid at ideal temperature (30°C) presented in Figure 4.25.

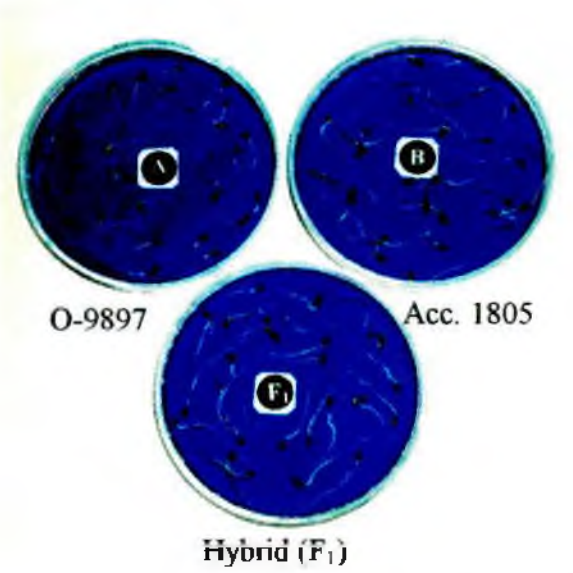


Figure 4.25: Normal germination of parents and their hybrid (F_1) seeds at 30°C

The seeds were also evaluated through germination at low temperature condition (16°C) in the incubator and the result shown in Figure 4.26.

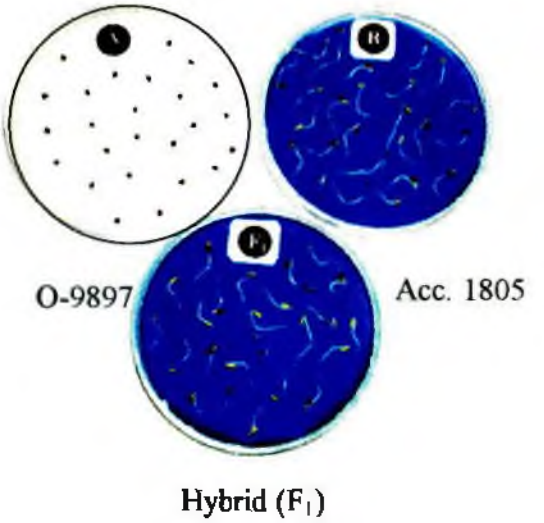


Figure 4.26: Germination of parents and hybrid (F_1) seeds at low temperature.

The F_2 seeds were obtained by selfing F_1 and assessed for their germination at low temperature (16°C) in an incubator (Figure 4.27).

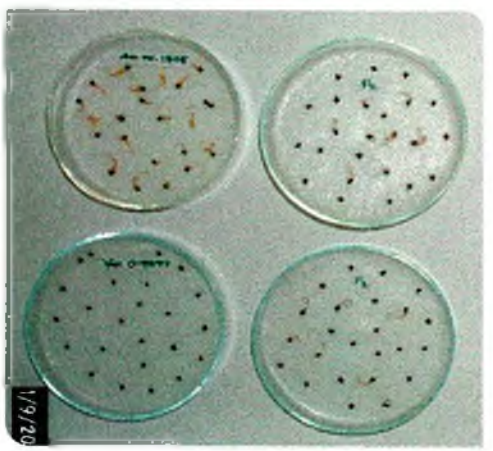


Figure 4.27: Germination of parents and F_2 seeds at low temperature (16°C)

The F_2 populations were grown differentially as low temperature tolerant and sensitive in the growth chamber in earthen pots. DNA from 114 F_2 populations and parents were isolated following CTAB method (describe in section 3.1.3.1) and were evaluated using 40 RAPD primers. A dendrogram was constructed using a particular number of F_2 populations and their parents (Figure 4.28).

The DNA obtained from differentially grown populations was evaluated with RAPD primer OPG-05 for tracking the amplified DNA fragment associated with low temperature tolerance (Figures 4.29a, 4.29b and 4.29c).

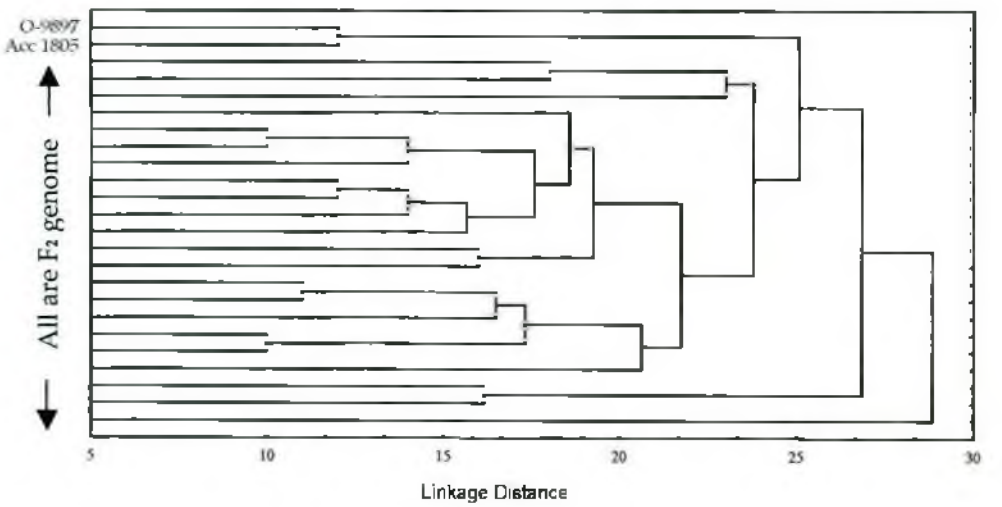


Figure 4.28: A dendrogram of 2 parents (O-9897 and acc. no. 1805) and 22 F_2s with RAPD data.

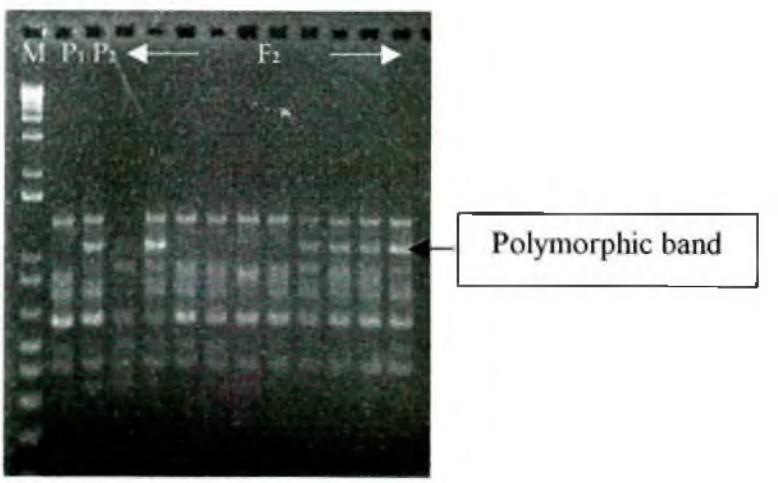


Figure 4.29a: Polymorphic band associated with low temperature tolerance amplified with RAPD primer OPG-05

Legend: M: Marker (1kb+), P_1: Seed parent, P_2: Pollen parent, F_2 Segregating population

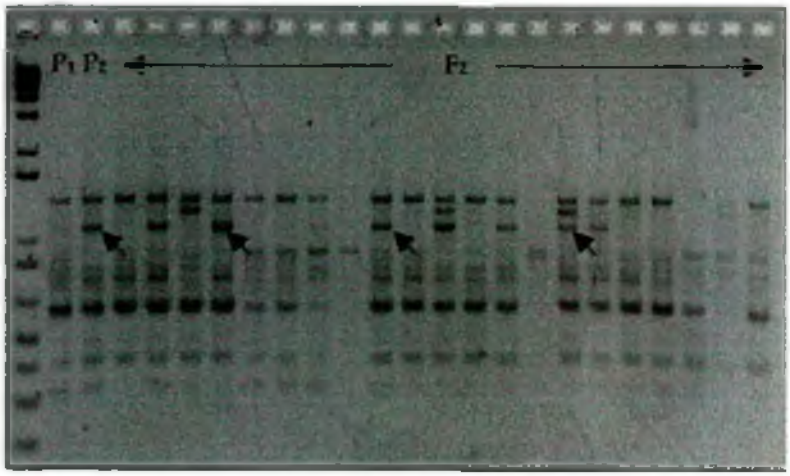


Figure 4.29b: Polymorphic band associated with low temperature tolerance amplified with RAPD primer OPG-05

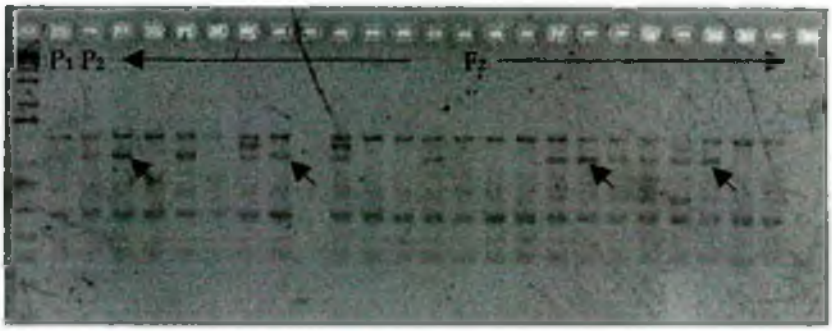


Figure 4.29c: Polymorphic band associated with low temperature tolerance amplified with RAPD primer OPG-05
Arrow showing the position of DNA fragment associated with low temperature tolerance

The band indicating association with low temperature tolerant was isolated, cloned (Figure 4.30) and sequenced (Appendix VI) for the development of sequence characterized amplified region (SCAR) marker.

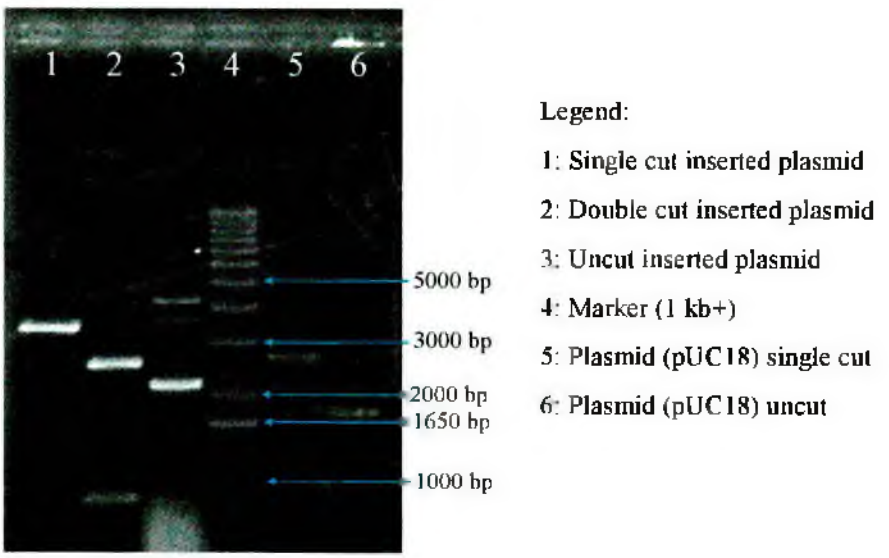


Figure 4.30: Plasmid inserted with cold tolerant DNA fragment

However, a Southern analysis of the tolerant and sensitive parents, using the DNA fragment associated with low temperature tolerance as probe, failed to differentiate the two parents (Figure 4.31). The two parents were also amplified with SCAR primer (Appendix VI) but no distinction was observed between the parents (Figure 4.32).

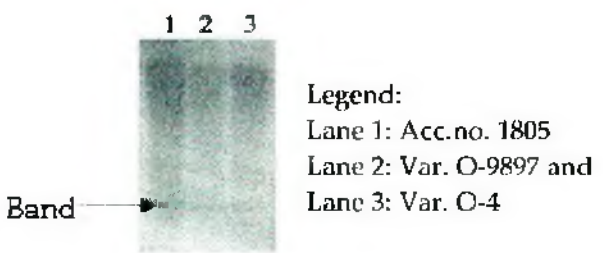
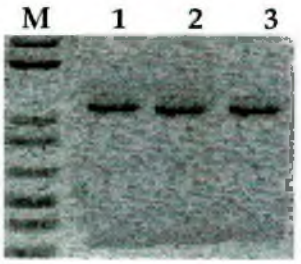


Figure 4.31: Southern blot hybridization, genomic DNA digested with *Eco*RI and DNA fragment associated with low temperature tolerance used as probe (DIG labeled)



Legend:
M: Marker (1kb+)
Lane 1: Acc. no. 1805
Lane 2: Variety O-9897
Lane 3: Variety O-4

Figure 4.32: Two parents (accession no. 1805 and variety O-9897) and a low temperature sensitive variety (O-4) amplified with SCAR primers.

On bioinformatics analysis of the DNA sequence associated with low temperature tolerance, a splice site was computationally predicted, with an acceptor site present at the beginning of 153 bp long open reading frame (ORF), presented in Figure 4.33.

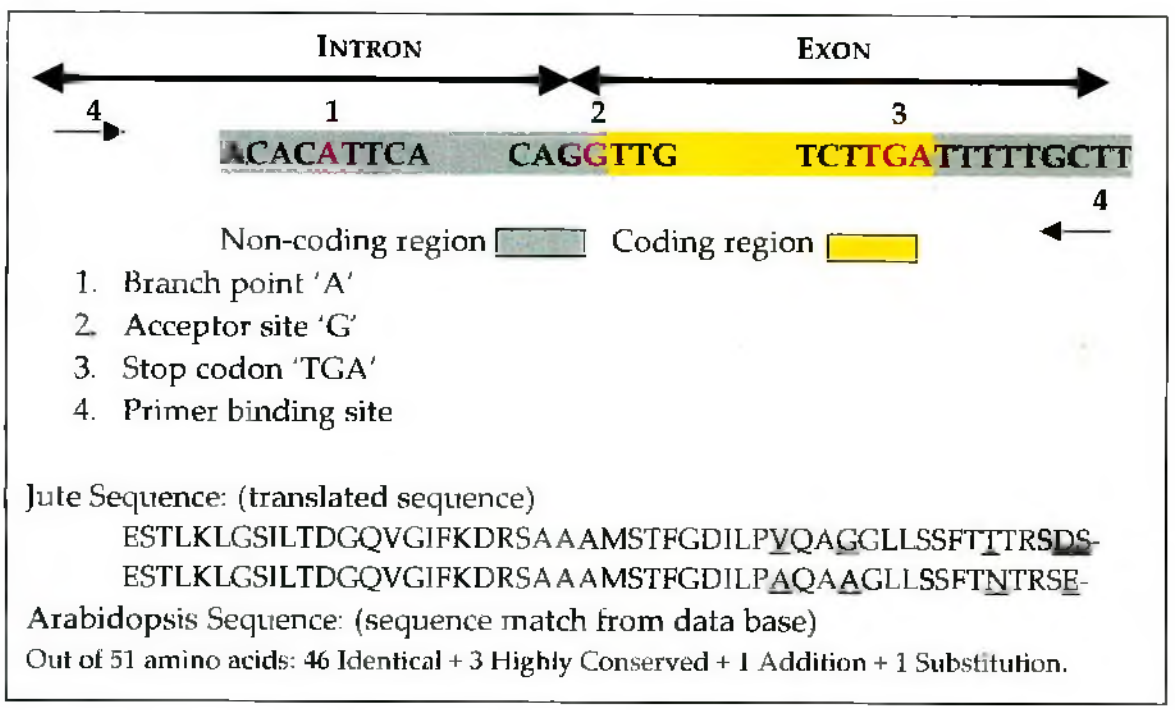
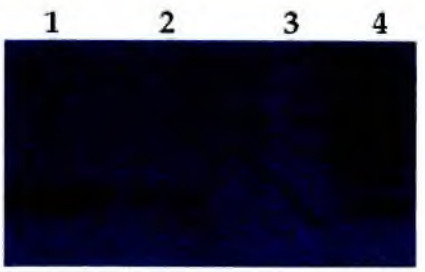


Figure 4.33: Homology study of the translated products of the low temperature tolerant DNA fragment with *A. thaliana*.

The sequence obtained from the DNA fragment associated with low temperature tolerance was analyzed with bioinformatics tools with the available database in the Genbank. Analyses showed that the fragment was part of a gene (terminal exon). Another effort was made to know the upstream sequence to rescue the total gene sequence through Inverse PCR (Figure 3.1). However, all attempts failed in getting the upstream sequences of the fragment.

For expression studies, total RNA was isolated from 3 day old seedlings of acc.no. 1805 and was passed through oligo-dT column to extract the mRNA. Expression of the gene was verified by RT-PCR (reverse transcription-PCR) using primers designed from the predicted exon (Figure 4.34).



Legend
Lane 1: RT+ PCR
Lane 2: Normal PCR
Lane 3: Control
Lane 4: Ladder

Figure 4.34: Expression of the predicted exon using mRNA isolated from acc.no. 1805

The F_2 populations were evaluated with other RAPD primers and the amplified products were scored and a genetic linkage map was constructed using MAPMAKER version 3.0 (Figure 4.35). Linkage groups were established at a LOD score of 3.0 and a maximum distance 50 cM by two-point analysis using the 'group' command. The characteristics of the linkage group with more than 1 locus was presented in Table 4.9. The 18 linkage groups with their locus/loci number(s) are shown in Table 4.10.

Table 4.9: Characteristics of the intraspecific genetic linkage group with more than one locus of jute.

Linkage group	Length (cM)	Number of markers	Average distance between markers (cM)
Linkage group-16	15.90	2	15.90
Linkage group-17	241.70	11	24.17
Linkage group-18	206.10	12	18.73
Total	463.70	25	19.60

Linkage group with single locus: Linkage group no. 1 to 15 is not mentioned in the table.

Table 4.10: Eighteen linkage groups with their locus/loci.

Linkage group	Total locus	Locus number/Primer/Size (bp)			
1	1	3. OPQ-17/450			
2	1	8. OPAB-01/660			
3	1	10. OPAB-03/1650			
4	1	12. OPAB-01/1000			
5	1	14. OPAB-08/1350			
6	1	17. OPG-11/900			
7	1	26. OPH-10/850			
8	1	29. OPH-12/550			
9	1	30. OPH-17/1200			
10	1	32. OPQ-06/950			
11	1	33. OPQ-06/650			
12	1	37. OPQ-08/500			
13	1	38. OPQ-16/750			
14	1	39. OPQ-16/450			
15	1	40. OPQ-16/400			
16	2	1. OPQ-17/760	27. OPH-10/700		
17	11	23. OPAB-20/700	25. OPH-10/1000	15. OPAB-08/700	24. OPAB-20/570
		20. OPAB-12/1050	22. OPAB-20/950	13. OPG-07/100	28. OPH-10/670
		5. OPE-17/900	21. OPAB-20/1330	2. OPQ-17/700	
18	12	36. OPQ-08/1350	34. OPQ-06/600	7. OPG-05/1100	11. OPAB-01/1350
		9. OPAB-01/150	18. OPG-10/200	35. OPQ-06/500	31. OPH-17/750
		19. OPG-13/1050	16. OPAB-08/650	4. OPE-17/950	6. OPG-05/1650

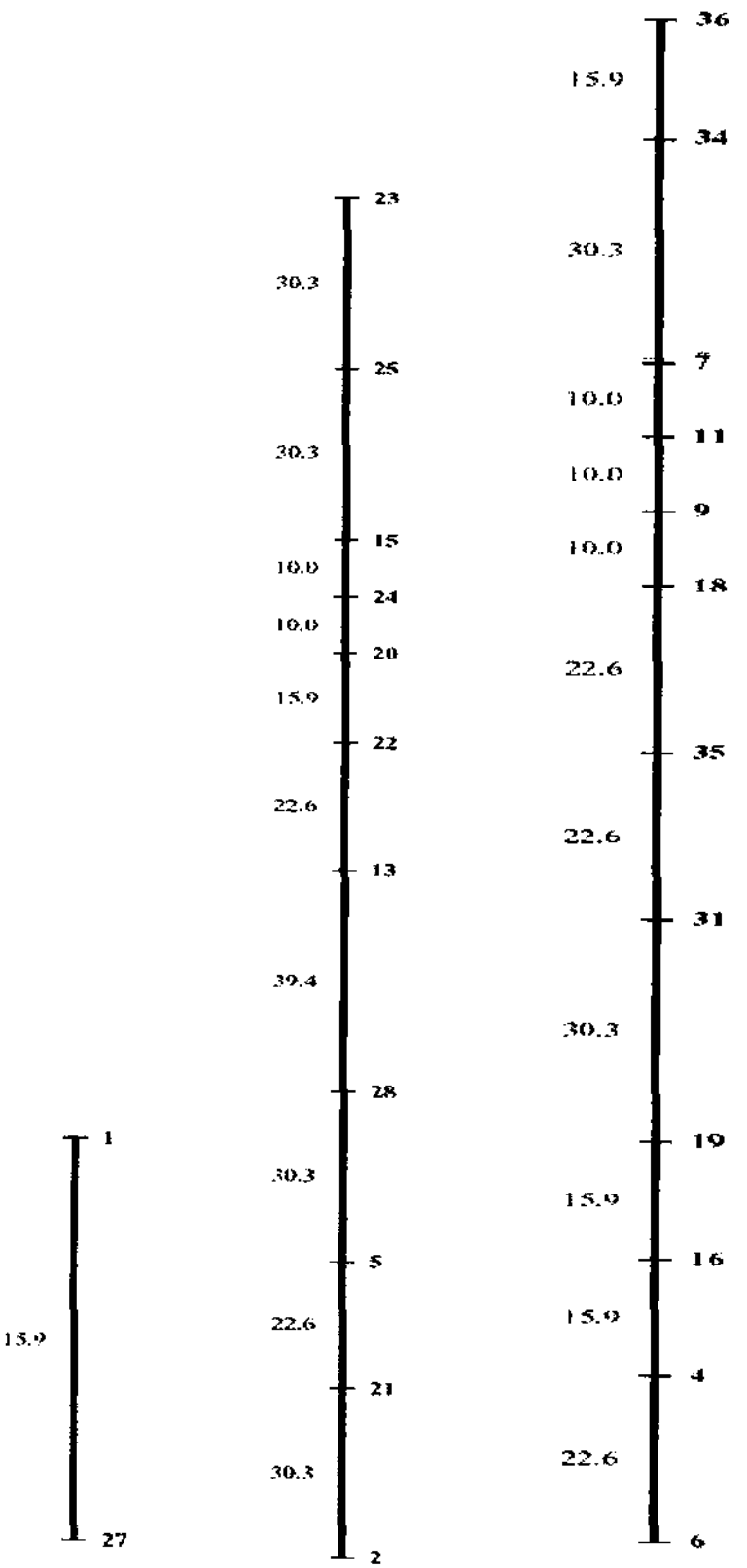


Figure 4.35: Three linkage groups representing 25 RAPD loci developed from F_2

4.5.2. DISCUSSION

It was observed in Figure 4.18 that the two varieties (O-4 and O-9897) failed to germinate at 16°C i.e. in low temperature stressed condition, in pots in the growth cabinet. But the genotypes in the other four pots not only germinated but also showed a reasonable growth in the low temperature environment. It may be remembered that the base temperature for jute is 20°C. So it was very convincing that among the six genotypes the accessions no. 1540, 1805, 1852 and 2015 were low temperature tolerant and varieties O-4 and O-9897 were sensitive. The same materials were also verified by Begum (1997) and found similar result.

A through investigation of the six genotypes at molecular level was done. As no DNA level information is available on jute, easy and readily affordable random amplified polymorphic DNA (RAPD) technique was performed with 40 random decamer primers. Noticeable discrepancies among the genotypes were observed. A particular polymorphic band of about 1200 bp amplified with primer OPG-05 was found in all the four low temperature tolerant accessions which was absent in the two varieties (Figure 4.19).

An opposite band pattern was also observed when the genotypes were amplified with RAPD primer OPAB-18 (Figure 4.20), where a particular band was found only in the sensitive genotypes but not in the tolerant one. This band was not further characterized.

A dendrogram was constructed using the software STATISTICA version 3.0 to evaluate the genetic diversity among the six genotypes (Figure 4.22). Similarly, amplified fragment length polymorphism (AFLP) technique was also carried out to evaluate the genetic relatedness among the six genotypes (Figure 4.21) and a phylogenetic tree was constructed (Figure 4.23). Both the dendrogram showed very similar clustering and sub-clustering pattern with little discrepancies in respect of their genetic distance.

It may be mentioned that similar clustering pattern has been observed and discussed in section 4.5.1. The two dendrograms were composed of two major clusters, where the sensitive varieties O-4 and O-9897 were in one cluster and the rest (four low temperature tolerant accessions 1540, 1805, 1850 and 2015) were in the other cluster. Among the major clusters, the order of the genotypes in the sub-clusters was exactly similar in both the dendrogram. Similar clustering pattern had also been observed by Hossain et al. (2003) in jute.

In marker analysis, AFLP is more acceptable because of its reproducibility than RAPD. In kenaf, more polymorphic bands were observed (Cheng, 2004) in AFLP than RAPD. But in the present study, it was observed that RAPD system was more effective than AFLP in jute genome for detecting polymorphism. Ashraf et al. (manuscript submitted) had also observed similar. By evaluating a number of jute genome sequences (as obtained from jute genomic library constructed commercially for SSR primers), it was observed that the restriction sites for *Eco*R1 were very low in comparison with other genomes. Obaid (2005) had made similar observations. This could be a cause for lower polymorphisms in AFLP system in jute genome since *Eco*RI digested DNA was used in the AFLP study.

From this evaluation, a popular *Corchorus olitorius* jute variety O-9897 and accession no. 1805 were selected as seed and pollen parent with respect to their low temperature sensitive and tolerant character, respectively (Figure 4.24). The phenotypes of the parents have been cited in Table 2. A cross was made between the parents and hybrids were produced. The viability of the hybrid seeds (F_1) along with their parents was evaluated through germination test at 30°C. It was observed from Figure 4.25 that the hybrid seeds germinated well and were good in quality, which indicated that there was no barrier in germination of hybrid seed. It was also observed from the Figure 4.26 that only the tolerant parent (accession no. 1805) and all hybrid seeds were capable of germinating at low temperature conditions (16°C) and the sensitive parent (variety O-9897) failed to

germinate at that temperature. The result indicated that the low temperature tolerant trait is a dominant character. The segregating F_2 seeds were evaluated at low temperature and partial germination of F_2 seeds were observed (Figure 4.27).

The DNA obtained from the parents and differentially grown (tolerant and sensitive) segregating F_2 populations were thoroughly evaluated with RAPD primer OPG-05 and tracked for the expected DNA fragment associated with low temperature tolerance. It was observed from Figures 4.29a, 4.29b and 4.29c that a particular band of about 1200 bp in size consistently co-migrated with a band of same size in the low temperature tolerant parent (accession no. 1805) and selected low temperature tolerant F_2 populations. This band was always absent in the sensitive parent (variety O-9897) and its populations. The observation strengthened the idea that this particular stretch of DNA could be associated with low temperature tolerant character. The F_2 populations along with their parents were also evaluated with other RAPD primers looking for further marker/s associated with low temperature tolerance but no other consistent marker was found. All PCR products amplified with different RAPD primers were scored (gel picture not presented) to construct linkage group analysis. A portion of the data with 22 F_2 populations along with their parents was analyzed and a dendrogram was constructed using the programme STATISTICA version 3.0 (Figure 4.28). From the dendrogram, it was observed that the genetic distance among the F_2s were higher than the variability between the parents. In few cases, the variability had reduced but in most of the cases increased in comparison with parents. It is a molecular proof that artificial cross creates genetic variability that was observed and reported by several plant breeders (Mendel, 1865; Hossain, 1996); indicating an opportunity for selecting desired population to design a variety with useful traits.

It may be mentioned that the co-segregating DNA fragment may be a part of low temperature tolerant gene or non-coding fragment associated with low temperature tolerant trait. For further confirmation, the fragment was isolated

from gel and used as probe to differentiate between the tolerant and sensitive parents through Southern analysis. The Southern technique could not differentiate the parents. This meant that the nucleotide sequence of both tolerant and sensitive parents in the amplified regions were very similar.

The isolated DNA fragment associated with low temperature tolerance was successfully cloned using vector pUC18 in the bacteria *Escherichia coli,* strain dH5α (Figure 4.30). The desired clone was sequenced commercially. The two parents were amplified and evaluated with SCAR primers repeatedly, giving reproducible results. That is a band of 1200 bp in size was found in both sensitive and tolerant parents. This lack of polymorphism between the tolerant and susceptible genotypes with the SCAR primers suggests that the original polymorphisms with the 10 base RAPD primers were due to small mismatches at the priming sites (Paran and Michelmore, 1993) resulting in no amplification product at this site. Whereas, the longer and more specific SCAR primers (21 to 22 bases) were not affected by small mismatches at the priming sites. Hence, amplification products were produced on both the tolerant and susceptible genotypes. Two possible mechanisms could be proposed; one is that there might be a combination of SNPs (both in intron and in exon), which are responsible for such differential phenotype. Secondly, there may be alternate splicing products, which could lead to different products formed in different genotypes. A similar result was recorded in Oat by Tanhuanpaa et al. (2006). In their study, RAPD marker ($OPI\text{-}11_{450}$) differentiated the genotypes 'Aslak' and 'Kontant'. The marker was sequenced and converted into a SCAR marker. The SCAR marker was, however, monomorphic.

To study the differences between the parents at nucleotide level in the amplified products, the amplified fragments from both parents were isolated, sequenced and compared. An SNP (single nucleotide polymorphism) was found between these

sequences. This could be used to develop SNP markers in jute as developed in cucumber and wheat (Robbins et al., 2006; Khlestkina et al., 2006).

A bioinformatics analyses with this sequence revealed the presence of an open reading frame (ORF) that has been shown to be expressed as determined using reverse transcription PCR (RT-PCR) (Figure 4.34). This indicates that the region is present in close proximity to the locus determining low temperature tolerance. From the evidence of strong linkage observed, this ORF itself could be responsible for the trait. The translated product of its sequence revealed a strong homology, to that of an *Arabidopsis thaliana* expressed gene coding for low-density lipoprotein B like protein. Out of 51 amino acids, 46 were identical, 3 highly conserved, 1 addition and 1 substitution in comparison with translated product of *Arabidopsis thaliana* (Figure 4.33). This is a significant finding, as lipoproteins are known for its role in cold tolerance in other organisms such as rye and other plants (Steponkus et al., 1993).

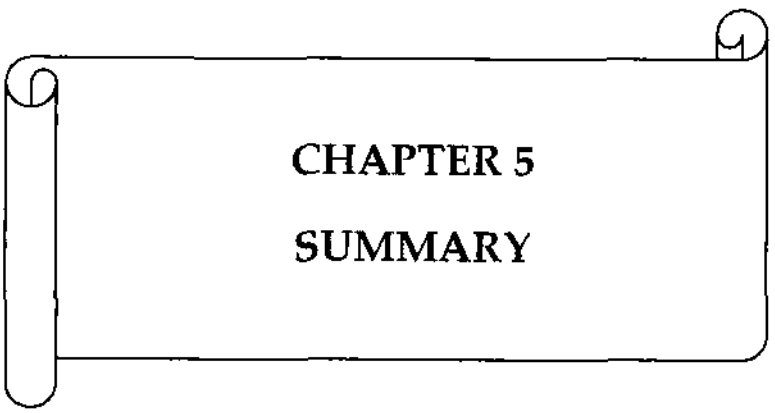
An effort was made to rescue the upstream sequence to know the total sequence of the gene using inverse PCR (technique presented in Figure 3.1). This technique was repeated under different PCR conditions, but no PCR products were found. But it is an effective technique. Total sequence of the promoter of fungal resistant gene (HMG I/Y) of pea was determined by Druffel et al. (2005) using inverse PCR. So further investigation is needed to address, whether ligation is a problem or long PCR is required or other effective technique like primer walking (Ashoub and Abdalla, 2006) could be used.

The first and very preliminary linkage map of the jute genome was constructed using software MAPMAKER version 3.0 (Stat Soft. 1994) considering 40 RAPD markers and their different combinations with an F_2 population developed from a cross between jute cultivars O-9897 and accession no. 1805 as low temperature

sensitive and tolerant respectively. Linkage analysis at a LOD score of 3.0 and a maximum distance 50 cM revealed 18 linkage groups.

Among the 18 linkage groups, 15 groups (group no. 1 to 15) contained single locus and the remaining three groups were designated as Linkage group-16, 17 and 18 contained 2, 11 and 12 loci respectively (Figure 4.35). The analysis revealed that the distance of 2 loci in linkage group-16 was 15.90 cM (Table 4.9). A total of 11 and 12 loci were mapped in a length of 241.70 and 206.10 cM respectively. This gave a marker density of 24.17cM in linkage group-17 and 18.73 cM in linkage group-18 between adjacent markers in these groups (Table 4.9). The average marker density obtained in the study (with 40 loci) seems to be high. However with 135 loci the first RELP map for rice had more intense marker density (10.35 cM) as had been observed by McCouch et al. (1988).

The unique chromosome number of *Corchorus* spp. is seven (2n=14); so seven linkage groups were expected to represent the genome. But for linkage group analysis, this effort was very limited and the total number of loci (40) was also low. In the software (Map maker version 3.0), it was indicated that, if the distance between two adjacent loci were more than 50 cM, there it would be a split point. More markers are needed to be mapped to merge smaller linkage groups to larger ones. In this way, the total of 40 loci of the 18 linkage groups may be in a single chromosome i.e. in a single linkage group in the saturated mapping. It was mentioned by Chawla (2002) that the total number of linkage groups should always be more than its real number till saturation. Due to the unavailability of co-dominant markers for jute, dominant markers (RAPD) were used for mapping. Dominant markers are unable to distinguish heterozygote from homozygote; however, they allow many polymorphic markers to be quickly identified, which is useful for mapping the genome such as legume crops (Menndez et al., 1997; Eujayl et al., 1998; Laucou et al., 1998; Santra et al., 2000) and also to extend the existing linkage map of rye (Masojc et al., 2001).



CHAPTER 5

SUMMARY

SUMMARY

Bangladesh is the homeland of quality jute. It is a dicotyledonous, self-pollinated and diploid (2n=14) Fibre yielding plant of the genus *Corchorus*, Family Tiliaceae. Jute Fibre is obtained from the bark of the two commercially cultivated species, namely *Corchorus capsularis* (white jute) and *Corchorus olitorius* (tossa jute). Jute is a short-day plant. It needs long day light for vegetative growth and short-day for flowering. The yield and quality of tossa is better than white jute. The centre of origin of white jute is said to be Indo-Burma including South China, and Africa for that of tossa.

The global demand for traditional products of jute is declining day by day due to invasion of synthetic Fibre. There is a golden opportunity to revive the past glory of jute through the production of paper pulp from green jute, which will solve the problem of retting and ensure the bulk use of green jute.

Bangladesh holds the largest gene bank of jute and allied Fibre (JAF) crops where, 5936 accessions are preserved and yet to be characterized. As jute is a self-fertilized crop, its natural genetic variability is very narrow; this makes the plant breeders helpless in the development of this crop. Molecular techniques could be a suitable alternative to improve the crop. Jute is a new crop in the field of molecular biology. So any kind of molecular information would be helpful for its improvement. As it is believed that jute cannot survive only of its traditional use, a bulk use must be ensured and production of paper pulp from green jute could be a suitable alternative. If this programme becomes a success, then at least two crops will be required in a year to meet the mill demand for green jute round the year and one crop will have to produce in the winter season. To produce jute in the winter season, it is essential to develop low temperature tolerant DNA marker for marker-aided selection and finally to develop a low temperature tolerant jute variety through molecular approach.

Isolation of quality DNA from jute is very difficult because of the presence of polysaccharides and other metabolites. Two modified methods (cited as Method-I and Method-II) were evaluated using four types of plant materials (seed, seedling, young leaf and old leaf) from two species of jute. It is interesting to note that no DNA was found from seed samples from both the species if isolated following Method-I (lack of phenol). From the observation, it may be concluded that Method-I is suitable for isolating DNA from seedlings and leaves but not from the seeds. On the other hand, Method-II (using phenol) is effective for all samples, however, compromising on the quality of DNA isolated from leaf samples. If the sample is limiting, as in the case of dead seeds, then Method–II is likely to be more suitable. Seedling is best for isolating quality DNA.

DNA fingerprinting or molecular identity of jute genotypes, particularly for the released varieties, is very important to protect biopiracy. Eighteen genotypes of two species of jute, *Corchorus capsularis* and *C. olitorius,* were used for fingerprinting analysis. Amplification of the isolated genomic DNA from each of the 18 genotypes, revealed a variety of RAPD patterns. SSR analysis was also performed with 16 genotypes in the same way as RAPD analysis. The result was very similar to that of RAPD. Two independent dendrograms were constructed using RAPD and SSR data. Two major clusters representing two species were resolved among the genotypes that were examined in the study. All *C. capsularis* genotypes belonged to one and the *C. olitorius* genotypes to the other cluster in both the dendrograms. The genetic distance among the genotypes within cluster correlated with little discrepancies with field data. This study holds enormous prospect for the development and application of molecular markers for jute. Further studies with more number of primers are highly recommended to generate a linkage map that spans the entire length of the jute genome.

The yield and quality of *C. olitorius* jute is better than *C. capsularis* but very sensitive to waterlogged condition. Developing a waterlogging tolerant DNA marker is very important but it was not possible to proceed with the selected materials. However,

it was decided to develop DNA markers for leaf structure (serrated versus entire leaf) and texture (matte versus glossy leaf). The serrated and glossy leaf characters are important for supporting a plant to be drought tolerant. The DNA obtained from the parents and homozygous lines, developed from cross between variety O-4 and Incisifolius, were analyzed with RAPD, ISSR and STMS primers. No consistent marker associated with desired traits was observed.

An effort was made to determine the yield performance of 4 homozygous lines along with their parents. The data such as plant height, base diameter, leaf area per plant, dry weight of different plant parts (leaf, stem and bark) per plant, flowering records such as flowering at 60 and 120 days after sowing, number of plants not flowered at harvest time and harvest index were recorded, analyzed statistically and the means were compared by DMRT. The result showed that among the 10 parameters, 3 treatments: base diameter, stem and bark dry weight were significant at 0.05 level and 6 treatments: plant height, leaf area, leaf dry weight, flowering at 60, 120 DAS and percentage of plants not flowered at harvesting time were highly significant (0.01). Only one treatment, harvest index, was found insignificant. Though the bark dry weight represents the Fibre yield (about 50% of the bark is Fibre) and was found highly significant, the harvest index was insignificant as it is a ratio. It may be concluded that the variety developed by Bangladesh Jute Research Institute is a rigid one and not so easy to supersede.

Another effort was made to develop molecular marker for low temperature tolerance. Two *C. olitorius* genotypes, variety O-9897 and accession no. 1805 were selected as low temperature sensitive and tolerant, respectively, following a thorough morphological and molecular investigation. In RAPD analysis a particular band was found in tolerant accession when amplified with primer OPG-05. A cross was made between O-9897 and accession no. 1805 as seed and pollen parent, respectively, and hybrid (F_1) was produced. It was observed that only the tolerant parent and hybrid seeds were capable of germinating at low temperature

conditions (16°C) where the sensitive parent failed. The result indicated that the low temperature tolerant trait is a dominant character.

The DNA obtained from the parents and differentially grown (low temperature tolerant and sensitive) segregating F_2 populations were thoroughly evaluated with RAPD primer OPG-05 for tracking the expected DNA fragment associated with low temperature tolerance. It was observed that a particular band of about 1200 bp in size consistently co-migrates with a band of same size in the low temperature tolerant parent and selected F_2 populations. This band was always absent in the sensitive parent and F_2s. The observation strengthened the idea that this particular stretch of DNA is associated with low temperature tolerant character.

This co-segregating DNA fragment may be a part of low temperature tolerant gene or non-coding fragment associated with low temperature tolerant trait. For further confirmation, the fragment was isolated from the gel and used as probe to differentiate the tolerant and sensitive parents through Southern analysis, but the technique failed to differentiate the parents. It means that the nucleotide sequence of both tolerant and sensitive parents in the amplified regions were very similar. The isolated DNA fragment associated with low temperature tolerance was successfully cloned and sequenced commercially. From the sequence, SCAR primers were designed. The two parents were amplified and evaluated with SCAR primers repeatedly, but also failed to discriminate the parents. This lack of polymorphism between the tolerant and susceptible genotypes with the SCAR primers suggests that the original polymorphisms with the 10 base RAPD primers were due to small mismatches at the priming site resulting in no amplification product at this site (Paran and Michelmore, 1993). Whereas, the longer and more specific SCAR primers (21 to 24 bases) were not affected by small mismatches at the priming sites. To study the differences between the parents at nucleotide level in the amplified products, the amplified fragments from both parents were isolated, sequenced and compared. An SNP was found between these sequences.

A bioinformatics analysis with this sequence was carried out. A strong homology (>90%) was observed at nucleotide level with genome *Arabidopsis thaliana*. This was shown to be a part of a gene (a terminal exon). The translated product of its sequence revealed a 153 bp region showing a strong homology, to that of an *Arabidopsis thaliana* expressed gene coding for low density lipoprotein B like protein. Out of 51 amino acids, 46 were identical, 3 highly conserved, 1 addition and 1 substitution was observed in comparison with translated product of *Arabidopsis thaliana*. This was a significant finding, as lipoproteins are known for its role in cold tolerance in other organisms.

An effort was made to rescue the upstream sequence to know the total sequence of the gene using inverse PCR with SCAR primer but no amplification was found. Further bioinformatics analysis of the sequence revealed the presence of an ORF that is shown to be expressed. This indicates that the region may be present in close proximity to the locus determining low temperature tolerance. From the evidence of strong linkage observed, this ORF itself is found to be responsible for the trait, two possible mechanisms could be proposed; one is that there might be a combination of SNP (both in intron and in exon), which are responsible for such differential phenotype. Second, it may alternate splicing products, which could lead to different products formed in different genotypes. It was evaluated that the gene responsible for low temperature tolerance was expressed as determined by RT-PCR.

The first and very preliminary linkage map of the jute genome was constructed with RAPD markers using two parents and their F_2 populations. Linkage analyses at a LOD score of 3.0 and a maximum distance 50 cM revealed 18 linkage groups. Among the 18 linkage groups, 15 groups (group no. 1 to 15) contained single locus and the remaining three groups designated as Linkage group-16, 17 and 18 contained 2, 11 and 12 loci respectively. The unique chromosome number of *Corchorus* spp. is seven (2n=14); so seven linkage groups should have been obtained to represent the genome. But for linkage group analyses, the effort was very limited and the total number of loci (40) was also low.

CHAPTER 6

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CHAPTER 7

APPENDIX

APPENDIX

Appendix I: List of 40 decamer RAPD primers with their sequences

Name	Sequence	GC content (%)	Name	Sequence	GC content (%)
OPAB-01	GTA G	70	OPH-03	AGA CGT CCA C	60
OPAB-03	TGG CGC ACA C	70	OPH-04	GGA AGT CGC C	70
OPAB-05	CCC GAA GCG A	70	OPH-05	AGT CGT CCC C	70
OPAB-06	GTG GCT TGG A	60	OPH-07	CTG CAT CGT G	60
OPAB-08	GTT ACG GAC C	60	OPH-10	CCT AGG TCA G	60
OPAB-10	TTC CCT CCC A	60	OPH-12	ACG CGC ATG T	60
OPAB-12	CCT GTA CCG A	60	OPH-13	GAC GCC ACA C	70
OPAB-18	CTG GCG TGT C	70	OPH-14	ACC AGG TTG C	60
OPAB-20	CTT CTC GGA C	60	OPH-15	AAT GGC GCA G	60
OPG-03	GAG CCC TCC A	70	OPQ-01	GGG ACG GAT G	70
OPG-05	AGT CGT CCC C	70	OPQ-05	CCG CGT CTT G	70
OPG-06	GTG CCT AAC C	60	OPQ-06	GAG CGC CTT G	70
OPG-07	GAA CCT GCG G	60	OPQ-07	CCC CGA TGG T	70
OPG-08	TCA CGT CCA C	60	OPQ-08	CTC CAG CGG A	60
OPG-09	CTG ACG TCA C	70	OPQ-16	AGT GCA GCC A	60
OPG-11	TGC CCG TCG T	70	OPQ-17	GAA GCC CTT G	60
OPG-13	CTC TCC GCC A	60	OPQ-20	TCG CCC AGT C	70
OPG-15	ACT GGG ACT C	70	OPE-17	CTA CTG CCG T	60
OPG-16	AGC GTC CTC C	60	OPN-02	ACC AGG GGC A	70
OPH-02	TCG GAC GTG A	60	OPN-13	AGC GTC ACT C	60

Appendix II: List of 47 SSR primers (Forward & Reverse) with sequence

Primer	Forward and Reverse Sequence		GC content (%)
SSR-01	Forward	CTT TCT TGA CCC AAA CAA TGC CA	43.5
	Reverse	GGA TGA TGA AAA ACG AAG TGC CTA	41.7
SSR-02	Forward	GTT TAT CCA ACC AAT ACC AAC CA	39.1
	Reverse	TGC CTC GTT GCT GGA CAT TGC A	54.5
SSR-03	Forward	GAA ATA ATT GTC ACA CTG CTC C	45.5
	Reverse	ATT CCA CGC TCC TTG TCA CC	55.0
SSR-04	Forward	CAA AAG TAG TGA AGA ACA TGA CCA	37.5
	Reverse	GGC AAA TTC TGA TAT ACG CCT GA	43.5
SSR-05	Forward	AGT GAC TTA TAG TCT AAT TAG TGA	29.2
	Reverse	ACA GAT AGG ATG TTA ACG GGA	42.9
SSR-06	Forward	CTA TCT CCC ATT GTA CCT GCA	47.6
	Reverse	GGC AGA TTG TGT GAG ACT ATC A	45.5
SSR-07	Forward	AAT GTA TAT GAA CCA TAG TGG TAC A	32.0
	Reverse	TTA TCA CAA AGT AGC AGA CTA ACA	33.3
SSR-08	Forward	GTA TAG ATA AAG AAA ATC CTA ACA	29.2
	Reverse	GCA GAA ATT GTG TGA GAT CAT CA	39.1
SSR-09	Forward	TTA CAT TAT ATA ATG TCC AGC CA	30.4
	Reverse	AGT GGC TAC TGG TTC CTA CA	50.0
SSR-10	Forward	GAA CAT CAA GAC TGA GTA AGA CCT A	40.0
	Reverse	TTG AGG ATT TTA ATA TGC ATG CA	34.8
SSR-11	Forward	TTA CTG CCC TGT TAA TGT TCC TCA	37.5
	Reverse	GGA TGG ATT AAC TTT TGA TTG GCA	37.5
SSR-12	Forward	CGC TCG CCT AAG TGA AGG CA	60.0
	Reverse	ATA AAA TAC AAG GGA CAC TTA GCA	33.3
SSR-13	Forward	TTA GGA GTC ATT TCT AAC AAG AC	34.8
	Reverse	AAT CCC TCC AGC TTT CTC GA	50.0
SSR-14	Forward	TTG TTT CCT CAG TGC TGA CTC A	45.5
	Reverse	CTC TGC TAT ATC ATC CGT AGC A	45.5

SSR-15	Forward	GAG AGG AAT GAT GCT GAG ATT CA	43.5
	Reverse	GAC ACC CTC CGC CAT TCT CA	60.0
SSR-16	Forward	TGG AAC CTG AGC ATC TCT CCA GA	52.2
	Reverse	CTT TTT CTT GTT CAG GGA CCT GA	43.5
SSR-17	Forward	AGA GTT TGC AAC AAG GTA GCC A	45.5
	Reverse	TGG CTA CTT AAA CTT AGT TGT GTG CA	38.5
SSR-18	Forward	GCT GTT GTC TCT CTA TTG GTG A	45.5
	Reverse	TTC CAC GCT CCT TGT TGC CA	55.0
SSR-19	Forward	TAT GAA GGT GAA CTA CTT GTC ACA	37.5
	Reverse	AGC TTC CAT TTC GAA CAT TCC A	40.9
SSR-20	Forward	GTA AAG CAC AGG ATT AGT CCC A	45.5
	Reverse	GGA AAG TGA ACC TCT AGT AGA TGA	41.7
SSR-21	Forward	AAT CAA ATT GAG AAT GGA CAT GCA	33.3
	Reverse	GAA AGG CAA ATG CGC TTG TTG A	45.5
SSR-22	Forward	CTG TTT GTC AAT ACT CTC TTT TGA GTC A	36.0
	Reverse	GTC CAA AAC ATC GTG CAG TGT GA	47.8
SSR-23	Forward	GGC CCT TCT AAT TAA CCT CCA	47.6
	Reverse	AGT TTT GTT TCC AGA TAT TGC TCA	33.3
SSR-24	Forward	GTA CAT CTG ATT AAT ATC CTC TA	30.4
	Reverse	CTA TGT AAA CTT ACT TGA ACG ACA	33.3
SSR-25	Forward	AAT GGC AAG CAA CTT CAT CCC	47.6
	Reverse	ATC GAG TAT AAA CAG AGT AAT GAC	33.3
SSR-26	Forward	GAA TAT TTA ACT CTA TCC ACC ACA	33.8
	Reverse	GCT CCT GCC TGT AAT TAC ACA	47.6
SSR-27	Forward	TTG TGT GCA AAC ACG AGT GCA	47.6
	Reverse	GGT AGC CAT GTT TAC TTC CTG A	45.5
SSR-28	Forward	AGA GAC GAG TAA ACA TAA AAG TCC	37.5
	Reverse	CTG GCG AAG CCT TAA AAA TGC A	45.5
SSR-29	Forward	CTG AAT GAA AGA TTG CTT TTA ATC C	32.0
	Reverse	CAT GCA TCA TTT GCA TTG CAT GCA	41.7

SSR-30	Forward	GAG TGA TTA GAG GGC AGC CA	55.0
	Reverse	TGC AAC AAA GTA TCC AAA TCG AC	39.1
SSR-31	Forward	GAT TTT TGG TAT GCA TGG GGA	42.9
	Reverse	CTG TTT AAG AAA TAC TCC AAA TGG	33.3
SSR-32	Forward	TTC ACT TTG CAT TAA AGA ACA CCA	33.3
	Reverse	GGG ATA AGG AAG CTT CCA TGC A	50.0
SSR-33	Forward	GTT GAT ATC TTT ACG GTG TAT AGA	33.3
	Reverse	GTG ATT TTG GTT ACT ATG ACC CA	39.1
SSR-34	Forward	GTG GAT CAT ATG TAA TTT CTC CAA	37.5
	Reverse	AGC TGG TTG TTG ATC TTC ATC TG	43.5
SSR-35	Forward	GTA TTT CTG CTG AGG GGC TTC A	50.0
	Reverse	CTA TCG TTG GTG AAT GTA TAT ACA	33.3
SSR-36	Forward	AAA AGA TAT CTT ACT GCT CAA GTC	33.3
	Reverse	TAT TAA TTA GAC TTG TCT GTC TAT C	28.0
SSR-37	Forward	AAT GTG AAA TCC AAT TAA AGC ACA	29.2
	Reverse	CCA AGA TTG TAG CAA CAA ACC A	45.5
SSR-38	Forward	ACC AAG TAT GAT CTG ACC TCT	42.9
	Reverse	AGC TAA AAA CAA CAC AAA AAT ATC TTG C	28.6
SSR-39	Forward	TTC ATA GCC GTT TGT GCA ATC A	40.9
	Reverse	ATC AAA AGA GTG GAG ATA TTG AAC	33.3
SSR-40	Forward	ACG ATA ATG CCC TCG CTT AG	50.0
	Reverse	GAT GCA GAT TGG TAG AGA GTC A	45.5
SSR-41	Forward	GGA AAA CAG CCT ATT GGG GAC A	50.0
	Reverse	CAT TCC TCC ATT GTT GAA GGA	42.9
SSR-42	Forward	GGA AAT ACC CAC ACT GTT CCA	47.6
	Reverse	GCA ATC AAA GTG TAT TTG GAT GAC A	36.0
SSR-43	Forward	TTA GCA GAA CAG CAG CAA GCA	47.6
	Reverse	TAA TTT CAG GTT TTG AGT ACT CCA	33.3
SSR-44	Forward	CAT TTT GTT AAC CTT CCT CAC	38.1
	Reverse	GAA TTC TAA TTG TGT GGT TTC	33.3

SSR-45	Forward	AAA GAG CTT TGA GAG ATG GTC C	45.5
	Reverse	CCT AGC ATA TTG TTC AAG TCC	42.9
SSR-46	Forward	TCG TCC CCC AGG ATA CAA C	57.9
	Reverse	AGT CGT CCC CTA TAT AAA GGG	47.6
SSR-47	Forward	AGT CGT CCC CAT TGA TAA TGG	47.6
	Reverse	AGT CGT CCC CTC ATC ATT TAC G	50.0

Appendix III: List of 15 ISSR primers with sequence

Primer	Sequence
ISSR-01	T AGAGAGAGAGAGAGAG
ISSR-02	C TGTGTGTGTGTGTGTG
ISSR-03	A GTCTCTCTCTCTCTCTC
ISSR-04	TC CAACAACAACAACAA
ISSR-05	AG CTTCTTCTTCTTCCTTCTT
ISSR-06	G CCACCACCACCACCACCA
ISSR-07	CT AAGAAGAAGAAGAAGAAG
ISSR-08	C GGGGGGGGGG
ISSR-09	TA AGAGAGAGAGAGAGAG
ISSR-10	TC GGCGGCGGCGGCGG
ISSR-11	ACACACACACACACAC T
ISSR-12	GTGTGTGTGTGTGTGT A
ISSR-13	GAGAGAGAGAGAGAGA T
ISSR-14	GAGAGAGAGAGAGAGA A
ISSR-15	CGGCGGCGGCGGCGG T

Appendix IV: Primers for the SSR or STMS PCR

Primer	Sequence	Tm°C	Product size (bp)
J003 F	TTT ATC TCT TTC GGC ATC ATC A	45	241
J003 R	GCA CAT TGC ACG TGA CTC AT	45	
J004 F	TGG TTG CAT TGT TTG CAT TT	45	201
J004 R	TGC AGA AGA CTC CCA ATT CC	45	
J008 F	GGA CCA AAA CAG GTG CAT TT	45	200
J008 R	AGG GTG ATT TTG TTG GCA CT	45	
J042 F	AAA TGA CCT GTT GAT GCC AAT	45	161
J042 R	TCA TTC CAC TCA ACC CAA CA	45	
J047 F	CAA CAA GAA CAA CAA CCT CA	45	182
J047 R	CTA GGC CGC AAG TTT TCA TT	45	
J121F	TGG GAA ATT AGG GTT TGG AG	45	241
J121R	CGA GAA TGA AAA CCC CTC AA	45	

Appendix V: List of AFLP primers (EcoR1 and Mse1) with sequence

EcoR1 group	Sequence (5` to 3`)
E-AA	CTT GTA GAC TGC GTA CC *AATTC AA*
E-TT	CTT GTA GAC TGC GTA CC *AATTC TT*
E-AG	CTT GTA GAC TGC GTA CC *AATTC AG*
E-AT	CTT GTA GAC TGC GTA CC *AATTC AT*
E-TC	CTT GTA GAC TGC GTA CC *AATTC TC*
E-TG	CTT GTA GAC TGC GTA CC *AATTC TG*
E-TA	CTT GTA GAC TGC GTA CC *AATTC TA*
E-AC	CTT GTA GAC TGC GTA CC *AATTC AC*

Mse1 group	Sequence (5` to 3`)
M-CAA	GAC GAT GAG TCC TGA G *TAA **CAA***
M-CAC	GAC GAT GAG TCC TGA G *TAA **CAC***
M-CAG	GAC GAT GAG TCC TGA G *TAA **CAG***
M-CTG	GAC GAT GAG TCC TGA G *TAA **CTG***
M-CTA	GAC GAT GAG TCC TGA G *TAA **CTA***
M-CTC	GAC GAT GAG TCC TGA G *TAA **CTC***
M-CTT	GAC GAT GAG TCC TGA G *TAA **CTT***
M-CAT	GAC GAT GAG TCC TGA G *TAA **CAT***

Appendix VI: Sequence (Forward and Reverse) of DNA fragment associated with low temperature tolerance

>FORWARD SEQUENCE:

AATAACTACATATAGGGCGAATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTG
TGATGGATATCTGCAGAATTCGGCTTAGTCGTCCCCTCATCATTTACGTACAAGCTGGAA
GTATATGCACCCCTATCCAATGGAAACAAAGCATGCATCTAAATTTGGTGGGAGGACAA
AGAGCTAAGGACCAAGCAAAAATCAAGAATCTGATCTGGTGGTGGTAAATGAAGAAAG
AAATCCCCCAGCTTGTACAGGTAAAATGTCACCAAATGTTGACATGGCAGCTGCTGATCT
ATCCTTGAATATGCCCACTTGCCCATCCGTTAGTATAGATCCCAACTTTAAAGTGCTCTCT
CCGAATTTACTTCCAACCTGGCCAGTCCAAAGGAAATCAATATCAAAACCTTGAATGTGT
TACAAACAGATAGCAAAACATCTAATCCACTTTACAATTGCAGTAAATATTCATAACTTG
CAAATTAGCTGCTTTACATAAGGCTAATTCCCAAGGAAGGTTCAAACCTTCATACGATCT
CATTTCAAGATCAAAATTTTCTACTTTGTCCATTATAAGATCAAACATTTCCGACAACACT
GAAGGGGTATTTTAGCATGCCTTTCATGCTCATTTGAGTCTCTTAAATATGTGAGAAAGTA
GATTTAGTGAAAAGCCAGACACAGCTCTACTTAAGGTTGACATGATTATCAGGACTTCCT
TGTTGATGAGTAAAACAATCCGACCTTCTTATGTAACAAGTCCAAATTCTTAGCTGGCTTC
CTCCAGTTTAGGATTGCCTTTTTCCAACAAGGACGTCAGATGCTAGTCAGCATTAATGGG
AATTGTGTTTGCCCTTCCCCTTAACTTAATGGGACCACTAAAACCCTTTTTTTGGCCAAAC
CCACTAAAACGAATTTCTTTAACATGTTAATCCTCACTGCATACTTTTTACACTGAAATAA
AGTTGTGTAAACCTACCTTCTGTCTTTTATCTATTAACTAGCTAGTCTTTACAATTTTCAGT
AAGATTATATATGGTTTTGTAATTCCTCTGATTCAAATTTGTTTCTCAGGGTAAATCATATT
AATACCCCTTGTGATTTTCCGTTTCATGATTTTGTAAATCTATGTTTCTTTTTGCCCCTTGCA
CCAAAACAAGAAACACCCTTGGGGTTTAACCTT

>REVERSE SEQUENCE

CCGAATGCTTGGTACGAGCTCGGATCCCTAGTAACGGCCGCCAGTGTGCTGGAATTCGGC
TTAGTCGTCCCCTATGATAATGGAGTAGAAGTATAAACCCCAAGGGTGTTTCCTGTTTTGG
TGCAAAGGGCAAAAAGAAACATAGATCTACAAAATCATGAAAACGGAAAATCACAAGG
GGTATTAATATGATTTACCCTGAGAAAACAATTTTGAATTCAGAGAACTTACAAAACCAT
ATATAATCTTACTGAAAATTGTAAAGACTAGCTAGTTAATAGATAAAAGACAGAAGGTA
CGTTTACACAACTTTATTTCAGTGTAAAAAGTATGCAGTGAGGATTAACATGTTAAAGAA
ATTCGTTTTAGTGGGTTTGGCCAAAAAAAGGGTTTTAGTGGTCCCATTAAGTTAAGGGGA
AGGGCAAACACAATTCCCATTAATGCTGACTAGCATCTGACGTCCTTGTTGGAAAAAGG
CAATCCTAAACTGGAGGAAGCCAGCTAAGAATTTGGACTTGTTACATAAGAAGGTCGGA
TTGTTTTACTCATCAACAAGGAAGTCCTGATAATCATGTCAACCTTAAGTAGAGCTGTGTC
TGGCTTTTCACTAAATCTACTTTCTCACATATTTAAGAGACTCAAATGAGCATGAAAGGC
ATGCTAAAATACCCCTTCAGTGTTGTCGGAAATGTTTGATCTTATAATGGACAAAGTAGA
AAATTTTGATCTTGAAATGAGATCGTATGAAGGTTTGAACCTTCCTTGGGAATTAGCCTTA
TGTAAAGCAGCTAATTTGCAAGTTATGAATATTTACTGCAATTGTAAAGTGGATTAGATG
TTTTGCTATCTGTTTGTAACACATTCAAGGTTTTGATATTGATTTCCTTTGGACTGGCCAGG
TTGGAAGTAAATTCGGAGAGAGCACTTTAAAGTTGGGATCTATACTAACGGATGGGCAA
GTGGGCATATTCAAGGATAGATCAGCAGCTGCCATGTCAACATTTGGTGACATTTTACCT
GTACAAGCTGGGGGATTTCTTTCTTCATTTACCACCACCAAGATCAGATTCTTGATTTTTG
CTTGGTCCTTAGCTCTTTGTCCCTCCACCAAATTTAGATGCATGCTTTG

SCAR primer: Forward AGTCGTCCCCTATGATAATGG (21b)

Reverse AGTCGTCCCCTCATCATTTACG (22b)

Note: Highlighted sequence indicates the cite of SCAR primer

Appendix VII: Acquaintance with Apparatus used in the Laboratory

Name	Description	Source
Eppendorf tubes	Size 1.5 ml, colorless	Fisher Scientific, USA.
Micropipette tips	Labsystems,	Finland.
PCR tubes	Size 0.5 ml	Perkin Elmer Cetus, USA.
Gloves	Disposable	Vinyl medical gloves, USA
Glass wares	Pyrex brand	USA
Micropipettes	0.5-10, 0.5-10µl 5-20µl, 10-100µl, 50-1000µl	Labsystems, Finland.

Gel kit and power	Model H5 Horizontal	BRL, Life Technologies Inc. USA.
Autoclave machine	Model HL-42A	E Hirayama Mfg Corp. Japan.
Microcentrifuge Machine	Eppendorf centrifuge 5810R	Germany
Vortex	Model 1190-1	Labline Instruments, USA.
Reciprocal shaking bath	Model 25	Precision Scientific.
Circulating water bath	Model260	Precision Scientific.
P^H meter		Orion Research, USA.
Tube rack		SIGMA, USA.
Balance Switzerland		Mettler AE 100,
Magnetic stirrer		Coming, UK.
Incubator shaker	Innova 4300	New Brunswick Scientific Nalgene,
Centrifuge tube		USA.
Refrigerated super speed centrifuge	Model RC 5B	Sorvall
Freeze drier	Model 12525	VirTis Comp.USA.
Refrigerator	-80°C	Barka Profiline
Microwave		Emerson, Korea.
Biodoc System	Kodak EDAS 290	Japan
Spectrophotometer	Analytikjena Specord 50	Germany